

Chapter 2

Signalling of Apoptin

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Abstract The virus-derived protein Apoptin has the ability to induce p53-independent apoptosis in a variety of human cancer cells while leaving normal cells unharmed. It thus represents a potential anti-cancer therapeutic agent of the future but a proper understanding of Apoptin-induced signalling events is necessary prior to clinical application. The tumor-specific nuclear translocation and phosphorylation of Apoptin by a cellular kinase such as protein kinase C seem to be required for its function but otherwise the mode of tumor selectivity remains unknown. Apoptin has been shown to interact with several cellular proteins including Akt and the anaphase-promoting complex that regulate its activity and promote caspase-dependent apoptosis. This chapter summarizes the available data on tumor-specific pathways sensed by Apoptin and the mechanism of Apoptin-induced cell death.

Keywords Cancer • Tumor selectivity • Molecular therapy • Apoptosis • p53 family • Protein kinase C (PKC) • Targeted therapy • DNA damage

2.1 Introduction to Apoptin

Apoptin is among the first tumor selective anti-cancer genes that have been isolated. This small viral protein induces p53-independent tumor cell-specific cell death. In this chapter we will discuss cellular pathways that are important for cell death initiation by Apoptin and possible mechanisms which regulate its tumor selectivity.

Apoptin was originally identified as the apoptosis-inducing protein derived from Chicken Anemia Virus (CAV), a single-stranded DNA virus of the Gyrovirus genus [1]. Infection with the virus gives rise to a severe disease in young chicken characterized by lymphoid atrophy, anemia, thrombocytopenia and intramuscular hemorrhages with mostly fatal consequences [2, 3]. The major targets of the virus include myeloid progenitor cells in the bone marrow and T lymphocyte precursor cells in the thymus. When infected these cells undergo apoptosis causing depletion of mature erythroid, myeloid and lymphoid cells resulting in the characteristic disease symptoms [4]. The CAV genome contains three partially overlapping open reading frames encoding viral proteins from a single polycistronic mRNA: VP1 (capsid protein), VP2 (protein phosphatase, scaffold protein) and the death-inducing protein VP3 [3]. Expression of VP3 alone was shown to be sufficient to trigger cell death in chicken lymphoblastoid T-cells and myeloid cells but not in chicken fibroblasts and it was therefore renamed Apoptin [5].

Recently, several viruses resembling the structure and overall organization of CAV have been isolated from human specimens and have now been included in the Gyrovirus genus [6–9]. Like CAV the first identified human Gyrovirus (HGyV) contains a VP3 protein homologous to Apoptin which exhibits similar features as



Fig. 2.1 Structure and sequence of Apoptin. The positions of key functional domains of the protein such as the nuclear localization sequence (NLS), nuclear export signal (NES) or the Thr-108 phosphorylation site are indicated. The *lower panel* shows the sequence of Apoptin; the corresponding amino acids of indicated domains are *highlighted*

CAV-Apoptin that are described below [10]. However, the importance of these viruses in human disease and the therapeutic potential of HGyV-Apoptin remain to be determined.

Since its discovery in the early 1990s Apoptin has become of interest in cancer research due to its ability to induce apoptosis in a variety of human tumor cells while leaving normal cells relatively unharmed [11–14]. Overall more than 70 cell lines from different tumor types, including melanoma, lymphoma, colon carcinoma and lung cancer, have displayed sensitivity to Apoptin-induced cell death. In addition SV40-transformed cells or UV-irradiated cells from patients with hereditary cancer-prone disorders are susceptible to Apoptin expression [11, 15, 16]. Thus, Apoptin seems to sense an early event of oncogenic transformation and induces cancer-specific apoptosis regardless of tumor type and presents a potential future anti-cancer therapeutic agent.

Several studies have been investigating strategies for the efficient delivery of Apoptin to the tumor *in vivo*, some of which will be discussed elsewhere in this book. In order to develop Apoptin into an effective anti-cancer drug it is important to understand Apoptin-induced signalling events. In addition, the identification of tumor-specific pathways activating Apoptin will help to develop novel and specific anti-cancer treatments. Hence this chapter focuses on the cellular pathways initiated by Apoptin and the mechanisms which determine Apoptin tumor cell selectivity.

2.2 Structure of Apoptin

Apoptin is a small protein of 14 kDa rich in proline, serine, threonine and basic amino acids and does not share any sequence homology with known cellular proteins described so far (Fig. 2.1). It comprises a C-terminal bipartite nuclear localization sequence (NLS) spanning amino acids 82–88 (NLS1) and 111–121 (NLS2) as well as a nuclear export signal (NES) which has been mapped to either the N-terminus or C-terminus of the protein. Both of these domains are required to facilitate nuclear shuttling of the protein [17–19]. Moreover, Apoptin contains several potential threonine and serine phosphorylation sites, including threonine 108 (Thr-108) within its C-terminus. These allow for modification by cellular kinases to regulate Apoptin activity and the interaction of Apoptin with other

proteins [20]. A short leucine-rich stretch (LRS) in the N-terminal domain (amino acids 33–46) is important for self-association of Apoptin and its binding to other cellular proteins such as promyelocytic leukemia protein (PML) [21].

Recombinant Apoptin fused to maltose binding protein (MBP-Apoptin) aggregates into stable globular complexes comprising 30–40 non-covalently attached Apoptin monomers *in vivo*. These multimers retain the ability to induce apoptosis and are stable in tumor cells but seem to be unstable and eventually diminish in normal cells [22, 23]. The formation of these complexes is facilitated through interactions of the hydrophobic regions located in the N-termini of Apoptin monomers (amino acids 1–69) and there seems to be little exchange of Apoptin subunits between multimers. Spectroscopic analyses did not reveal any ordered α -helical or β -sheet secondary structure of Apoptin monomers; however they adopt a stable conformation within the complexes. The three dimensional structure of Apoptin multimers is characterized by hydrophobic patches on the surface as well as the exposure of the C-terminal domains of Apoptin monomers, both of which may affect its binding to other cellular factors [24]. Importantly, Apoptin multimers have been shown to form superstructures with DNA with a preference for DNA strand ends *in vitro* and localize to DNA-dense heterochromatin and nucleoli *in vivo* [25]. However, the functional consequences of DNA binding for the cell and for the virus will have to be revealed by further studies.

2.3 Cell Type-Specific Localization of Apoptin

The mode of Apoptin's tumor cell-specific cytotoxicity so far remains unknown although several mechanisms have been proposed. One of the major differences observed is the subcellular distribution of Apoptin that correlates with its ability to induce apoptosis. In tumor cells or transformed cells Apoptin is expressed predominantly in the nucleus whereas in normal cells it is mainly localized in the cytoplasm (Fig. 2.2).

Importantly, nuclear translocation is at least partially required for the ability of Apoptin to induce cell death. Several deletion mutants of Apoptin defective in nuclear translocation thus induce apoptosis less efficiently than wild type Apoptin [14, 17, 26]. Moreover, targeting of Apoptin to the endoplasmic reticulum (ER) or the mitochondria instead of the nucleus reduced its ability to trigger cell death [12]. However, forced expression of Apoptin into the nucleus of normal cells on its own did not result in apoptosis, showing that additional factors are important for the activation of Apoptin. In addition, both the C-terminal and N-terminal domains of Apoptin are able to induce apoptosis on their own, albeit less potently than full-length Apoptin, even though only the C-terminus contains a NLS [17]. Thus nuclear localization is partially required but not sufficient for Apoptin-induced cell death. In a study by Wadia et al. nuclear localization of Apoptin in cancer cells was suggested to depend on the intracellular protein levels of Apoptin instead of the tumorigenic status of the cell [27]. However, nuclear import remained significantly

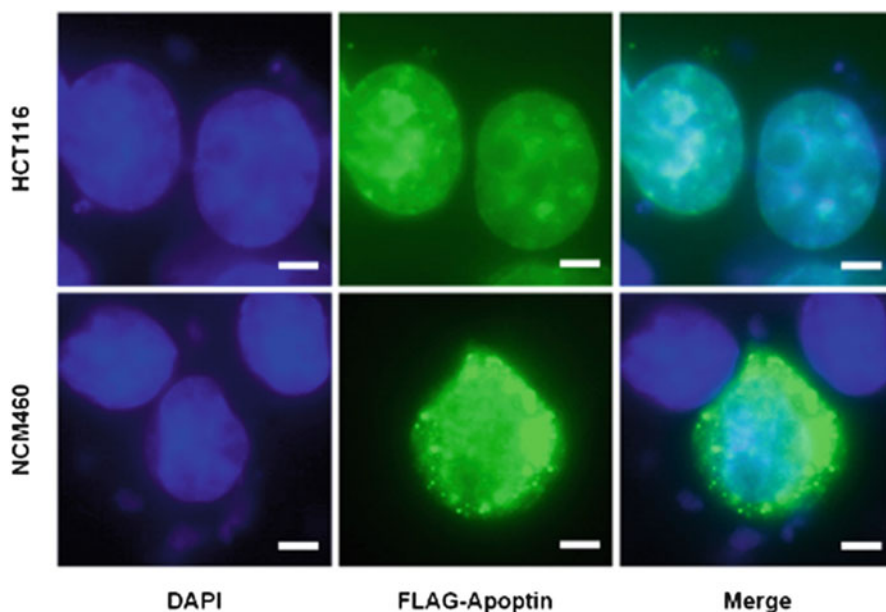


Fig. 2.2 Subcellular distribution of Apoptin in normal and tumor cells. HCT116 colorectal cancer cells and NCM460 normal colon mucosa cells were transfected with FLAG-Apoptin. At 48 h post-transfection cells were fixed and stained with a primary mouse anti-FLAG and secondary FITC anti-mouse antibody for detection of FLAG-Apoptin. In addition, nuclei were detected by counterstaining with DAPI. Scale bars indicate 20 μ m

higher in transformed cells compared to untransformed fibroblasts under all transfection conditions tested. Furthermore, their study employed a C-terminal fragment of Apoptin lacking a putative nuclear export signal as well as the Apoptin multimerization domain which most likely affects nuclear shuttling compared to the full length protein. Moreover, the use of Apoptin tagged with green fluorescent protein (GFP-Apoptin) might favor Apoptin translocation to the nucleus even in normal cells. Despite this, GFP-Apoptin was still shown to accumulate in the nucleus to a greater extent in transformed cells compared to their non-transformed counterparts [28, 29].

The nuclear shuttling of Apoptin is regulated by nuclear localization and nuclear export signals that have been identified in mutagenesis studies. Mutation of either of the predicted nuclear localization sequences in the C-terminus resulted in reduced translocation to the nucleus, showing that both sequences form a bipartite NLS are required for efficient nuclear localization of Apoptin [17, 19, 30]. This NLS seems to be active in both normal and tumor cells as inhibition of Crm1-mediated export from the nucleus caused nuclear accumulation of Apoptin in normal cells, suggesting that the cell type-specific localization of Apoptin may be regulated by nuclear export rather than import [18]. While agreeing on the importance of Apoptin's C-terminus to trigger nuclear translocation, two separate

studies have proposed different sequences as potential nuclear export signals. Poon et al. described a NES within the C-terminus of Apoptin (amino acids 97-105) mediating Crm1-recognized nuclear export in normal cells. In their study mutations within the N-terminal LRS (amino acids 33-46) resulted in reduced nuclear accumulation of Apoptin in Saos-2 osteosarcoma cells, suggesting it as a nuclear retention sequence [19]. In contrast, a study by Heilman et al. reported that mutation of the putative N-terminal NES (amino acids 37-46) caused localization of Apoptin to the nucleus in normal cells. Expression of deletion mutants containing only the N-terminal NES resulted in Crm1-dependent nuclear export of Apoptin into the cytoplasm of H1299 non-small cell lung carcinoma cells [18].

In summary, the cell-type specific localization and nuclear shuttling of Apoptin is believed to be controlled by a C-terminal bipartite NLS as well as a NES located in either the C-terminal or the N-terminal half of the protein. Furthermore, the localization of Apoptin can also be affected by the interaction with cellular proteins resulting in its nuclear or cytoplasmic retention.

Although it is clear that nuclear translocation is important for Apoptin-induced killing of tumor cells, the link between subcellular distribution and induction of cell death is still missing. Despite its ability to bind DNA Apoptin does not act as a direct transcriptional repressor or activator but might still indirectly affect the activity of other cellular transcriptional regulators [17].

Interestingly, the induction of DNA damage response (DDR) signalling was shown to trigger nuclear localization of Apoptin and apoptosis induction even in non-transformed primary cells [31]. Conversely, inhibition of DDR signalling, e.g. through knockdown of ataxia telangiectasia mutated kinase (ATM) or DNA-dependent protein kinase (DNA-PK), resulted in cytoplasmic localization of Apoptin in tumor cells. Since many cancer cells are characterized by constitutive DNA damage signalling these data suggest a possible link between DDR, nuclear localization and induction of cell death by Apoptin in tumor cells.

Furthermore, in normal cells Apoptin localizes to the cytoplasm, forms aggregates and becomes epitope shielded, eventually leading to its elimination [23]. Early after injection into VH10 fibroblasts MBP-Apoptin could be detected as larger aggregates in the cytoplasm but later became undetectable and neutralized due to epitope shielding even though the protein was still present. However, eventually MBP-Apoptin was completely degraded in normal cells. In line with that, treatment with proteasomal inhibitors such as Bortezomib has been shown to stabilize Apoptin in normal but not in tumor cells [32]. However, the relevance of this differential sensitivity to proteasomal degradation depending on the cell type still needs further evidence.

In addition, nuclear translocation might be required for interaction with nuclear binding partners in the nucleus such as the anaphase promoting complex or PML bodies that are discussed below.

In summary, even though the role of nuclear translocation for Apoptin-induced cell death has been extensively investigated the precise link to apoptotic pathways remains unclear.

2.4 Tumor-Specific Phosphorylation of Apoptin

Further studies on Apoptin also revealed another interesting property of the protein that provides a potential link to tumor-specific signalling pathways. In cancer cells but not in normal cells Apoptin becomes phosphorylated on threonine 108 (Thr-108) by a tumor-specific but as yet unknown cellular kinase [20]. The phosphorylation on Thr-108 could be detected in vitro and in vivo both in tumor cell lines and human tumor tissue lysates but not from healthy individuals. In addition, transient expression of the SV40 large T antigen (SV40 LT) was shown to trigger Apoptin phosphorylation in normal cell lines [16] suggesting that the Apoptin kinase can be activated by a transforming signal.

Apoptin phosphorylation was suggested to drive nuclear accumulation of Apoptin through inactivation of the adjacent putative NES in tumor cells. A gain-of-function point mutation of Thr-108 to a phospho-mimicking glutamic acid (T108E) led to increased nuclear translocation of Apoptin and cell death induction in VH10 normal fibroblasts [19, 20, 30]. In contrast, another study claims that phosphorylation of Thr-108 is dispensable for Apoptin's tumor-specific nuclear localization and partially also its apoptotic activity [33]. An unphosphorylatable Apoptin mutant or a C-terminally truncated protein lacking the Thr-108 phosphorylation site still retained a partial ability to accumulate in the nucleus and induce apoptosis. This might be explained by the fact that Apoptin contains two distinct pro-apoptotic domains and that Thr-108 phosphorylation is essential only for regulation and activation of the C-terminal death domain. Moreover, Apoptin might be phosphorylated alternatively on the adjacent threonine residues Thr-106 and Thr-107. In support of that, another study showed that both Thr-108 and Thr-107 were required to be mutated to completely abolish Apoptin-induced cell death [29]. The potential mechanism of Apoptin activation through phosphorylation and subsequent nuclear translocation is summarized in Fig. 2.3.

The tumor cell-specific activation of Apoptin through phosphorylation might be due to the presence of a cancer-associated pathway resulting in increased activity of a cellular kinase. Thus, several studies have attempted to identify the kinase or kinases responsible for mediating the phosphorylation of Apoptin in tumor cells. Identification of such kinases has immense importance not only for unravelling the function of Apoptin but may also provide novel targets for the development of selective anti-cancer drugs.

Maddika et al. have reported a sustained activation of the pro-survival PI3K/Akt pathway through direct interaction of Apoptin with the p85 subunit of phosphoinositide 3 kinase (PI3K) [34, 35]. Akt (or protein kinase B) is recruited and activated downstream of the PI3K-induced generation of lipid messengers and regulates processes including cell survival, cell cycle and metabolism [36–38]. In their study, expression of Apoptin triggered sustained activation and nuclear translocation of Akt which was required for Apoptin-induced cell death. However, neither PI3K nor Akt were able to phosphorylate Apoptin directly. Instead, interaction with Apoptin was shown to result in Akt-dependent activation of

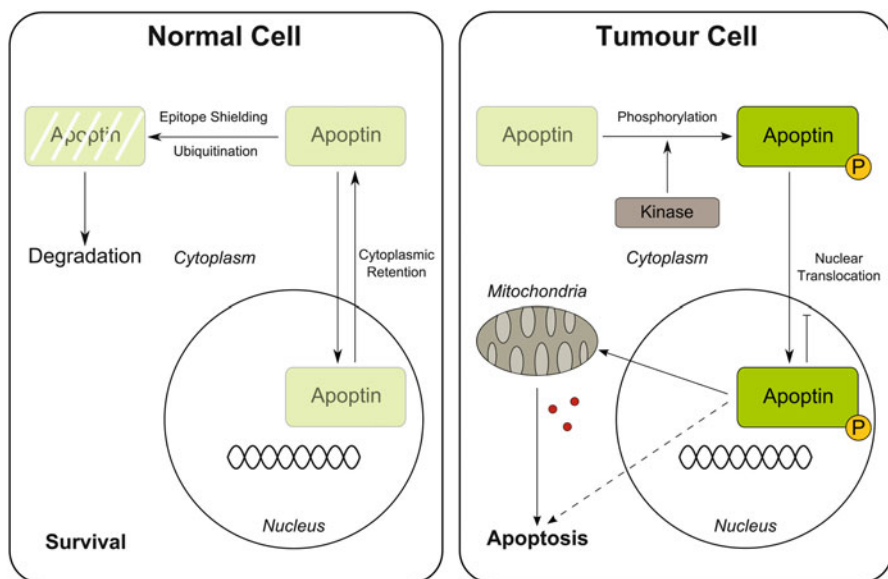


Fig. 2.3 Model of Apoptin activation in tumor cells versus normal cells. In tumor cells Apoptin is phosphorylated by a cellular kinase and translocates to the nucleus where it accumulates. Subsequently Apoptin activates the apoptotic machinery either through the mitochondria or other pro-apoptotic signalling pathways resulting in cell death. In contrast, in normal cells Apoptin shuttles back to the cytoplasm, becomes epitope shielded and eventually degraded in a proteasome-dependent manner

cyclin-dependent kinase 2 (CDK2) which in turn mediated the phosphorylation of Apoptin on Thr-108 in PC3 prostate cancer cells. Pharmacological inhibition or siRNA knockdown of either PI3K/Akt signalling or CDK2 protected cells from Apoptin-induced cell death [39].

In 2010 Jiang et al. proposed the β isozyme of protein kinase C (PKC β) as a potential Apoptin kinase in a model of human multiple myeloma (MM) cell lines [40]. Dexamethasone-resistant MM1.R cells displayed higher susceptibility to Apoptin-induced cell death than the parentally matched MM1.S cells, correlating with increased Apoptin phosphorylation in MM1.R cells. Microarray expression analysis and RT-PCR revealed a differential expression pattern of several kinases including PKC β which showed a ninefold higher mRNA level in MM1.R cells compared to MM1.S. Since recombinant PKC β was able to phosphorylate MBP-Apoptin in an in vitro kinase assay, further experiments were conducted to confirm the link between PKC β and Apoptin in vivo. Pharmacological inhibition of PKC β but not of Akt as well as shRNA-mediated knockdown of PKC β significantly reduced Apoptin phosphorylation in MM1.R cells. Further immunoprecipitation studies also identified a physical interaction between Apoptin and PKC β in vitro and in vivo, supporting an important role for PKC β in regulating Apoptin activity. This study also showed that Apoptin expression results in PKC β upregulation and

activation as well as its translocation to the nucleus of HCT116 colon carcinoma cells together with Apoptin. Besides, expression of Apoptin also induced cleavage of PKC δ , a known pro-apoptotic PKC isoform that is able to activate caspases and in turn gets activated through proteolytic processing by effector caspases. However, whether PKC δ cleavage and activation is a causal factor or merely a consequence of Apoptin-induced cell death requires further investigation.

PKC is a group of serine-threonine kinases that comprises at least 12 known isoforms sharing a common structure and mechanism of activation [41]. They were originally identified as the receptors for tumor-promoting phorbol esters in the early 1980s [42] and many PKC isoforms can contribute to tumorigenesis and invasive or metastatic phenotypes of cancers. However, each isoform exerts different cell type-dependent effects on cellular pathways controlling apoptosis or cell survival. For example, upregulation of PKC β 2 seems to promote early colon carcinogenesis through increased proliferation and invasion of epithelial cells as well as proliferation in general [43–45] while PKC δ is mediating cell death acting as a tumor suppressor in many cell types [46, 47]. In multiple myeloma upregulated PKC signalling, in particular of PKC β , has been implicated in pathogenesis and the PKC inhibitor Enzastaurin was shown to inhibit the survival and proliferation of MM cell lines [48]. Thus, in the study by Jiang et al. Apoptin senses a tumor-specific survival pathway and potentially redirects it towards induction of apoptosis by a yet unknown mechanism.

Recently, inactivation of the protein phosphatase PP2A through expression of the transforming SV40 small T antigen (SV40-ST) or RNA interference was shown to be sufficient to activate Apoptin in normal VH10 fibroblasts [49]. Thus, this study proposes the differential activation of phosphatases rather than kinases in regulating Apoptin's tumor selective phosphorylation and function. Inactivation or downregulation of PP2A has been linked to oncogenic transformation and it might represent a central regulator for Apoptin activation. However, further studies will be necessary to establish the role of PP2A, or dephosphorylation in general, for the regulation of Apoptin activity.

In summary, while it is clear that Apoptin phosphorylation on Thr-108 is important for its death-inducing ability, a single tumor-specific Apoptin kinase could not be identified so far. Most likely several cellular kinases are involved in phosphorylating Apoptin in cancer cells, depending on the tumor type. A common denominator seems to be the fact that Apoptin is able to turn established pro-survival pathways such as PI3K/Akt or PKC signalling, which are often upregulated in cancers, into a death-inducing signal. In addition, recent evidence also points to a potential role of protein phosphatases in regulating Apoptin phosphorylation and function.

2.5 Interacting Proteins

To exert its tumor-specific cytotoxicity Apoptin needs to become activated to be able to trigger cellular signalling pathways. Several cellular proteins have been identified to interact or co-localize with Apoptin and affect its cytotoxic function or

Table 2.1 Apoptin interaction partners

Protein	Biological function	References
Protein Kinase C β (PKC β)	Binding of Apoptin to PKC β results in PKC β -dependent phosphorylation and activation of Apoptin and nuclear translocation of PKC β	[40]
Fas-associated protein with death domain (FADD)	Overexpression causes co-localization of FADD and Apoptin in cytoplasmic death effector filaments, potentially interfering with death receptor signalling	[12]
Bcl-10	Apoptin and the NF- κ B regulator Bcl-10 co-localize in cytoplasmic filaments with a yet unknown consequence	[12]
Protein Kinase G (PKG-I)	High levels of PKG-I in normal cells correlate with lower Apoptin activation, however the precise role for PKG-I during Apoptin-induced cell death remains unclear	(unpublished data)
PI3-Kinase (PI3K) and Akt	Apoptin binds to the p85 subunit of PI3K, activating PI3K which results in sustained activation and nuclear translocation of Akt. This triggers activation of CDK2 which in turn phosphorylates Apoptin	[34, 35, 39]
Heat shock cognate protein 70 (Hsc70)	Binding of Apoptin triggers nuclear translocation of Hsc70 which might be required for Apoptin-induced Akt activation and downregulation of p65	[50, 51]
Promyelocytic leukemia protein (PML)	Apoptin is sumoylated and targeted to nuclear PML bodies. However, the interaction with PML is not required for the cytotoxic function of Apoptin	[21]
Anaphase promoting complex 1 (APC1)	Apoptin binds to APC1, disrupting the APC/C and resulting in G2/M arrest and apoptosis	[18, 52]
Hip-1 protein interactor (Hippi)	In normal cells but not in tumor cells Apoptin and Hippi interact in the cytoplasm	[53]
Death effector domain-associated factor (DEDAF)	Co-expression of Apoptin and DEDAF results in enhanced apoptosis compared to expression of either protein alone	[54]
Peptidyl-prolyl isomerase-like 3 (Ppil3)	Overexpression of Ppil3 can promote cytoplasmic localization of Apoptin	[55]
N-myc interacting protein (Nmi)	Nmi was identified as an Apoptin binding partner but no functional studies are available yet	[56]
Breast cancer associated gene 3 (Bca3)	Bca3 enhances Apoptin phosphorylation and cytotoxic function by a still unknown mechanism	[49]

cellular localization. Many of these interacting partners have been shown to either directly activate Apoptin or to be involved in initiating signalling pathways which lead to induction of apoptosis (Table 2.1). These include PKC, p73, anaphase promoting complex 1 (APC1) or PI3K and Akt. For others the importance of Apoptin binding still needs to be further investigated such as heat shock cognate protein 70 (Hsc70), Bcl-10 or N-Myc-interacting protein (Nmi).

2.5.1 Protein Kinase C (PKC)

Apoptin was shown to interact with the PKC β isozymes in vitro and in vivo [40]. PKC β 1 and PKC β 2 could be detected in complexes immunoprecipitated from MM1.R multiple myeloma cells expressing GFP-Apoptin using an anti-GFP antibody. Other cellular kinases including MERTK, Akt, PI3K/p85, PKC α and PKC δ were not found to interact with Apoptin in this model. The interaction of PKC β with Apoptin resulted in PKC β -dependent phosphorylation and activation of Apoptin as well as translocation of PKC β together with GFP-Apoptin to the nucleus. This suggests a novel mechanism in which Apoptin expression induces PKC activation consequently leading to Apoptin phosphorylation and induction of apoptosis, either through Apoptin or the activation of other apoptotic signalling pathways.

2.5.2 Bcl-10 and Fas-Associated Protein with Death Domain (FADD)

At early stages of expression in Saos-2 cells Apoptin localizes to cytoplasmic filaments resembling so-called death effector filaments (DEF). These DEFs are usually generated upon overexpression of proteins from the death domain superfamily that are involved in the extrinsic pathway of apoptosis. In Saos-2 cells overexpression of GFP-tagged Fas-associated protein with death domain (FADD) or Bcl-10 resulted in their co-localization with Apoptin in cytoplasmic filaments [12].

FADD functions as an adaptor molecule required for the formation of the death inducing signalling complex (DISC) in response to apoptotic signals activating death receptors on the cell surface such as Fas or tumor necrosis factor receptor 1 (TNFR1) [57]. The interaction of FADD with the receptor through its C-terminal death domain (DD) results in exposure of the N-terminal death effector domain (DED) and subsequently recruitment of pro-caspase-8 to the DISC to initiate the proteolytic caspase cascade [58]. Thus Apoptin might potentially be involved in modulating components of the extrinsic apoptotic pathway but the significance of this association with FADD remains unclear.

Bcl-10 contains an N-terminal caspase recruitment domain (CARD) and has been shown to trigger apoptosis as well as the activation of nuclear factor kappa b (NF- κ B). Together with other proteins it acts as an adapter protein during signalling from antigen receptors to the IKK complex and NF- κ B stimulation, resulting in lymphocyte activation [59]. While NF- κ B has been well established to promote cell survival and proliferation, recent studies also suggest a pro-apoptotic and tumor suppressor role for NF- κ B. Amongst others NF- κ B was shown to be involved in p53- and Fas-mediated apoptosis as well as cell death in response to certain viral infections [60–62]. Interestingly, Apoptin induces luciferase expression from an NF- κ B-responsive element in a reporter assay that could be blocked by overexpression of inhibitor of NF- κ B (I κ B α) [63]. This finding suggests the activation of a pro-apoptotic NF- κ B pathway in response to Apoptin expression.

Co-expression of Apoptin with Bcl-10 reduced the level of NF- κ B activation induced by Bcl-10 alone which might indicate differential activation of pro- or anti-apoptotic NF- κ B responses. However, the significance of the co-localization of Apoptin with Bcl-10 as well as the role of NF- κ B still requires further investigation.

2.5.3 Protein Kinase G (PKG-I)

Recent data from our laboratory suggest a protective effect of protein kinase G-I (PKG-I) expression in normal cells against Apoptin-induced cell death (Daryl Cole, PhD Thesis). Using a pair of colon cell lines it was demonstrated that the resistance of normal colon mucosa cells (NCM460) to Apoptin correlated with high levels of PKG-I as well as low expression of PKC β 1. In addition, in normal human 1BR3 fibroblasts the siRNA-mediated knockdown of PKG-I triggered nuclear translocation and activation of Apoptin (Fig. 2.4). Several studies have shown a loss of PKG-I expression during the process of tumorigenesis in tumor tissues compared to normal cells [64, 65]. Thus, reduced PKG-I levels in cancers might represent a tumor-specific alteration that affects the function of Apoptin. However, the precise effect of PKG-I on Apoptin-mediated signalling remains to be determined.

2.5.4 PI3-Kinase (PI3K) and Akt

Apoptin associated with the p85 subunit of PI3 Kinase (PI3K) in several tumor cell lines by binding through its proline-rich region to the SH3 domain of p85. This interaction resulted in the activation of PI3K which seems to be important for Apoptin's pro-apoptotic function [35]. Apoptin mutants lacking the proline-rich region failed to bind and activate p85 and displayed a reduced ability to induce apoptosis. In addition, siRNA-mediated downregulation of p85 caused an impaired nuclear accumulation and cytotoxic function of Apoptin. Apoptin was also shown to interact with the downstream PI3K target kinase Akt in PC3 prostate cancer cells. Binding of Apoptin to Akt (and PI3K) triggered sustained activation and nuclear translocation of Akt which was suggested to promote cell death instead of survival [34]. Neither Akt nor PI3K were able to directly phosphorylate and activate Apoptin. However, activated Akt has been described to phosphorylate several nuclear target proteins including cyclin-dependent kinase 2 (CDK2) which was shown to phosphorylate Apoptin and regulate its activity [39]. These results suggest an important role for sustained activation of the PI3K/Akt pathway through direct binding by Apoptin, which in turn promotes Apoptin-induced apoptosis in a CDK2-dependent manner.

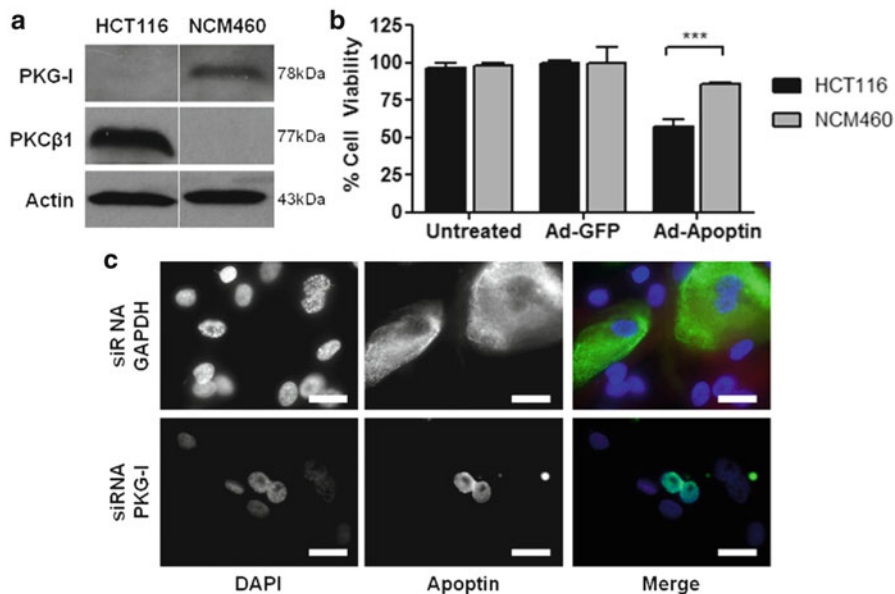


Fig. 2.4 Role of PKG-I in Apoptin-mediated cell death. (a) The expression levels of PKG-I and PKCβ1 respectively in HCT116 colorectal cancer cells and NCM460 normal colon mucosa cells were determined by Western blot analysis (blots were cut and combined at the indicated line). (b) NCM460 and HCT116 cells were left untreated or infected with Ad-Apoptin or Ad-GFP at an MOI of 10. After 48 h cell survival was analyzed by MTT assay. The results are shown as percentage of viable cells normalized to untreated control cells. Error bars indicate standard deviation from experiments performed in triplicate (***) $p < 0.0005$). (c) 1BR3 normal human fibroblasts were transfected with siRNA to knock down PKG-I or control siRNA, infected with Ad-Apoptin and fixed for immunofluorescence. Apoptin was visualized with FITC-conjugated antibodies and nuclei were counterstained with DAPI

2.5.5 Heat Shock Cognate Protein 70 (Hsc70)

Recently, Chen et al. proposed a role of heat shock cognate protein 70 (Hsc70) for Apoptin-induced cell death. Hsc70 is a constitutively expressed member of the heat shock protein 70 protein family and is involved in the regulation of multiple cellular processes. Chen et al. were able to show an interaction of both exogenous and endogenous Hsc70 protein with Apoptin in a cell line of spontaneously transformed chicken fibroblasts [51]. In the same cells Apoptin expression triggered translocation of Hsc70 from the cytoplasm to the nucleus and Hsc70 was required for Apoptin-induced downregulation of p65, a member of the NF-κB transcription factor family. However, whether this holds true for human cells and is a general molecular mechanism for Apoptin function remains to be established. In a concurrently published study they confirmed the interaction of Apoptin and Hsc70 as well as Hsc70 nuclear translocation in transformed human embryonic kidney (HEK293T) cells [50]. Moreover, downregulation of Hsc70 was shown to inhibit

Apoptin-induced phosphorylation and activation of Akt. These data suggest a potential role of Hsc70 in the activation of PI3K/Akt by Apoptin described above but the importance of such interactions requires further investigation.

2.5.6 Promyelocytic Leukemia Protein (PML)

In tumor cells Apoptin displays a distinct expression pattern forming subnuclear aggregates. These resemble so-called PML bodies, nuclear matrix domains organized by the promyelocytic leukemia protein (PML). Poon et al. demonstrated that Apoptin co-localization with PML bodies in Saos-2 cells is dependent on its N-terminal LRS (amino acids 33-46) with a yet unknown functional consequence [19]. PML bodies have been implicated in many cellular processes such as cell cycle regulation, apoptosis and anti-viral responses [66, 67]. In addition to PML, several other proteins including regulators of apoptosis are physiologically recruited to PML bodies. In many cases targeting to PML bodies is achieved by covalent attachment of small ubiquitin-like modifier (SUMO) to lysine residues known as sumoylation [68]. Indeed, Janssen et al. showed that Apoptin was sumoylated at several leucine or isoleucine residues within the LRS, interacted with PML protein and associated with PML bodies in U2OS osteosarcoma cells [21]. However, the pro-apoptotic activity of Apoptin was not affected by knockout of PML or mutation of the LRS which eliminates Apoptin sumoylation. While sumoylation and association with PML bodies do not seem to be required for Apoptin-induced apoptosis it was speculated that these modifications might be relevant for CAV replication.

2.5.7 Anaphase Promoting Complex 1 (APC1)

Teodoro et al. have identified the APC1 subunit of the anaphase-promoting complex/cyclosome (APC/C) as an Apoptin binding protein using mass spectrometry [52]. The APC/C functions as an E2 ubiquitin ligase and targets cell cycle proteins for proteasomal degradation to promote the transition from metaphase to anaphase [69]. In human cancer cells lacking functional p53 but not in normal cells Apoptin associated with APC1, disrupting the APC/C and inhibiting its function. Overexpression of Apoptin and its association with APC1 was shown to result in G2/M arrest and apoptosis similar to siRNA-mediated downregulation of APC1 in p53-deficient cells. The interaction of Apoptin with APC1 was mediated by a domain located within the C-terminus of Apoptin that partially overlaps with the bipartite NLS (amino acids 80-121). Moreover, Apoptin was shown to trigger the formation of PML bodies in H1299 non-small cell lung carcinoma cells and lead to the sequestration of APC/C components in these PML bodies [18]. In a recent study Apoptin was found to co-localize with MDC1, an APC-associated

mediator of DNA damage signalling, in PML bodies resulting in proteasome-dependent degradation of MDC1 [31]. Taken together these results provide evidence that APC/C could be a potential target for anti-cancer drug development in addition to suggesting a novel mechanism for Apoptin-induced cell cycle arrest and apoptosis.

2.5.8 *Hip-1 Protein Interactor (Hippi)*

Cheng et al. identified Hippi (Hip-1 protein interactor and apoptosis co-mediator) as an Apoptin binding partner in a yeast two-hybrid screen [53]. The complex of Hippi and huntingtin interacting protein 1 (Hip-1) can recruit and activate pro-caspase-8 and thereby induce apoptosis via the extrinsic pathway albeit independently of death receptors [70]. This might contribute to neuronal cell death in Huntington disease as expansion of the polyglutamine repeat on huntingtin was shown to reduce Hip-1 binding to huntingtin which liberates it for binding to Hippi and subsequent caspase-8 activation [71]. In confirmation of the initial screen, Apoptin and Hippi were shown to interact both in vitro and in vivo. The respective binding regions were mapped to the self-multimerization domain of Apoptin and the C-terminal half of Hippi which contains a pseudo-DED motif. In normal cells Apoptin and Hippi were shown to interact and co-localize in the cytoplasm whereas in cancer cells Apoptin translocated to the nucleus while Hippi remained in the cytoplasm. The binding of Apoptin to Hippi in normal cells might reduce the sensitivity of cells to caspase-8-mediated apoptosis. However, Apoptin-induced cell death seems to be independent of caspase-8 activation as cells deficient in caspase-8 remain sensitive to Apoptin [72]. Otherwise, binding of Hippi to Apoptin might sequester Apoptin in the cytoplasm and prevent its nuclear accumulation in normal cells, but overexpression of Hippi in cancer cells is not able to retain Apoptin in the cytoplasm. Thus, the significance of this interaction remains to be discovered.

2.5.9 *Death Effector Domain-Associated Factor (DEDAF)*

Death effector domain-associated factor (DEDAF) interacts with several DED-containing proteins such as FADD, pro-caspase-8 and pro-caspase-10 to promote apoptosis [54]. Like Apoptin, transient overexpression of DEDAF resulted in the induction of apoptosis in several tumor cell lines but not in normal fibroblasts [73]. Apoptin itself was shown to interact with DEDAF in tumor cells in the nucleus and overexpression of DEDAF increased the rate of cell death induced by Apoptin. Whether this is due to DEDAF-mediated transcriptional repression and whether DEDAF is required for Apoptin-induced apoptosis is still unclear.

2.5.10 *Peptidyl-Prolyl Isomerase-Like 3 (Ppil3)*

The binding of Apoptin to peptidyl-prolyl isomerase-like 3 (Ppil3) was shown to regulate Apoptin localization in normal and tumor cells [55]. Ppil3 belongs to the cyclophilin family which catalyzes the cis-trans isomerization of peptide bonds at proline residues and can regulate protein stability. Members of this family are involved in mitochondrial maintenance, apoptosis and cell cycle progression [74]. Ppil3 was initially identified as an Apoptin binding partner in a yeast two-hybrid screen and confirmed using an in vitro GST pull-down assay. Overexpression of Ppil3 resulted in increased retention of Apoptin in the cytoplasm. This was dependent on the proline-109 residue of Apoptin and binding of Ppil3 in this region was suggested modify the activity of the nearby NES and/or NLS to regulate Apoptin localization. However, the importance of Ppil3 for Apoptin pro-apoptotic function is currently unclear.

2.5.11 *N-Myc Interacting Protein (Nmi)*

Using a yeast two-hybrid system, N-Myc-interacting protein (Nmi) was identified as an Apoptin binding partner [56]. Nmi is an interferon-inducible protein and can enhance the activity of Myc proteins [75]. Transcription factors of the Myc protein family are key regulators of cell proliferation and differentiation and their upregulation can contribute to tumorigenesis [76]. This interaction might account for Apoptin's tumor-selective activity as Nmi expression levels in normal tissues are low while it is upregulated in transformed cells. However, so far no further functional studies on Nmi involvement during Apoptin-triggered cell death are available.

2.5.12 *Breast Cancer Associated Gene 3 (Bca3)*

In a recent study the breast cancer associated gene 3 protein (Bca3) was identified as an Apoptin binding partner in normal human fibroblasts [49]. Overexpression of Bca3 was shown to enhance Apoptin phosphorylation and increase apoptosis induction in Saos-2 cells. However, the significance of Apoptin interaction with Bca3 for Apoptin-induced cell death remains to be discovered. Bca3 (or AKIP1) interacts with several other cellular proteins including protein kinase A (PKA) and Tap73 to modify cellular signalling pathways [77–79]. The authors of the present study argue that Bca3 could negatively affect PKA-mediated activation of the phosphatase PP2A resulting in decreased Apoptin phosphorylation. Furthermore, Bca3 might also promote NF- κ B activation in a PKA-dependent or PKA-independent manner and enhance the transcriptional activity of Tap73 in response to Apoptin expression.

2.6 Mechanism of Apoptin-Induced Cell Death

2.6.1 *Apoptotic Pathways*

Apoptosis, or programmed cell death, is a physiological process that is essential during the development and the life of multicellular organisms. Inhibition of apoptosis allows for cell proliferation in an uncontrolled fashion, resulting in the persistence of potentially dangerous and unwanted cells. When undergoing apoptosis, cells display a characteristic morphology clearly distinct from that of healthy or necrotic cells [80]. To execute the apoptotic program, cells need to carry out a series of tightly regulated intracellular events. All apoptotic stimuli result in the activation of caspases, specific cysteine proteases that reside as inactive zymogens and require activation by proteolytic processing [81]. The initiator phase is marked by the recognition of an apoptotic stimulus and activation of initiator caspases which in turn activate effector caspases that specifically cleave cellular substrates leading to cell disintegration. The extrinsic pathway of apoptosis is triggered by the engagement of death receptors of the tumor necrosis family such as TNF-R1 or CD95/Fas by their corresponding ligands [82]. Ligand binding induces receptor trimerization and a conformational change exposing the receptor's death domain. This results in recruitment of adapter proteins such as FADD containing both a death domain and a death effector domain (DED). Pro-caspase-8 can associate with this DED to form the DISC which triggers its proteolytic activation [83]. Activated caspase-8 can then directly cleave effector caspases (type I pathway) or trigger the intrinsic apoptotic pathway through cleavage of the BH3-only protein Bid (type II pathway) [84].

The intrinsic or mitochondrial pathway is activated by various stress stimuli including DNA damage, growth factor deprivation or ER stress. A hallmark of the intrinsic pathway is the permeabilization of the mitochondrial outer membrane (MOMP) which leads to the release of numerous pro-apoptotic factors from the mitochondrial intermembrane space into the cytoplasm [85]. These include cytochrome c, which associates with apoptotic protease-activating factor (Apaf-1) and pro-caspase-9 to trigger formation of the apoptosome. In this complex caspase-9 is activated and can in turn cleave caspase-3 and other effector caspases [86]. The main regulators of the intrinsic pathway controlling MOMP belong to the Bcl-2 protein family [87]. Stress signals are sensed by BH3-only proteins, which accumulate to overcome the anti-apoptotic effect of pro-survival Bcl-2-like proteins. Consequently, the pro-apoptotic proteins Bax and Bak can be activated to oligomerize on the outer mitochondrial membrane leading to the formation of pores and MOMP.

2.6.2 Induction of Apoptotic Pathways by Apoptin

The precise mechanism of Apoptin-induced cell death is not clearly understood although several key events and molecular players have been identified and discussed. Expression of Apoptin ultimately results in the activation of effector caspases, in particular caspase-3, to execute the apoptotic program. Treatment with the pan caspase inhibitor zVAD-fmk or co-expression of the viral caspase inhibitor p35 reduced the sensitivity of cancer cells to Apoptin in two separate studies, indicating that caspase activation is required for Apoptin-induced cell death [88, 89]. However, Danen-van Oorschot et al. argue that activation of caspase-3 occurs only in Apoptin-expressing cells at late apoptotic stages and might thus represent a consequence of apoptosis induction rather than a cause [89]. Furthermore, other effector caspases such as caspase-6 or caspase-7 are able to compensate for caspase-3 deficiency in MCF-7 cells during Apoptin-induced apoptosis.

To investigate whether Apoptin triggers apoptosis through the extrinsic pathway Maddika et al. tested the sensitivity of Jurkat cells deficient in key regulators of the death receptor pathway including caspase-8 and FADD to treatment with Apoptin fused to the HIV-TAT protein transduction domain (TAT-Apoptin) [72]. A previous study had shown that Apoptin co-localizes with Bcl-10 and FADD in cytoplasmic filaments in Saos-2 cells suggesting a role for the extrinsic pathway in Apoptin-induced cell death [12]. However, Jurkat cells lacking functional caspase-8 or FADD remained sensitive to Apoptin and they could not detect activation of caspase-8, indicating that Apoptin probably mediates cell death independently of death receptor signalling. Nevertheless, since several Apoptin binding partners are known to be involved in death receptor signalling the possibility that in other cellular systems Apoptin induces activation of the extrinsic pathway should not be excluded.

In the same study by Maddika et al. treatment with TAT-Apoptin caused a loss of the mitochondrial membrane potential and release of cytochrome c, both representing key events in the intrinsic pathway [72]. Knockdown of the adapter protein Apaf-1 blocked Apoptin-induced cell death in transformed fibroblasts [88]. Taken together these data indicate that Apoptin-triggered apoptosis is executed via the classical mitochondrial pathway and might thus be regulated by members of the Bcl-2 protein family. However, the role of the anti-apoptotic protein Bcl-2 in Apoptin-induced apoptosis remains controversial. Several studies suggest that Apoptin triggers cell death independently of the Bcl-2 status and that overexpression of Bcl-2 rather sensitizes cells to induction of cell death by Apoptin [90, 91]. Another study demonstrated that overexpression of the pro-survival protein Bcl-x_L did not impair Apoptin-induced cell apoptosis in a head and neck squamous cell carcinoma cell line [92]. By contrast, Burek et al. showed that co-expression of either Bcl-2 or Bcl-x_L with Apoptin protected MCF-7 cells from cell death while the pro-apoptotic family member Bax promoted Apoptin-induced apoptosis [88]. These opposing effects of Bcl-2 on cell death triggered by Apoptin seem to be cell type-specific and might be due to the differential activity of other cellular signalling factors that control Bcl-2 function. One of these potential factors

is Nur77, an orphan receptor that has a regulatory role in both cell growth and apoptosis. Upon various apoptotic stimuli phosphorylated Nur77 translocates from the nucleus to the mitochondria where it activates the intrinsic apoptotic pathway either directly or indirectly, e.g. by changing the conformation of Bcl-2 to promote apoptosis instead of suppressing it [93–95]. Indeed, treatment of MCF-7 cells with TAT-Apoptin triggered Nur77 shuttling to the mitochondria [72]. In addition, inhibition of Nur77 expression protected cells against Apoptin-induced killing. While this indicates a potential role for Nur77 in the cell death pathway induced by Apoptin, the actual mechanism of Nur77 in this apoptotic program so far remains unclear.

2.6.3 Role of the Tumor Suppressors p53 and p73

Another protein that is known to transmit apoptotic signals from the nucleus to the mitochondria is the tumor suppressor p53. Apart from inducing the transcription of pro-apoptotic target genes like p53-upregulated modulator of apoptosis (PUMA) or Noxa, p53 can also translocate to the mitochondria upon stress stimuli and exert a direct pro-apoptotic function there by interacting with members of the Bcl-2 protein family [96]. However, Apoptin is able to induce apoptosis even in cells that lack functional p53 such as Saos-2 cells [14]. This p53-independent function of Apoptin is an important feature for its potential as a future anti-cancer therapeutic agent because many tumors contain mutated, non-functional p53.

Another p53 family member, p73, shares significant homology with p53, particularly in the DNA-binding domain. It is expressed as multiple isoforms that result from alternative splicing in its C-terminus as well as the use of two distinct p73 promoters which generates either transactivation-competent (TA) or N-terminally truncated (Δ N) forms. While TAp73 isoforms are able to induce the transcription of genes involved in apoptosis and cell cycle arrest, Δ Np73 isoforms function as dominant negative inhibitors of TAp73 or p53 activity through a negative feedback loop [97, 98]. In response to genotoxic stress TAp73 isoforms become stabilized and activated while Δ Np73 levels decrease, changing the equilibrium between pro-apoptotic and anti-apoptotic isoforms to induce apoptosis. This process is controlled by a network of post-translational modifications of p73 such as phosphorylation, acetylation and ubiquitination [99].

Two recent studies provide evidence for an important role of specific p73 isoforms during the regulation of p53-independent Apoptin-induced cell death. Apoptin expression in p53-deficient H357 head and neck squamous cell carcinoma cells resulted in increased protein levels of TAp73 α and reduction in the expression of Δ Np73 α , probably through selective protein stabilization [100]. Furthermore, exogenous expression of TAp73, in particular TAp73 β , but not Δ Np73 further enhanced the sensitivity of Saos-2 cells to Apoptin-induced cell death (Fig. 2.5).

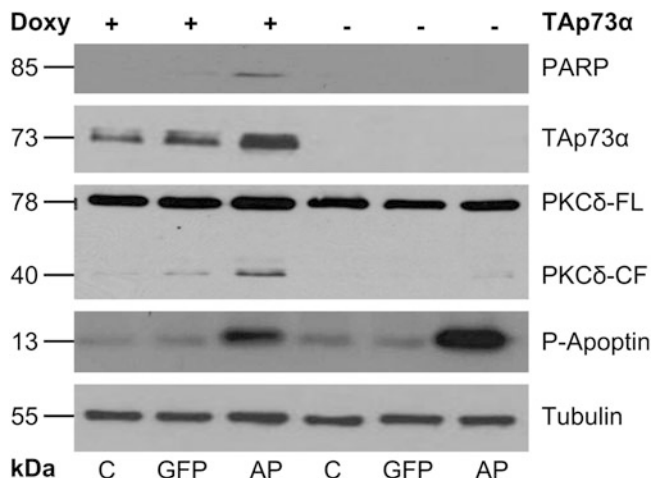


Fig. 2.5 Importance of p73 isoforms for Apoptin-induced cell death. TAp73α Saos-2 inducible cells were infected with either Ad-GFP (GFP) or Ad-Apoptin (AP) at an MOI of 10 or left untreated in the presence or absence of doxycycline to induce expression of TAp73α. Cells were collected after 24 h post-infection and subjected to Western blot analysis with the indicated antibodies

Apoptin-mediated activation of TAp73 isoforms also led to increased expression of the pro-apoptotic BH3-only protein PUMA resulting in the activation of the intrinsic pathway of apoptosis in response to Apoptin.

A recent study in our laboratory further highlighted the importance of p73 and PUMA for Apoptin-induced cell death. While knockout of p53 did not affect the sensitivity of HCT116 cells to Apoptin, siRNA-mediated downregulation of p73 or inhibition of PUMA significantly decreased the levels of apoptosis induction [101]. Further investigations revealed that the initial Apoptin-induced stabilization of TAp73α resulted in p73-mediated induction of p73-induced RING2 protein2 (PIR2). PIR2 had previously been identified as a transcriptional target of p73 and functions as an ubiquitin ligase to mediate the preferential polyubiquitination and degradation of ΔNp73 isoforms [102]. Expression of Apoptin led to the PIR2-mediated proteasomal degradation of ΔNp73 isoforms, relieving the inhibitory effect of ΔNp73 on TAp73 and thus facilitating the induction of apoptosis. These data suggest a novel mechanism of Apoptin-induced changes in the equilibrium between pro-apoptotic and anti-apoptotic p73 isoforms to induce cell death in cancer cells. However, the signalling pathway linking Apoptin expression to the initial activation of TAp73 has not yet been identified. Stabilization of TAp73 might involve phosphorylation by kinases such as PKCδ and PKCβ or components of the DDR, which have already been implicated in Apoptin signalling (Fig. 2.6).

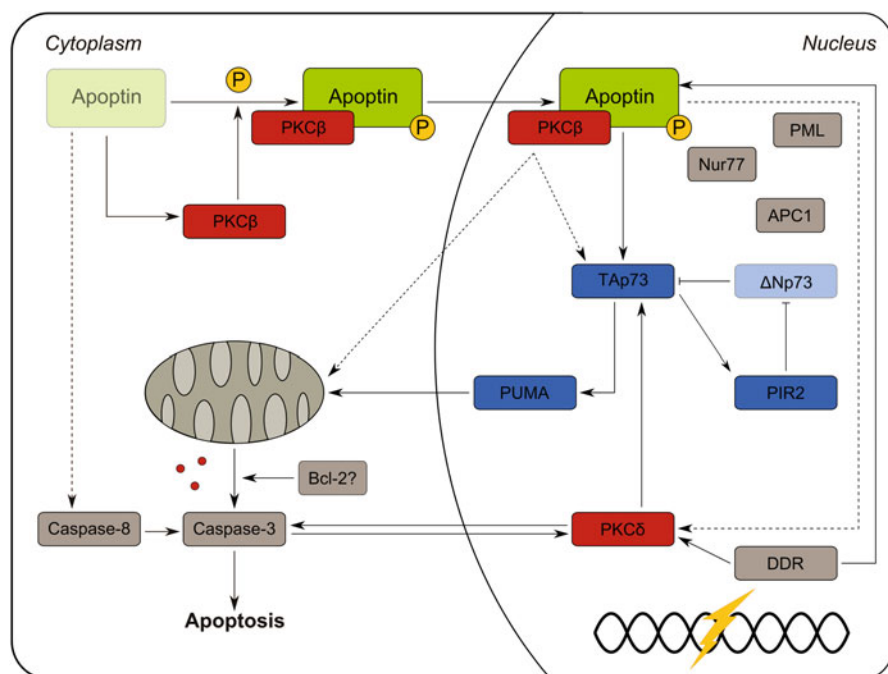


Fig. 2.6 Proposed model of Apoptin action in tumor cells. In tumor cells Apoptin expression induces activation of protein kinase C β (PKC β) which in turn binds to and phosphorylates Apoptin. Both proteins translocate to the nucleus where Apoptin triggers activation of pro-apoptotic TAp73 isoforms. This results in transactivation of the ubiquitin ligase PIR2 which selectively promotes the degradation of Δ Np73 isoforms to relieve their inhibitory effect on TAp73. Stabilized TAp73 can then induce the expression of pro-apoptotic proteins such as PUMA that transmits the apoptotic stimulus to the mitochondria to trigger cytochrome c release and subsequent caspase activation and cell death. A constitutively activated DNA damage response (DDR) in cancer cells supports this model by activation of PKC δ , which might also be activated by Apoptin itself, and promoting nuclear translocation of Apoptin. Whether Apoptin also induces apoptosis through a caspase-8-dependent pathway and whether PKC β can directly activate TAp73 or other pro-apoptotic pathways is currently under investigation. In addition, several other proteins including Nur77, PML and APC1 have been implicated in Apoptin-induced cell death

2.6.4 Induction of G2/M Arrest

Cell cycle arrest at the G2/M checkpoint allows for the repair of damaged DNA prior to the initiation of mitosis and may eventually lead to apoptosis of arrested cells. Teodoro et al. reported that Apoptin expression in p53-deficient cells resulted in G2/M arrest and apoptosis through association with the APC/C and inhibition of its function [52]. In addition, Apoptin was shown to induce accumulation of Saos-2 cells in G2/M phase, linking it to the activation of pro-apoptotic p73 isoforms [101]. These data provide a novel mechanism of Apoptin-induced cell

death due to G2/M arrest that might be induced by interference of Apoptin with DDR signalling, the APC/C or other signalling components still to be identified.

2.6.5 Mitotic Catastrophe

Mitotic catastrophe is considered to be a protective mechanism against genetic or chromosomal instability and thus suppresses tumor progression [103]. A recent study by Lanz et al. suggests that Apoptin triggers mitotic catastrophe and subsequently cell death by sensing inaccuracies in the spindle assembly checkpoint [104]. A majority of Saos-2 cells expressing Apoptin displayed abnormal spindle formation and slower progression through mitotic phases compared to control cells. This eventually resulted in cell death either during mitosis or the following interphase that showed characteristics of apoptosis such as membrane blebbing and DNA fragmentation. The fact that Apoptin interacts with and inactivates the APC/C as discussed above might provide a link from Apoptin to the final induction of cell death through mitotic catastrophe. However, in this study the apoptosis-inducing activity of Apoptin was not confined to cycling cells and mitotic catastrophe might thus represent only one aspect of Apoptin-induced cell death.

2.6.6 Ceramide Signalling

As a further potential mechanism for Apoptin-mediated apoptosis Liu et al. have proposed that Apoptin signalling involves modulation of the sphingolipid-ceramide metabolism [105, 106]. Expression of GFP-Apoptin in a prostate cancer cell line resulted in elevated ceramide levels with a concurrent decrease in sphingomyelin levels. Apoptin-treated cells displayed increased acid sphingomyelinase (ASMase) and decreased acid ceramidase (ACDase) activities respectively compared to control cells, both of which result in accumulation of ceramide. Downregulation of ceramide by inhibition of ASMase attenuated Apoptin-induced apoptosis while co-treatment with a ceramide analogue increased the induction of cell death. Ceramide is upregulated in response to several cellular stress signals such as tumor necrosis factor α (TNF α) stimulation or irradiation and functions as a second messenger activating pro-apoptotic pathways. To promote their survival cancer cells often upregulate enzymes that metabolize ceramide such as ACDase. A study on primary prostate cancer tissues revealed increased ACDase levels in a majority of samples compared to normal tissues, making it a potential therapeutic target. Indeed, combination treatment of tumor cells with Apoptin and an ACDase inhibitor induced enhanced cell death both in vitro and in vivo.

2.6.7 Summary

In summary, a number of known pro-apoptotic pathways and proteins have been implicated in Apoptin-induced cell death. The specific mechanism of cell killing by Apoptin might depend on the tumor type, the cellular background regarding oncogenic genetic alterations and the mode of Apoptin delivery to the cell. While it is established that Apoptin triggers caspase-dependent apoptosis of tumor cells, the precise mechanism resulting in caspase activation remains unclear and the possibility of caspase-independent cell death induction cannot be excluded.

2.7 Conclusive Remarks

A major goal of cancer treatment is the eradication of malignant cells while minimizing damage to normal cells. A number of proteins such as TRAIL and HAMLET have been reported to selectively kill tumor cells by sensing essential molecular and cellular changes during carcinogenic transformation and redirecting these pathways towards cell death. Since its identification in the early 1990s as the apoptosis-inducing component of CAV, Apoptin has become of interest due to its ability to induce p53-independent cell death specifically in transformed cells. Apoptin is activated by a cancer-associated pathway and has been linked with several cellular signalling pathways leading to apoptosis although the precise mechanism of Apoptin-induced cell death remains unclear. Further investigation of the pathways contributing to the cytotoxic function and tumor-selective mode of Apoptin will help to identify novel targets for the development of anti-cancer therapies.

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