

Mechanisms of Apoptin-induced cell death

Suna Zhou · Mingxin Zhang · Jia Zhang ·
Hui Shen · Ermek Tangsakar · Jiansheng Wang

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Abstract Apoptin, a 13.6-kD protein encoded by chicken anemia virus, is paid more and more attention, since it selectively induces apoptosis in tumor cells while abolishes cytotoxic effect in normal cells. In addition, Apoptin shows different localization in tumor cells and normal cells: it predominantly accumulates in nucleus of tumor cells, whereas in normal cells, it is detected mainly in cytoplasm. There are various mechanisms implicated in the program of Apoptin-mediated cell death. Up to now, the interpretations have been recognized including that the particular domains control nucleocytoplasmic shuttling of Apoptin, phosphorylation on specific residue and varies relevant signaling contribute to Apoptin's activity, and the partners interacted with Apoptin regulate activity or subcellular localization of Apoptin. In this review, we make a comprehensive survey of the existing evidence about mechanisms of Apoptin's action, which might provide scientific basis to make progress in novel targeted tumor therapy.

Keywords Apoptin · Selectively · Apoptosis · Mechanisms

Introduction

Chicken anemia virus (CAV) is a small virus that causes cytopathogenic effects in young chicken thymocytes via CAV-induced apoptosis. As three overlapping open

reading frames are comprised, proteins VP1 (51.6 kDa), VP2 (24.0 kDa), and VP3 (13.6 kDa) are encoded in the genome [1]. Importantly, expression of VP3 alone is sufficient to induce apoptosis in a variety of cells, which is also renamed as Apoptin [1–3]. It is interesting to note that Apoptin selectively induces apoptosis in diverse cancer cells including hepatoma, osteosarcoma, melanoma, cholangiocarcinoma, colon carcinoma, lung cancer, breast cancer, prostate cancer, cervix cancer, gastric cancer, and so on [4–13], but has no effect on cell death of normal cells. To date, the mechanisms of Apoptin-induced tumor-specific cell apoptosis are not clearly understood. Based on the existing evidence reported in studies, the mechanisms of Apoptin will be summarized in detail in this report.

Nucleocytoplasmic shuttling of Apoptin in tumor and normal cells

Tumor cells are sensitive to Apoptin-induced apoptosis, whereas the corresponding normal cells are resistant. Almost all the research subscribe to an interpretation that the tumor-selective cytotoxicity of Apoptin refers to that, in tumor cells, Apoptin shows predominantly nuclear localization, but in normal cells, it is detected mainly in cytoplasm. A possible cause for Apoptin's distinctive subcellular localization is two principal sequences, including nuclear localization signals (NLSs) and nuclear export signals (NESs).

Apoptin is a 121-amino acid protein, which shows no homology to any known cellular proteins [14]. The C-terminus of Apoptin contains a bipartite-type nuclear localization signal: NLS1 located at amino acids (aa) 82–88, and NLS2 located at aa 111–121 [15, 16]. Moreover, the residues aa 33–46 in Apoptin's sequence is

S. Zhou · M. Zhang · J. Zhang · H. Shen · E. Tangsakar ·
J. Wang (✉)

Department of Thoracic Oncosurgery, First Affiliated Hospital
of Medical School, Xi'an Jiaotong University, Yanta West Road
No. 277, Shaanxi 710061, China
e-mail: wangjshxjtu@gmail.com

known as a putative NES [17], as shown in Fig. 1. The C-terminus of Apoptin (residues aa 74–121), out of the context of the full-length protein, represents a unique tumor cell-enhanced nuclear targeting module [16]. Danen-Van et al. used a mutagenesis strategy to explore the role of nuclear localization for Apoptin-induced cell death in tumor cells, and they found a strong correlation of nuclear localization with cell-killing activity [15]. It is worthwhile to note that they employed a construct (NLS-Apoptin) to enforce nuclear localization of Apoptin in normal human skin fibroblasts (VH10), but NLS-Apoptin was unable to induce apoptosis in VH10 cells. It implies that nuclear localization is not sufficient for Apoptin's killing activity, and other events must be involved. Surprisingly, Wadia et al. [18] regulated the expression levels of GFP-Apoptin^{70–121} in either transformed 3T3ras fibroblasts or primary human foreskin fibroblasts and found an amazing switch from a predominant nuclear localization at high levels to distribution throughout the cell under low levels in both cells. Therefore, they drew the conclusion that NLS of Apoptin is not tumorigenic selective, but rather concentration dependent.

In addition, sequence analysis shows that Apoptin contains a putative CRM1-mediated NES (aa 33–46). Further investigations found that the NES is not a real NES, but in fact a cytoplasm retention signal, which is responsible for cytoplasm retention of Apoptin in normal cells, by using a specific inhibitor of the CRM1-mediated export process [17]. Then, another study uses quantitative approaches and isogenic tumor/nontumor cell pairs to conduct a comprehensive research on the most attractive domains on Apoptin. They proved that NES (aa 33–46) contributes to nuclear accumulation rather than to cytoplasmic retention or nuclear export and renamed it as the leucine-rich sequence (LRS). The actually responsible NES lies within aa 97–105 and is recognized by CRM1 [19]. Nucleocytoplasmic transport of Apoptin in tumor and normal cells is determined by a set of unique targeting signals, which comprise a bipartite-type tumor cell-specific nuclear targeting signal (NLS1 and NLS2), a CRM1-recognized NES by regulating the activity of the threonine 108

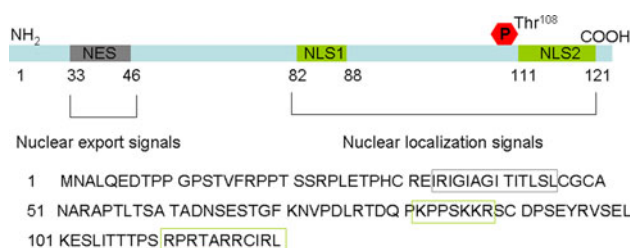


Fig. 1 The sequence of Apoptin: NES located at amino acids 33–46 is underlined or marked in *green*. Two NLSs at amino acids 82–88 (NLS1) and 111–121 (NLS2) are also *underlined* or marked in *blue*

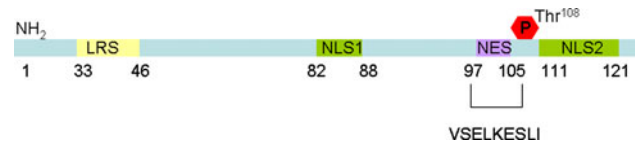


Fig. 2 The functional domains of Apoptin: LRS located on amino acids is marked in *white*, NLS1 at 82–88 and NLS2 at 111–121 are marked in *blue*, NES at 97–105 is marked in *red*, Thr-108 adjacent to NES is marked in *yellow*

phosphorylation site (Thr-108), and an LRS by functioning as a nuclear retention sequence (as shown in Fig. 2).

Another interesting finding was proposed by Heckl [20]. These experiments used fluorescein isothiocyanate (FITC) and dansyl-labeled conjugates, which comprise the truncated C-terminal part of Apoptin (aa 81–121) with either phosphorylated or nonphosphorylated Thr-108, to study the subcellular localization of Apoptin in the tumor/normal cell pairs from the brain, prostate, and bladder. Surprisingly, all the conjugates show nuclear accumulation and cause cell death in both tumor and nontumor cells. These results contrast to a previous report, which described that enforcing nuclear uptake of Apoptin did not induce apoptosis in healthy cells (VH10) [15]. The debate may be due to differences in the composition of the cell membrane, which exists between the tumor and nontumor cell type pairs in diverse researches, and also may refer to some other unknown cell line-specific factors. And more importantly, the NLS2 is principal to nucleocytoplasmic shuttling of the FITC-labeled Apoptin^{81–121} peptide. It is confirmed by altering five arginines to serines in NLS2, which almost completely abolished the cellular and nuclear uptake of Apoptin^{81–121}.

The threonine 108 phosphorylation site (Thr-108)

As we know, the above-mentioned C- and N-terminal apoptosis domains are not sufficiently responsible for the tumor-specific killing effects of Apoptin. Therefore, a further tumor-selective activation process is required. Early in 2002 [21], a speculation, that phosphorylation on residue Thr-108 appears to assist tumor-specific nuclear accumulation of Apoptin, has been proposed. In the report, by the results that the phosphorylation of Apoptin on Thr-108 was detected specifically in different original tumor and transformed cells, it has been proved that the activation of Thr-108 is relevant to a transformed or tumorigenic environment. To further assess a link between Thr-108 and Apoptin function, they constructed a phospho-mimic Apoptin mutant (mutating Thr-108 to a glutamic acid as T108E) in human diploid VH10 fibroblasts. The result showed that T108E not only translocates to the nucleus but also induces apoptosis in normal cells. Hence, it is

presumed that modification on Thr-108 may act as a bifunctional switch, which includes the transportation to nucleus and the induction of apoptosis. In further in-depth studies, Lee et al. [22] and Heilman et al. [23] obtained similar results that Apoptin with Thr108 phosphorylation is not required for its tumor-specific nuclear accumulation, in contrast to the work by Poon et al. [19]. Through replacing both Thr-108 and Thr-107 with alanines to abolish the Thr108 phosphorylation site of HA-Apoptin, they examined the subcellular localization of this mutant Apoptin in HeLa cells and corresponding normal cells by immunofluorescent microscopy. It was found that the special mutant did not affect its different subcellular localization in normal and tumor cells. Moreover, abolishing the Thr-108 phosphorylation of Apoptin affected its apoptotic activity partially. One possible explanation for such consequences is that Apoptin Thr-108 phosphorylation may only be required for the apoptotic activity mediated through the C-terminal apoptosis domain. However, Apoptin exerts its apoptotic effect through both N- and C-terminal apoptosis regions.

Apoptin-mediated relevant signaling

Apoptin induce apoptosis in a P53-independent way

In spite of that mostly chemotherapy and radiotherapy have been verified to induce tumor cell apoptosis in a p53-dependent manner [24, 25], Apoptin can induce apoptosis in human tumor cells without the involvement of p53 [26]. In contrast to the tumor suppressor p53, Bcl-2, belonging to a family of Bcl-2-like proteins, acts as an apoptosis inhibitor [27]. However, the activity of Apoptin-induced apoptosis is even enhanced by overexpressing Bcl-2 [28]. A potential interpretation may be that Apoptin can activate caspases to cleave Bcl-2 into a proapoptotic form of Bcl-2 [29]. Similarly, Bcl-xL, another antiapoptotic protein, cannot inhibit Apoptin-induced apoptosis in head and neck squamous cell carcinoma (HNSCC) [30]. It is concluded that the mechanism of Apoptin is independently associated with p53 and Bcl-xL and also enhanced by Bcl-2.

Apoptin-induced apoptosis relevant to caspases

Caspase-mediated signaling pathway plays an important role in the process of apoptosis. In one study, Apoptin is confirmed to not activate the upstream caspases 1 and 8 in osteosarcoma cells by using several specific inhibitors. More importantly, utilizing immunofluorescence, they found that activation of the downstream caspase 3 was essential for rapid Apoptin-induced apoptosis [31]. Research about the action of Apoptin in cholangiocarcinoma has come up with

similar results. When the upstream caspases were blocked by a broad-spectrum inhibitor p35, the cell death caused by Apoptin was delayed rather than completely abolished [32]. In radioresistant SQD9 human head and neck squamous carcinoma cell lines, Apoptin induced apoptosis accompanied by the release of mitochondrial cytochrome c and subsequent activation of caspase 3 [33].

The phosphatidylinositol 3-kinase (PI3-K)/Akt pathway

The phosphatidylinositol 3-kinase (PI3-K)/Akt pathway is well known as the regulator of cell survival, proliferation, growth, transcription and translation [34]. On the contrary, Maddika et al. [35] applied mass spectrometry to analyze the proteins specifically bound to Apoptin and identified p85 (the regulatory subunit of PI3-K) and Akt (a kinase downstream of PI3-K). Further investigations verify that Apoptin induce cell death in tumor cells through the constitutive activation of PI3-K and Akt. Moreover, when Akt translocated to the nucleus together with Apoptin during Apoptin-induced cell death, nuclear-Akt acted as an apoptosis stimulator rather than a repressor. It is presumed that the PI3-K/Akt pathway acts as a pro-cell death pathway in Apoptin-induced cell death. Later, Maddika et al. make a further research on the mechanism of Apoptin's anticancer toxicity. The most important is that they identified cyclin-dependent kinase-2(CDK2), which is modulated by Akt through p21^{Cip1/Waf1} [36], is crucially required for Apoptin-induced cell death by phosphorylating Apoptin. They found cells with CDK2-knockdown were markedly insensitive to Apoptin. Thus, the activation of CDK2 triggered by Akt may interpret the mechanism of Apoptin-selective toxicity in tumor cells [37].

The sphingomyelin–ceramide pathway

Liu et al. have proposed that Apoptin-mediated cell death involves the modulation of the sphingomyelin–ceramide pathway lately [38]. They detected that the accumulation of tumor suppressor ceramide was upregulated by Apoptin expression in prostate cancer cells DU145, PC-3, and LNCaP. The tumor cells showed decreased sensitivity to Apoptin-induced apoptosis, which was ascribed to the downregulation of ceramide by inhibition of acid sphingomyelinase (ASMase) or by upregulation of acid ceramidase (ACDase, which deacylates ceramide to sphingosine) [38, 39]. Ceramide is known to induce cell death by either apoptosis or necrosis in tumors and either senescence or cell cycle arrest in normal cells [40]. In addition, numerous studies have indicated that ceramide serves as a second messenger for the induction of stress-activated protein kinases to maintain a balance between cell survival and death [41, 42]. Liu et al. treated cells with Ad-GFP-

Apoptin and then found that Apoptin increased ceramide levels by the activation of ASMase with a concomitant decreased sphingomyelin, which can hydrolyze into ceramide [38]. In short, activation of ASMase plays a role in Apoptin-induced cell death by generating ceramide via sphingomyelin hydrolysis. They have also reported that 10 of 15 primary prostate cancer specimens show upregulated ACDase activity compared to normal adjacent tissues. Furthermore, cotreatment with the ACDase inhibitor LCL20 significantly enhanced Apoptin-induced tumor cell-killing in vitro and vivo [39].

The mitochondrial/intrinsic pathway

There is an added research which demonstrated that Apoptin triggers apoptosis by activating the mitochondrial/intrinsic pathway, and it acted independently of the death receptor/extrinsic pathway. Furthermore, the orphan steroid receptor Nur77 transmits the Apoptin-induced death signal from the nucleus to the mitochondria in tumor cells, and using specific siRNA to inhibit Nur77 activity in MCF7 cells shows strong protection against Apoptin-induced cell death [43]. As reviewed by Marsden and Strasser, the mitochondrial/intrinsic death pathway is modulated by pro- and anti-apoptotic Bcl-2 family members [44]. Nevertheless, experimental data from Maddika et al. clearly underline the protective role of both Bcl-2 and Bcl-X_L against Apoptin-triggered apoptosis. Apoptin causes the loss of mitochondrial membrane potential, resulting in the release of cytochrome c and AIF via migration of Nur77 from the nucleus to the cytoplasm [43].

The partners interacting with Apoptin during Apoptin-induced apoptosis

Leliveld et al. showed that recombinant MBP-Apoptin protein was a globular multimer when inducing apoptosis. It is possible that the multimerization of Apoptin is essential for Apoptin's original function. There are several possible explanations such as aggregation may stabilize Apoptin, or the multimerization may facilitate certain partners (DNA, RNA, chromatin, nuclear pores, etc.) to bind Apoptin moieties [45]. In a later study, the same author reported that both Apoptin's N- and C-terminal halves separately bound DNA, albeit with reduced affinity compared to full-length Apoptin. Since both N- and C-terminal halves can also independently induce apoptosis, Apoptin's capacity to bind DNA is considered to be associated with its biological activity [46].

In another report [47], Apoptin interacted with death effector domain-associated factor (DEDAF), which in association with death effector domain (DED)-containing

pro-apoptotic proteins involved in the regulation of transcription. Interestingly, DEDAF and Apoptin are partially (about 75%) colocalized in the nucleus of tumor cells; however, both are detected predominantly in the nucleus of apoptotic cells. In normal cells, DEDAF exhibited a predominantly nuclear localization, while Apoptin was detected preferentially in the cytoplasm. Moreover, overexpression of DEDAF induced apoptosis in tumor cell lines, but not in normal fibroblasts or mesenchymal stem cells, which is similar to the action of Apoptin.

It is well documented [48] that a major Apoptin-associated polypeptide was APC1, a subunit of the anaphase-promoting complex/cyclosome (APC/C), and it is responsible for G2/M arrest and cell death induced by Apoptin in transformed cells lacking p53. Based on the data of gel-filtration analysis, Teodoro et al. suggested that Apoptin binds to APC1, resulting in the disruption of the APC/C complex and loss of its activity. Interestingly, APC1 only interacts with the C-terminal domain of Apoptin.

Through a yeast two-hybrid screen, Huo et al. [49] identified human peptidyl-prolyl isomerase-like 3 (Ppil3) as one of the Apoptin-associated proteins, which can retain Apoptin in cytoplasm even in tumor cells via a direct protein-protein interaction. Surprisingly, the expression level of intrinsic Ppil3 contributed to the nuclear-cytoplasmic distribution of Apoptin. It is proved by the result that the upregulation of Ppil3 held Apoptin in the cytoplasm of hepatocellular carcinoma (HCC) cell lines, and knockdown of Ppil3 could resume the nuclear localization of Apoptin. Another interesting finding is that the Apoptin P109A mutant could evidently impair the function of Ppil3 and hence infer that P109 of Apoptin is required for its Ppil3-dependent cytoplasmic retention.

p85, the regulatory subunit of phosphoinositide 3-kinase (PI3-K), was identified as another Apoptin-associated protein [35, 50]. Apoptin interacts with the SH3 domain of p85 in various tumor cells independent of cell type or species, through the PKPPSK sequence (aa 81–86) of Apoptin. This specific interaction may change the conformation of p85 to sequentially active PI3-K. Importantly, downregulation of the p85 expression by siRNA or inhibitors of PI3-K severely impairs both the cytotoxic activity and nuclear localization of Apoptin [50].

Luo et al. found that Apoptin interacted with heat shock cognate protein 70 (Hsc70) via its 30- to 60-amino acid region using yeast two-hybrid and immunoprecipitation approaches [51]. Hsc70, also called Hsp73 or Hsp70-8, as a specific molecular chaperone, is a member of the heat shock protein 70 (Hsp70) family [52]. Apoptin interaction with Hsc70 induces the translocation of Hsc70 from cytoplasm to nucleus in transformed HEK293T cells. In addition, Akt phosphorylation induced by Apoptin is markedly inhibited by Hsc70 knockdown. In general,

Table 1 Molecules that interact with Apoptin

Molecule	Influence by the interaction	References
N-Myc interaction protein (Nmi)	Unclear	[53]
Hip-interacting protein (Hippi)	Apoptin binds to the C-terminal half of Hippi and subsequently colocalizes with Hippi in the cytoplasm of normal cells, whereas in corresponding cancer cells, most Apoptin and Hippi were located in the nucleus and cytoplasm separately	[54]
Fas-associated death domain protein (FADD)	In tumor cells, Apoptin initially shows filamentous and partial cytoplasmic colocalization with FADD	[55]
Bcl10	Apoptin localizes to cytoplasmic filaments that colocalize with Bcl10	[55]
Promyelocytic leukemia protein (PML)	Apoptin colocalized with PML bodies, which was implicated in the execution of p53-independent apoptotic programs, in the nucleus of transformed cells	[56, 57]
Importin β 1	Apoptin is transported into the nucleus most likely through the action of the importin β 1 protein, which is a soluble cellular transport receptor	[16, 19]
Protein kinase C (PKC)	Apoptin-mediated cell death proceeded through the upregulation of PKC β and cleavage of the PKC δ catalytic domain	[58]
p73	The transactivation competent p73 and its target PUMA as important, p53-independent mediators of Apoptin-induced apoptosis	[58]

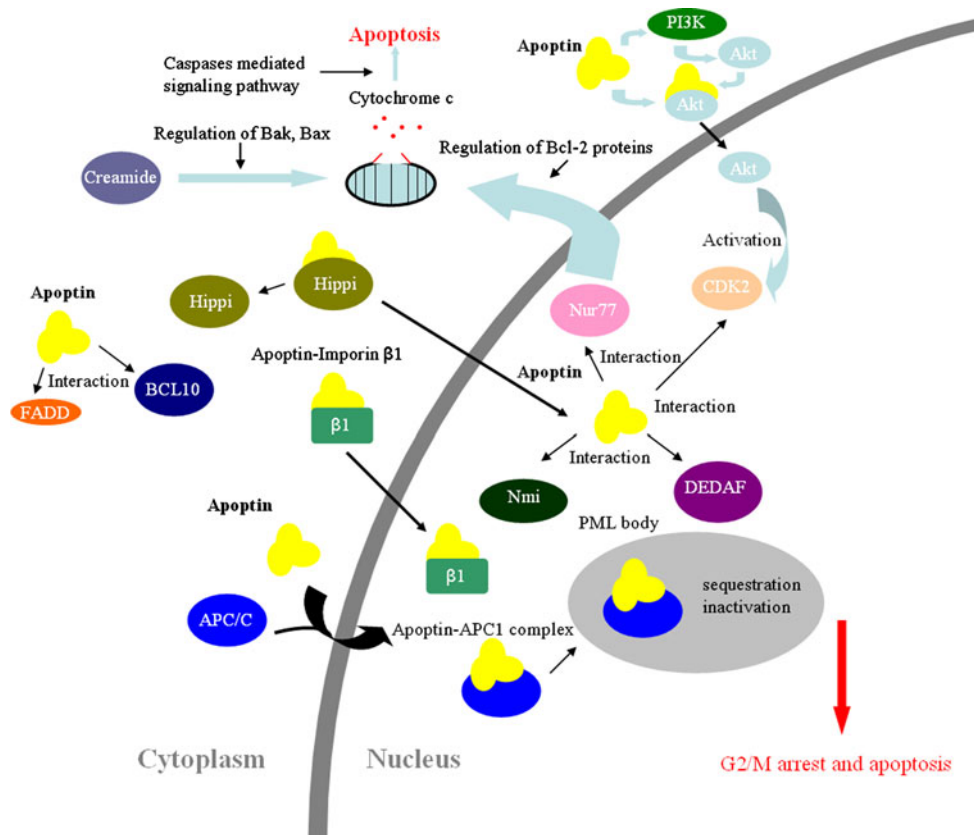


Fig. 3 The signaling and Apoptin-associated molecules are involved in Apoptin-induced apoptosis in tumor cells. Apoptin causes the loss of mitochondrial membrane potential, resulting in the release of cytochrome c by the sphingomyelin–ceramide pathway; then, cytochrome c activates caspase-mediated signaling pathway, induces apoptosis, and regulates subunit of PI3K, resulting in the activation and nuclear translocation of Akt. In the nucleus, Apoptin can

associate with different interaction partners including DEDAF (death effector domain-associated factor), Nmi (N-Myc-interacting protein), CDK2 (cyclin-dependent kinase-2), Hippi (Hip-interacting protein), Nru77 (the orphan steroid receptor), and PML (promyelocytic leukemia protein). In the cytoplasm, Apoptin can interact with FADD (Fas-associated death domain protein), APC/C (anaphase-promoting complex/cyclosome), importin β 1, and Bcl10

Hsc70 plays a role in Apoptin-induced phosphorylation of Akt and contributes to Apoptin-induced apoptosis in transformed cells [51].

Table 1 shows that there are also other molecules that interact with Apoptin and contribute to the nuclear localization of Apoptin or its tumor-selective killing.

Summary

The characteristic of Apoptin's tumor-selective killing activity is not determined by a single element. Based on a comprehensive investigation into mechanisms of Apoptin-mediated cell death, it is found that it is a complicated process involving Apoptin's nucleocytoplasmic trafficking, some apoptotic pathway, modification of conformation, interaction with other molecules or proteins, and so on. In tumor or transformed cells, the signaling and Apoptin-associated molecules are involved in Apoptin-induced apoptosis, as shown in Fig. 3. Recently, Apoptin has been used alone or combined with other treatments in tumor-bearing animal model due to its character [9, 10, 13, 57]. However, further research is warranted to better illustrate the mechanism of Apoptin-induced cell death for better clinical application in tumor therapy.

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