Apoptosis 2005; **10**: 717–724

Apoptin: Specific killer of tumor cells?

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In the early 1990s it was discovered that the VP3/Apoptin protein encoded by the Chicken Anemia virus (CAV) possesses an inherent ability to specifically kill cancer cells. Apoptin was found to be located in the cytoplasm of normal cells while in tumor cells it was localized mainly in the nucleus.[1] These differences in the localization pattern were suggested to be the main mechanism by which normal cells show resistance to Apoptin-mediated cell killing. Although the mechanism of action of Apoptin is presently unknown, it seems to function by the induction of programmed cell death (PCD) after translocation from the cytoplasm to the nucleus and arresting the cell cycle at G_2/M, possibly by interfering with the cyclosome.[2] In addition, cancer specific phosphorylation of Threonine residue 108 has been suggested to be important for Apoptin's function to kill tumor cells.[3]

In contrast to the large number of publications reporting that nuclear localization, induction of PCD and phosphorylation of Apoptin is restricted to cancer cells, several recent studies have shown that Apoptin has the ability to migrate to the nucleus and induce PCD in some of the normal cell lines tested. There is evidence that high protein expression levels as well as the cellular growth rate may influence Apoptin's ability to specifically kill tumor cells. Thus far both *in vitro* and *in vivo* studies indicate that Apoptin is a powerful apoptosis inducing protein with a promising prospective utility in cancer therapy. However, here we show that several recent findings contradict some of the earlier results on the tumor specificity of Apoptin, thus creating some controversy in the field. The aim of this article is to review the available data, some published and some unpublished, which either agree or contradict the reported "black and white" tumor cell specificity of Apoptin. Understanding what factors appear to influence its function should help to develop Apoptin into a potent anti-cancer agent.

Keywords: Apoptin; Bcl10; CAV; cyclosome; cytoplasmic filaments; DEDAF; FADD; Hippi; NF-κB; NMR; nuclear export; nuclear localization; TAT-PTD; tumor-specific apoptosis.

Abbreviations: Apoptin: Apoptosis-inducing protein, CAV: chicken anemia virus, PCD: programmed cell death, NLS: nuclear localization signal, NES: nuclear export signal, ER: endoplasmic reticulum, NMR: nuclear magnetic resonance, SV40 LT: simian virus 40 large T antigen, DED: death effector domain, Hippi: Huntingtin interacting protein 1 protein interactor, TRAIL: TNF-related apoptosis-inducing ligand.

LG was supported by a grant from the BBSRC (47/GTH12530).

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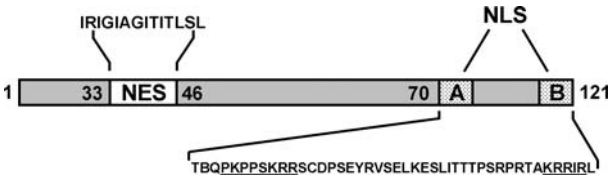
Apoptin: Structure and function

VP3, a 13.6 kDa serine-threonine rich protein of 121 amino acids, has been shown to have the ability to induce PCD in chicken thymocytes both *in vitro* and *in vivo* in a fairly extensive body of scientific literature going back to the early 1990s[4–7]. Because of its death-inducing abilities, the VP3 gene product was renamed Apoptin. Interestingly, the apoptotic activity of Apoptin is not restricted to chicken thymocytes. Apoptin also causes PCD in various human tumor and transformed cells.[1,8,9] Remarkably, *in vitro* experiments showed that Apoptin did not induce cell death in a range of normal human cell types.[1] Importantly however, co-expression of Apoptin with the SV40 large-T antigen in normal cells was sufficient to induce cell death, indicating that even brief expression of this viral transforming gene makes normal cells susceptible to Apoptin-induced PCD.[10] Furthermore, induction of PCD by Apoptin is independent of the p53 status and appears to be aided by the anti-apoptotic gene Bcl-2.[9] In contrast, the 19kDa anti-apoptotic adenovirus E1B protein appears to inhibit Apoptin-induced PCD in a cell-type dependent manner.[11]

The C-terminus of Apoptin contains a bipartite-type nuclear localization signal (NLS), which resides between amino acids (aa) 70 to 121. The N-terminus (aa 1–69) contains a putative nuclear export sequence (NES), located between aa 33 to 46 (Figure 1).[12] However, the importance of this sequence in the subcellular localization and

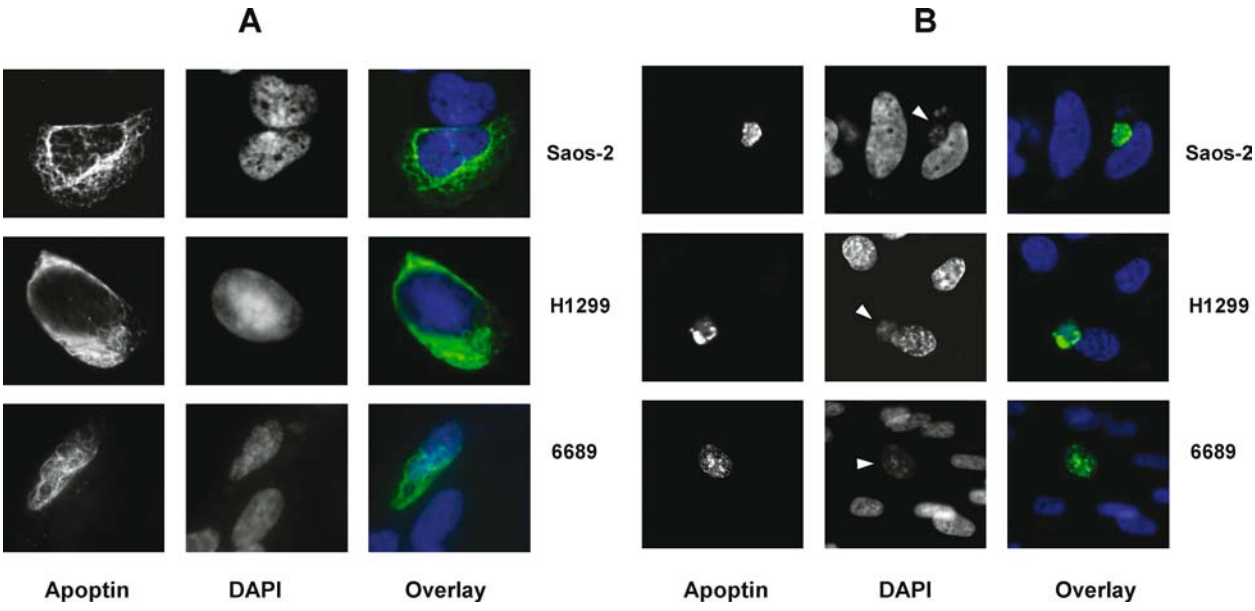
Figure 1. Apoptin structure with nuclear localization and export signals. NLS: nuclear localisation signal; NES: putative nuclear export signal.



induction of PCD by Apoptin remains to be confirmed. Expression of Apoptin in tumor cells shows at first a filamentous distribution in the cytoplasm followed by nuclear translocalization (Figure 2).[13] In contrast, Apoptin was found to remain located in the cytoplasm of normal cells.[1] These differences in localization were suggested to be the main mechanism by which normal cells show resistance to Apoptin-mediated cell killing. An Apoptin-mutant with an 11 aa deletion at the C-terminus, disrupting the C-terminal bipartite NLS, showed reduced nuclear localization, resulting in delayed and less efficient induction of PCD.[1,6,8,9] The ability of a series of Apoptin deletion mutants to localize to the nucleus was shown to strongly correlate with the efficiency of induction of PCD.[12] Furthermore, directing Apoptin to the ER or mitochondrial subcellular compartments almost completely abolished its PCD inducing ability.[13] However, forcing Apoptin into the nucleus of normal cells did not result in PCD, showing that the nuclear localization per se is not the only determining factor in Apoptin-mediated cell killing.[12]

Interestingly and perhaps unexpectedly, it has been demonstrated spectroscopically that the biologically active form of the recombinant Apoptin protein associates into highly stable roughly globular complexes of approximately 30–40 monomers which exchange very few of their member monomers once formed.[14,15] It is the hydrophobic N-terminal domain from aa 1–69 that has been shown to be the driving force for the creation of the complex. Interestingly, a tentative NES (aa 33–46) resides within the N-terminus of each monomer. Putatively, it may be buried or otherwise inoperative while sequestered within the complex. If so, this could make export of the complex from the nucleus very difficult. The C-terminus has an ordered and stable conformation which appears to form dimers or trimers within the complex. Phosphorylation of threonine 108 within the C-terminus is necessary for the apoptotic effect. Importantly, the complex apparently doesn't need to dissociate to induce apoptosis in transformed cells. In normal cells however, within the cytoplasm the (recombinant MBP-Apoptin) complex ultimately grows over time to form larger, more extensive globular bodies. Although these bodies have been shown not to be lysosomes, they are eventually eliminated.[16] The complex has several distinguishing characteristics. The individual Apoptin moieties within the complex don't appear to have any extensive α-helical or β-sheet secondary structure yet they appear to be structurally stable in their conformations. The complex also has a large number of hydrophobic patches. Clearly,

Figure 2. Filamentous expression, nuclear localisation and induction of cell-death by Apoptin. Cells were transfected with pCMV-Ap, fixed and stained using anti-VP3 antibody followed by anti-mouse-FITC (Green). DNA was counterstained with DAPI (Blue). (a) Filamentous expression at 1 day post-transfection. (b) Nuclear localisation and induction of PCD by Apoptin at 5 days post-transfection. Original magnifications: 1000 ×.



the extensive 3D structural arrangement of the Apoptin complex, with its large surface area characterized by exposed hydrophobic patches and C-terminal phosphorylation, must have an important determinant effect on which other proteins and cellular factors associate with Apoptin (and others with them indirectly). Indeed, it may well be that it is this ability to interact and co-locate with a wide range of other molecules, many of which are differentially expressed between different cell-types, that determines the sometimes apparently contradictory behavior of this protein. Structural studies of the complex by x-ray crystallography, NMR, or cryoelectron microscopy could be invaluable for adding significant information to this model.

Apoptin interaction with pro-apoptotic proteins

Apoptin appears to interact with a number of cellular proteins, including Bcl10, FADD,[13] Hippi[17] and DEDAF.[18] The physiological significance of these interactions remains unclear. DEDAF (death effector domain-associated factor) associates with a number of DED containing proteins but does not contain a DED itself and is predominantly localized in the nucleus. It is believed to be a translational repressor and has been shown to interact with FADD, procaspase-8 and procaspase-10, thus implicating itself in caspase-mediated apoptosis. DEDAF-induced cell death has been shown to be inhibited by the caspase inhibitor p35.[18,19] Interestingly, DEDAF has been shown to interact with Apoptin in tumor cells, while it does not co-localize with Apoptin in normal cells. Apoptin residue Ala41 has been tentatively identified as being responsible for DEDAF interaction (for a full discussion see references in[18]).

Hippi is the protein interactor of Huntingtin-interacting protein 1 (Hip-1) and the Hip-1-Hippi complex has been shown to induce apoptosis via the recruitment and activation of the cysteine protease caspase-8 (CASP8).[17,20] Typically Hip-1 is associated with the Huntingtin protein Htt but as this complex dissociates (*e.g.*, via expansion of the polyglutamine repeat on Htt), free Hip-1 forms the conjugate complex with Hippi. It is this complex which recruits procaspase 8 and subsequently activates it to CASP8 which is important in the induction of PCD. CASP8 contains two DEDs in its prodomain and its participation in PCD can be mediated by either Fas or the TNF receptor. Interestingly, Hip-1 contains a pseudo-DED motif. The C-terminal half of Hippi appears to bind within the self-multimerization domain of Apoptin, which causes colocalization in the cytoplasm of normal cells. Interestingly, the C-terminal half of Hippi contains a DED-like motif. In tumor cells, however, no Hippi-Apoptin interaction is detected with Hippi remaining in the cytoplasm while Apoptin translocates to the nucleus.[17] This strongly suggests the possibility that in normal cells Hippi helps prevent Apoptin nuclear transport and/or the Hippi-Apoptin complex prevents formation of the Hip-1-Hippi complex and subsequent activation of the proapoptotic CASP8.

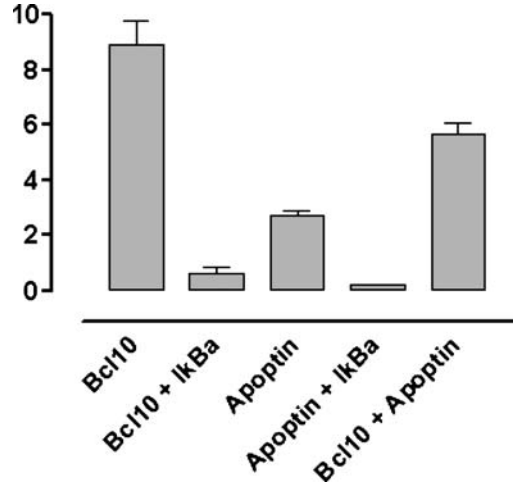
Apoptin has been shown to also colocalize with FADD (Fas Associating Protein with Death Domain), and even more strikingly with Bcl10,[13] both of which are involved in Fas and TNFR-1 mediated PCD. FADD is well known to be important in apoptosis and mediates the signaling of all identified death domain-containing members of the TNF receptor superfamily.[21] It has also been demonstrated *in vivo* that FADD and Fas interact through association of their death domains.[22] Chinnaiyan *et al*.[23] have shown that the interaction of the C-terminal death domains of FADD and Fas causes the exposure of the N-terminal effector domain of FADD and recruitment of CASP8 into the Fas signaling complex.[23] This subsequently activates a cysteine protease (*i.e.* caspase) cascade and cell death. Interestingly, Kabra *et al*.[21] also suggests that FADD, possibly through a death domain-containing receptor, is required for the proliferative phase of early T-cell development.

The Bcl10 gene produces a 233 aa protein and is the cellular homologue of the equine herpesvirus-2 gene (E10). Both the cellular and the viral proteins contain an N-terminal caspase recruitment domain (CARD) homologous with those found in several apoptotic molecules. Ruland *et al*.[24] reported that Bcl10 can both induce apoptosis and activate NF-κB in mice thus it principally functions as a positive regulator of lymphocyte proliferation that specifically connects antigen receptor signaling in B and T cells to NF-κB activation.[24] While Bcl10 is known to activate NF-κB, this possibly results in the generation of an anti-apoptotic signal. Until recently, NF-κB has not been considered as a tumor suppressor in human cancer, because the only studies reporting its perturbation indicated elevated or enhanced expression instead of being lost. Recently however, NF-κB has been shown to be important for p53 and Fas-mediated PCD as well as cell death induced after infection with certain viruses.[25–27] Surprisingly, Apoptin was found to cause stimulation of an NF-κB-responsive element in luciferase reporter assays (Figure 3).[42] The significance of this phenomenon in Apoptin-mediated induction of PCD remains to be elucidated.

Mechanism of killing

The mechanism by which Apoptin kills is not yet clear. Caspase 3 (CASP3), an apoptosis-related cysteine protease that catalyzes the proapoptotic activation of CDK2, has been shown to be activated by Apoptin. Mitochondria are also thought to be involved as indicated by cytochrome c

Figure 3. NF-κB-activation by Bcl10 and Apoptin. H1299 cells were co-transfected with the NF-κB-responsive promoter-luciferase construct and equal amounts of the pCMV-Bcl10 and pCMV-Ap, in the presence or absence of the IκBα-encoding construct. DNA amounts were normalised with empty vector DNA. Luciferase activity was measured at 24 hours post-transfection and is shown as fold induction as compared to basal luciferase activity level after transfection with empty vector DNA. Results shown are representative for 2 independent experiments. Error bars indicate standard deviation.



release.[28] In one study it was shown that Apoptin-induced PCD was diminished in MCF7 cells, which lack CASP3 activity, further signifying a role for CASP3 in Apoptin-induced PCD.[13] CASP3 activity is induced by the Fas apoptotic pathway both through cleavage of the CASP3 zymogen into its active subunits and by stimulating the denitrosylation of its active site thiol.[29,30]

Interestingly, in Huntingtin-related studies cytochrome c, released by mitochondria, was shown to interact with the Fas pathway proteins Apaf-1 and procaspase-9, which in turn activates CASP3 and CASP7, both executioner caspases important in PCD. In addition it was suggested that CASP8 increases cytochrome c release with subsequent CASP3 activity.[31,32] It is thought that the increase in CASP8 is due to the polyglutamine aggregation of Htt, however the mechanism involved is unknown (see discussion of the Hip-1-Hippi complex above).

As mentioned above, it has been shown that the nuclear localization is crucial for Apoptin's ability to induce PCD. The difference in the subcellular localization of Apoptin between normal and tumor cells is highly likely to depend on the specific posttranslation modification and/or interaction of Apoptin with specific cellular factors. Identification of Apoptin interacting proteins is therefore crucial in understanding the mechanisms involved in its nuclear transport and induction of PCD. There is evidence that an unknown cellular kinase phosphorylates the Apoptin NLS on Threonine 108 in tumor cells, thereby inducing nuclear translocation and the apoptotic effect.[3] It has also been reported that this, or a similar, kinase is upregulated by the N-terminal J domain of the SV40 LT antigen in normal cells, subsequently inducing nuclear translocation of Apoptin and PCD.[33] Though it has been widely reported that phosphorylation is required for Apoptin's ability to translocate to the nucleus and to induce PCD in tumor cells, a recent report suggests that the only requirement for nuclear localization is the cytoplasmic concentration of Apoptin.[34] Wadia *et al*.[34] could not detect any phosphorylation of Threonine 108 in 3T3 *Ras* transformed cells by orthophosphate labeling. Furthermore, mutation of Threonine 108 did not alter the accumulation of Apoptin in the nucleus in normal or *Ras*-transformed 3T3 cells. This study claims that the previously reported tumor-selective nuclear accumulation and differential killing of tumor cells by Apoptin is based not on tumorigenic status but on the higher expression levels achieved by transfection into tumorigenic cells, whilst transfection of normal cells in general results in lower expression levels. Indeed, transfection and microinjection of a serial dilution of Apoptin plasmid showed that the efficiency of Apoptin-induced cell death correlated with the protein expression levels.[13] It may be that reaching a critical threshold level of Apoptin sufficient to trigger cytoplasmic multimerization with subsequent nuclear translocation may be the important mechanism of apoptotic activity as has been suggested by Wadia *et al*.[34] In this scenario, increased Apoptin levels would serve as a sensor to induce viral host cell killing during the CAV life cycle.[34] It is not clear to what degree the results of Wadia *et al.* are in direct contradiction to the findings of Zhang *et al*.[16] and Leliveld *et al*.[14] (as discussed above) or rather if this points out a gap in the understanding of this complex process, which appears to be exceedingly sensitive to the local microenvironment within cells.

Another recent study has demonstrated that Apoptin associates with the anaphase-promoting complex (APC) and induces G_2/M arrest and PCD, regardless of the status of p53.[2] APC, also known as the cyclosome or the APC/C, is a complex of eleven proteins that guides the cell through mitosis. The function of the cyclosome is to organize ubiquitination of numerous regulatory proteins and target them for destruction in the proteasome, which controls key events in mitosis. In this study, Apoptin was shown to disrupt the cyclosome complex with the resultant degradation of several APC/C subunits including Cdc27 (APC3), and its largest subunit APC 1 (*ca.* 200 kDa), an essential component of the mitotic checkpoint apparatus. It is thought that phosphorylation is necessary for activation of the APC/C and APC1 and Cdc27 are both important targets for phosphorylation. Interestingly, Teodoro *et al*.[2] observed that while the canonical

molecular weight of monomeric Apoptin is only 13.6 kDa, it migrated at an apparent MW of ca. 200 kDa to >1 MDa in their gel filtration experiments, suggesting that Apoptin forms into multimeric complexes consistent with results reported by other investigators.[2,14,16]

Apoptin has also been shown to kill tumors *in vivo*. In nude mice, implanted subcutaneously with HepG2 cells, the survival of the animal was substantially enhanced by orthotopic administration of an Apoptin-expressing adenovirus. This study also demonstrated that 8×10^9 pfu injected intravenously in 4×10^9 pfu doses on 2 consecutive days was non-toxic to the animals during 5 weeks course of the experiment.[35]

Apoptin kills some normal cells

The p53-independent and tumor-specific properties associated with Apoptin make it a very important candidate for cancer therapy. However, as mentioned above, some recent reports have questioned the absolute tumor specificity of Apoptin, due to the induction of PCD by Apoptin in a number of normal cell types.[34]

In the study reported by Guelen *et al*.[13] 1BR3 normal human diploid fibroblasts and the early passage secondary culture of normal human embryonal lung fibroblasts 6689 were both shown to be sensitive to cell death induced by Apoptin and GFP-Apoptin.[13] In addition, normal breast epithelial and skin keratinocytes were killed by Apoptin (Guelen *et al*., unpublished data). Similar to the pattern observed in tumor and transformed cells, Apoptin and GFP-Apoptin were predominantly located in the nucleus of these normal cells. Furthermore, time-lapse fluorescence microscopy of non-transformed MDCK dog epithelial cells and lung embryo fibroblasts expressing GFP-Apoptin revealed that the fusion protein was located in the nucleus and was able to induce rapid cell death in both cell types[13] (see supplementary AVI video). Freshly isolated rat neural cells expressing GFP-Apoptin also showed nuclear localization and induction of PCD (Guelen *et al*., unpublished data). In addition, HB4a normal breast epithelial cells expressing a temperature sensitive SV40 LT were similarly killed by Apoptin at both the permissive and non-permissive temperature (Guelen et al., manuscript in preparation).[42]

Results obtained by Dr He in Beijing showed that infection of normal human marrow stromal cells (HMSC) and normal embryo lung fibroblasts with an adenoviral vector expressing a FLAG-tagged Apoptin protein resulted in mainly cytoplasmic location of Apoptin. However, in another HMSC cell line (derived from a normal 6 months foetus) either transfection with pcDNA/FLAG/Apoptin or infection with Ad/Apoptin resulted in predominantly nuclear localization of Apoptin. In both tumor and normal cells Apoptin caused significant chromatin condensation and G2 arrest. (Xiangjun He, Peking University Medical College, Beijing, China, personal communication, manuscript submitted).

In support of the effect of protein concentration, Guelen *et al*. (2004) showed that delivering Apoptin fused to the TAT protein transduction domain resulted in the induction of PCD in tumor cells. Nuclear translocation and induction of PCD in normal cells was not observed when Apoptin protein was delivered as a TAT-Apoptin fusion. This study concludes that the resistance of the normal cells to PCD induced by Apoptin may be the consequence of lower intracellular Apoptin levels achieved using protein transduction, as compared to transgene expression. Indeed, it was observed that addition of lower concentrations of TAT-Apoptin (400 nM or 200 nM) to Saos-2 cells resulted in lower intracellular levels of the protein and delayed PCD, although still virtually 100% of the cells were transduced.[13] There is a possibility that all cell types have an individual Apoptin protein level threshold above which they undergo PCD. Tumor cells in general may show a lower tolerance to the presence of Apoptin, which maybe due to reported increased stability of Apoptin in tumor cells.[36] Precise titration of TAT-Apoptin and monitoring the rate of induction of PCD in normal and tumor cells should provide insight into putative concentration dependency of Apoptin-mediated induction of PCD.

Furthermore, cell division time seemed to affect the speed by which cells were killed by Apoptin. Fast growing tumor cells such as Hela and 6689 normal embryonic fibroblasts, which have population doubling times (PDT) of 24 and 21 hours, respectively, were killed within two days. In contrast, Apoptin requires 5 days to kill slow growing Saos-2 and 1BR3N transformed cells, with a PDT of over 42 hours.[42] This may be due to the increased rate of protein synthesis in fast growing cells, resulting in the necessary concentration of Apoptin required to nucleate the active multimer, thereby inducing PCD earlier.

Conclusions

The induction of PCD specifically in tumor cells has great potential for cancer treatment. Enormous effort has been put into the investigation of genes and proteins, such as Apoptin, TRAIL[37] and MDA-7,[38,39] which seem to preferentially kill tumor cells, and this work is expanding rapidly.

Tumor specific induction of apoptosis by Apoptin has been reviewed at length previously.[36,40] In this review we have summarized recent reports from several groups, much of which supports the classic view of Apoptin as acting in a tumor specific manner. However, we also show that contrary to this view, under specific circumstances, such as high protein expression levels, Apoptin is able to kill some normal cells. The reasons for the observed

discrepancies in the activity of Apoptin as a tumor-specific cytotoxic agent are currently unclear and could be due to the use of different methods of transduction of the cells and on the many different cell types and cell lines used in both published and unpublished studies. For instance, it is clear that the use of various gene delivery methods such as transfection, viral infection, microinjection, as well as protein transduction, all seem to influence intracellular Apoptin protein levels in tumor and normal cells. In addition, most normal cells, particularly those of hematological origin, are very hard to transduce. Hence most studies on the induction of PCD have been limited to morphological analysis of only a small percentage of the transduced, Apoptin-containing cells, making conclusive interpretation of the results difficult. Since the intracellular concentration of Apoptin seems to influence its tumor cell specificity, delivery by methods which enable the accurate dosage of the internalized proteins, such as protein transduction[41] or expression from inducible vectors, should allow better control of Apoptin protein levels. This should also enable the determination of the concentration of Apoptin protein needed for the efficient and specific induction of Apoptosis in different cell types. We are currently using a doxycyclin inducible vector to investigate what Apoptin levels are required for the induction of PCD in tumor cells yet at which their normal counterparts remain resistant.

Though there are several discrepancies if not controversies in the literature, there are also emerging pictures of what the mechanism of action might be for Apoptin-induced PCD. Such discrepancies should be seen as gaps in our understanding, which present opportunities for future research that will shed light on what the actual mechanisms of action are. It would be naïve to try to construct an internally consistent mechanism from the information contained here, as it is likely that there are many other important interactions taking place than has been presented or even discussed in the literature to date. We present a scenario of where the sources of discrepancy might arise when comparing the native to the targeted models, which is internally consistent with the information presented in this review.

If we consider a putative native model of CAV, the chicken anemia virus, attacking a normal cell, we can identify several steps that characterize the key elements of how Apoptin might interact with the cellular environment under the conditions of viral invasion. The first response is for the cell to begin to produce a specific set of proteins to combat the viral attack (*e.g.* Fas and NF-κB pathway associated proteins, etc.) in addition to its normal constitutive proteins, depending upon the cell type. Next, CAV introduces its viral genes into the cytosol so that the cellular machinery can make viral proteins (including Apoptin), which will ultimately be assembled into new viral particles (virions). Once this is in motion, CAV Apoptin monomers will begin to accrete as their concentration increases, probably in a diffusion-controlled manner. Because of the surface morphology and electrostatic charge distribution of the emerging complex, which includes the presence of the aforementioned hydrophobic patches, certain cellular proteins will be recruited into the nascent Apoptin Multimeric Complex (AMMC), because they have specific domains and properties that are complementary to this surface. Importantly, these cellular proteins contain domains and active sites that will be important for the translocation of the AMMC and activation of PCD (*e.g.* DED and CARD). This set of cellular proteins will consist of both those normally produced as well as those activated in response to the viral invasion. It is very likely that some cellular proteins, once associated with the AMMC, will indirectly recruit others into the complex. It is also very likely that Apoptin will interact with other systems to induce proapoptotic events and cascades (*e.g.* the several caspases mentioned). It is important to remember that the AMMC is highly stable with 30–40 monomers and very few of the monomers exchange back into the cytosol. This argues for the construction of a well-ordered interrelationship between the members of the complex, though the structure itself may be somewhat fluid. This structure can then present its various constituents, including the protruding Apoptin C-terminal dimers and trimers, which contain the bipartite NLS, in an ordered manner to perform the tasks they were recruited for. Such an arrangement of its constituent elements is likely to provide both its defensive and offensive power. At a "critical mass"the AMMC achieves "Death Star" status and is fully operational. It has its full complement of Apoptin in association with all the necessary cellular proteins to be effective for both translocation into the nucleus with subsequent initiation of PCD. Once the "Death Star" translocates to the nucleus additional nuclear cellular proteins and complexes are recruited and/or attacked (*e.g.* APC/C, the Anaphase Promoter Complex or Cyclosome). In this phase, PCD destroys the cell, thereby releasing virions into the extracellular space whereupon they can attack other cells.

In the next scenario, Apoptin would be artificially transfected or otherwise delivered into a normal, unstressed cell with different results. Such a cell would be constitutively producing a specific set of normal proteins, depending upon the cell type. As Apoptin monomers accumulate in the cytosol they begin to accrete as their concentration increases. As in the first scenario, certain cellular proteins will be recruited into the nascent AMMC because they have specific domains and properties. Importantly, due to the lack of viral attack, the invasion response pathways will not be upregulated and the proteins involved will not be produced by the cell. As Apoptin continues to be produced, the AMMCs will continue to increase in size. In this scenario, however, the AMMC cannot

achieve "Death Star" status because key cellular proteins are missing from the complex. To be fully active, (i.e. for both translocation and initiation of PCD) the AMMC requires a full complement of Apoptin in association with a necessary and sufficient set of cellular proteins. Thus the AMMC continues to grow but it cannot translocate to the nucleus because it is not fully functional. Ultimately, the oversized nonfunctional globular AMMC bodies are eventually eliminated from the cell leaving the cell intact.

In the final scenario, Apoptin is artificially transfected or delivered into tumor or transformed or otherwise stressed cells. Such a cell is producing a specific set of proteins characteristic of its stressed condition (*e.g.* again from Fas and NF-κB pathways, etc.) in addition to its normal constitutive proteins depending upon the cell type. It is highly likely if not virtually certain that a subset of AMMC-specific proteins are being produced. As before, the Apoptin monomers will begin to accrete in a diffusion controlled manner as their concentration increases and certain cellular proteins will be recruited into the nascent AMMC, because they have specific domains and properties necessary for the functionality of the mature proapoptotic AMMC. Also as in scenario one, some associated cellular proteins will recruit others into the complex indirectly. Again as before, upon hitting "critical mass" the AMMC achieves "Death Star" status. It now has a full complement of Apoptin in association with all the necessary and sufficient cellular proteins to be effective for translocation to the nucleus and initiation of PCD. Thus, the Death Star now translocates to the nucleus, expropriates several needed nuclear cellular proteins and complexes then attacks critical components (e.g. the APC/C) with the result that apoptosis destroys the tumor cell.

Using this admittedly oversimplified view of the function and fate of Apoptin, depending on the system and target cells it is involved with, we can nonetheless rationalize much of the observations reported here and perhaps even identify several important knowledge gaps that might point us in the direction of useful experimental discovery. One example might be resolving the issue between elimination from the cell of large masses of aggregated Apoptin vs. the induction of PCD at higher Apoptin concentrations in normal cells. Another would be whether or not and/or under what circumstances Threonine 108 in the NLS of the C-terminus of Apoptin is phosphorylated and effects the functionality of the AMMC. It might even be that the canonical behavior of Apoptin with its virtually complete specificity for tumor- or otherwise transformed cells only exists for truly *in vivo* native cell populations. The wide heterogeneity of model cell systems may be due to the abnormal microenvironment induced within them in cell culture, in which the normal balance of cell proliferation and apoptosis can be disrupted to varying degrees.

However, only the determination of the mechanism by which Apoptin induces PCD and the identification of proteins with which it interacts will allow us to elucidate whether Apoptin is truly a global tumor specific apoptosis-inducing agent, or if it is only applicable to certain specific situations. As described above a number of proteins have been shown to interact with Apoptin but their significance in Apoptin's function remains unclear. What is clear is that this is an exceedingly complex system and many other proteins and agents of varying degrees of importance are yet to be discovered. We are currently using gene expression profiling by microarray analysis in matched normal and tumor cells expressing Apoptin from an inducible vector to identify cellular targets, whose expression levels are affected by Apoptin. Identification of such targets would allow additional insights into the behavior of existing pathways and lead to the discovery of novel pathways involved in Apoptin-induced apoptosis. Such information will be crucially important in designing Apoptin-based tumor specific therapeutic strategies for cancer.

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