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Importance of Nuclear Localization of Apoptin for Tumor-specific Induction of Apoptosis*

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The chicken anemia virus-derived protein Apoptin induces apoptosis specifically in human tumor and transformed cells and not in normal, untransformed cells. The cell killing activity correlates with a predominantly nuclear localization of Apoptin in tumor cells, whereas in normal cells, it is detected mainly in cytoplasmic structures. To explore the role of nuclear localization for Apoptin-induced cell death in tumor cells, we employed a mutagenesis strategy. First, we demonstrated that the C terminus of Apoptin contains a bipartite-type nuclear localization signal. Strikingly, further investigation showed that Apoptin contains two different domains that induce apoptosis independently, and for both domains, we found a strong correlation between localization and cell killing activity. Using inhibitors, we ruled out the involvement of de novo gene transcription and translation and further showed that Apoptin itself does not have any significant transcriptional repression activity, suggesting that Apoptin exerts its effects in the nucleus by some other method. To determine whether nuclear localization is sufficient to enable Apoptin to kill normal, untransformed cells, we expressed full-length Apoptin fused to a heterologous nuclear localization signal in these cells. However, despite its nuclear localization, no apoptosis was induced, which suggests that nuclear localization per se is not sufficient for Apoptin to become active. These studies increase our understanding of the molecular pathway of Apoptin and may also shed light on the mechanism of cellular transformation.

Apoptin is a protein derived from chicken anemia virus (CAV) (1), an avian pathogen that causes depletion of thymocytes and erythroblastoid cells in young chickens via the induction of apoptosis. This protein, one of three encoded by the viral genome of CAV, was shown to reproduce the cell killing activity in cultured cells (2). Apoptin has been shown to induce apoptosis not only in transformed chicken cells but also in human tumor cell lines derived from a wide variety of tissue origins, including tumor cells with common lesions such as loss of p53 or overexpression of Bcl-2, with no known exceptions to date (3, 4). Remarkably, however, all normal untransformed cells tested were resistant (5, 6). These characteristics make Apoptin a promising candidate for anti-tumor therapy, which is often thwarted by genetic lesions that render tumor cells resistant (7–9). Dissecting the mechanism of Apoptin-induced apoptosis may be helpful not only for the use of Apoptin as an anti-tumor agent but may also provide information about the characteristics of the transformation-specific state of a cell.

The mechanism of action for Apoptin is still unclear, but a clue may be found in its localization. In transformed and tumor cells, Apoptin is predominantly found in the nucleus, whereas in untransformed cells Apoptin is mainly localized to the cytoplasm (5). A further indication that localization may be important was obtained from studies in which Apoptin was transiently co-expressed with the strongly transforming SV40 large T antigen in previously untransformed cells. Remarkably, this treatment redirected Apoptin to the nucleus of normal diploid cells and simultaneously sensitized these cells to Apoptin-induced apoptosis (10). Furthermore, Apoptin was found to translocate to the nucleus and induce cell death in primary cells from certain cancer-prone individuals, but only after UV irradiation (11), further strengthening the correlation between localization and cell death.

The amino acid sequence of Apoptin (see Fig. 1) contains two basic stretches constituting putative nuclear localization signals (NLS) and a stretch of alternating isoleucines and leucines that resembles a nuclear export signal (12, 13). Deletion of the second of the two putative NLSs of Apoptin resulted in its diffuse localization all over the cytoplasm, with some protein still present in the nucleus (4). This truncated form of Apoptin had a reduced cell killing activity, suggesting that nuclear localization may be required for apoptosis induction. However, such a truncation could influence other aspects of the protein, e.g. its folding, which could also alter its apoptosis activity. Therefore it was essential to test more mutants of Apoptin.

In this study, we used mutagenesis to investigate further the relationship between nuclear localization of Apoptin and its cell killing activity. We determined the minimal region of Apoptin required for nuclear localization and showed that this region contains a bipartite-type NLS. Furthermore, we showed that distinct parts of Apoptin have independent, intrinsic cell killing activities and that for both domains, nuclear localization is strongly correlated with induction of cell death. However, redirecting Apoptin into the nucleus of normal, untrans-
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Plasmids—pCMV-VP3, encoding Apoptin, and pCMV-neo-Bam, the empty control plasmid, have been described before (2, 14). pCMV-Desmin encodes the muscle-specific cytoskeletal protein Desmin, which has no apoptotic activity and is used as a negative control (5). Several phGFPS65T-derived plasmids expressing fusions of green fluorescent protein (GFP) and parts of Apoptin have been generated. All of the fusion proteins have GFP at the N terminus of Apoptin. A schematic overview of all fusion constructs is given in Fig. 2. phGFPS65T expresses a variant of GFP from Aequorea victoria, which has been optimized for human codon usage, and serine 65 has been threonine to enhance the fluorescent signal (Clontech).

A DNA fragment encoding Apoptin was fragmented by pT716b-VP3 with NdeI/BamHI. This fragment was then digested with BsrI, resulting in fragments encoding Apoptin amino acids (aa) 1–69 and Apoptin aa 70–121. These fragments were ligated with the appropriate linkers in between the BsrGI and NotI sites of C-terminal of GFP, generating phGFPS65T-VP3-F (1–69), and phGFPS65T-VP3 (70–121). phGFPS65T-VP3-F was made by inserting a linker, which encodes a 4–aa spacer (Pro-Gly-Ala-Gly) and aa 111–121 of Apoptin, in between the BsrGI and NotI sites of phGFPS65T. PCR fragments of Apoptin aa 80–121, 90–121, and 100–121 were cloned into phGFPS65T to generate the plasmids phGFPS65T-VP3 (80–121), phGFPS65T-VP3 (90–121), and phGFPS65T-VP3 (100–121). All of the plasmids were sequenced to confirm the correct reading frame of the fusion proteins.

pCMV-NLS-VP3, encoding Apoptin fused to an SV40 large T-NLS (PPKKKRKV), was generated by ligating a linker, with ATG start sequence, in between the NdeI and BamHI fragment of NdeI/BamHI. This fragment was then digested with BsrGI, resulting in fragments encoding Apoptin amino acids (aa) 1–69 and Apoptin aa 70–121. These fragments were ligated with the appropriate linkers in between the BsrGI and NotI sites of C-terminal of GFP, generating phGFPS65T-VP3-F (1–69), and phGFPS65T-VP3 (70–121). phGFPS65T-VP3-F was made by inserting a linker, which encodes a 4–aa spacer (Pro-Gly-Ala-Gly) and aa 111–121 of Apoptin, in between the BsrGI and NotI sites of phGFPS65T. PCR fragments of Apoptin aa 80–121, 90–121, and 100–121 were cloned into phGFPS65T to generate the plasmids phGFPS65T-VP3 (80–121), phGFPS65T-VP3 (90–121), and phGFPS65T-VP3 (100–121). All of the plasmids were sequenced to confirm the correct reading frame of the fusion proteins.

Apoptin Contains a Functional, Bipartite-type NLS—Previous work has suggested that the nuclear localization of Apoptin may be correlated to its killing activity in tumor cells. In this study, we first set out to delineate the elements involved in nuclear localization of Apoptin. Apoptin contains two sequences that resemble NLSs based on the presence of positively charged NLS2 (38–52) and NLS2 (111–121) (Fig. 1). Deletion of aa 111–121 has already been shown to result in decreased nuclear localization, suggesting that NLS2

M. H. M. Noteboom, unpublished data.
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FIG. 1. Sequence of Apoptin. Amino acid residues are depicted in single-letter codes. A putative nuclear export signal located at aa 33–46 is underlined with a single line. Two putative nuclear localization signals at aa 82–88 (NLS1) and 111–121 (NLS2) are double underlined.

is involved in nuclear targeting (4). To test whether aa 111–121 of Apoptin can act independently as an NLS, this part was N-terminally fused to GFP, creating GFP-Apoptin(111–121) (Fig. 2A). An SV40 large T-derived NLS fused to the C terminus of GFP (GFP-NLS) was used as a positive control. The human osteosarcoma cell line Saos-2 was transfected with the GFP fusion constructs, and cells were fixed and analyzed by fluorescence microscopy for protein expression and localization in healthy cells. Wild type (wt) GFP was localized diffusely throughout the whole cell. In contrast, GFP-NLS was clearly located in the nucleus. However, GFP-Apoptin(111–121) was found dispersed throughout the whole cell, similar to what was seen with wt GFP, although GFP-Apoptin(111–121) did show a more punctate staining in the cytoplasm than did wt GFP (Fig. 2B). These data indicate that aa 111–121 (NLS2) of Apoptin are not sufficient to confer nuclear localization.

To resolve the minimal requirements for nuclear localization of Apoptin in tumor cells, we made more N-terminal GFP fusions with fragments of Apoptin (Fig. 2A) and determined their localization. Fusion of aa 70–121 or 80–121, containing both putative NLSs, effectively redirected GFP to the nucleus, whereas fusion of aa 90–121 or 100–121, which lack NLS1, did not. These data suggest that both NLSs are needed for efficient nuclear targeting. Fusion of GFP with aa 1–69 of Apoptin, which lacks both NLSs, almost completely abrogated nuclear localization (Fig. 2C). These data indicate that the N-terminal half of Apoptin is able to change the localization of GFP from being diffused throughout the whole cell to being predominantly cytoplasmic, which suggests that this part of Apoptin possesses either nuclear export or cytoplasmic retention activity. To test further the requirement for both NLSs for nuclear localization, we mutated aa 86–90 (KKRSC) to alanines, creating the mutant Ala86. Ala86 exhibited a partial but significant impairment of nuclear localization, together with the formation of aggregates in the cytoplasm (Fig. 2D). These data indicate that the NLSs of Apoptin are indeed interdependent, consistent with them functioning as a bipartite-type NLS characterized by two basic domains separated by a spacer (23).

Nuclear Localization Strongly Correlates with Apoptotic Activity—We observed that early after transfection (2–3 days) Apoptin is mainly localized in the nucleus of Saos-2 cells but hardly induces apoptosis, which is consistent with previous results (4, 24). This implies that Apoptin enters the nucleus before the onset of cell death. Here, we examined further the relationship between the localization of Apoptin and its apoptotic activity. To this end, the human osteosarcoma cell line Saos-2 was transfected with the GFP fusion constructs described above, along with GFP fused to wt Apoptin as a positive control. Five days post-transfection, the cells were fixed and analyzed by fluorescence microscopy for expression of the proteins and for apoptosis induction as determined by nuclear morphology. At this time point, the majority of cells expressing Apoptin will have become apoptotic (4, 19, 24).

We first examined whether fusion to GFP had any effect on the apoptotic activity of Apoptin and found that the full-length Apoptin protein fused to GFP-induced apoptosis to the same extent as did wt Apoptin in Saos-2 cells (data not shown). GFP by itself was mainly found in a diffuse pattern throughout the cell and only resulted in a background level of apoptosis induction (Fig. 3A) similar to that caused by the negative control protein, Desmin, which does not induce apoptosis (5, 24) (Fig. 4). GFP-Apoptin(1–69) induced cell death only slightly, albeit significantly (p < 0.01) (Fig. 3A). In contrast, GFP-Apoptin(70–121) displayed a more robust cell killing activity (Fig. 3A). However, apoptosis induction was markedly reduced as compared with GFP-Apoptin. These results show that the C-terminal half of Apoptin and, to a lesser extent, the N-terminal half both have intrinsic apoptotic activity, albeit less than full-length Apoptin.

To delineate further the C-terminal apoptosis domain, we determined the cell killing activity of smaller fragments of Apoptin fused to GFP. Whereas expression of GFP-Apoptin(100–121) did not result in significant cell death, GFP-Apoptin(90–121) caused 12% of the cells to undergo apoptosis, which is moderate but significantly higher (p < 0.01) than the effect of wt GFP (Fig. 3A). In contrast, GFP-Apoptin(80–121) caused a robust level of apoptosis (30%), although less than that induced by GFP-Apoptin (Fig. 3A). Thus, aa 80–121 are sufficient to induce the cell death conferred by the C-terminal apoptosis domain of Apoptin.

Next, we compared the cell killing activity of the different mutants to their localization. The localization was determined in cells not yet displaying apoptotic morphology, because in apoptotic cells hardly any distinction can be made between the nucleus and the cytoplasm. In apoptotic cells, Apoptin largely co-localizes with the DNA (data not shown; Refs. 4, 19, and 24). Strikingly, as shown in Fig. 3 (A and B), the mutants conferring the strongest nuclear localization, namely GFP-Apoptin(70–121) and (80–121), also induced the highest amounts of cell death. Furthermore, we examined the cell killing activity of the mutant Ala86, in which part of the bipartite NLS is disrupted. Ala86 not only displayed impaired nuclear localization but also significantly reduced apoptosis activity (p < 0.05) (Fig. 3, C and D). Taken together, these data show that a strong nuclear localization correlates with a more robust cell killing activity of parts of Apoptin, and, moreover, that a mutation that impairs nuclear localization of Apoptin also diminishes its cell killing activity.

Directing the N-terminal Domain to the Nucleus Greatly Enhances Its Cell Killing Activity—We were surprised to find that two separate domains of Apoptin were both able to kill tumor cells. However, the N-terminal domain, aa 1–69, induced much less apoptosis than the C-terminal domain, aa 80–121, or than wt Apoptin. Based on the results shown thus far, implying that induction of apoptosis depends on nuclear localization, this result may be explained by the mainly cytoplasmic localization of the N-terminal domain. Therefore, we generated a mutant encoding aa 1–69 of Apoptin fused at its N terminus to a heterologous NLS. Expression of this protein in Saos-2 cells not only resulted in increased nuclear localization but also in a stronger induction of apoptosis (Fig. 4), albeit less than wt Apoptin. The fact that nuclear targeting enhanced the cell killing activity of the N-terminal domain again suggests that nuclear localization is involved in the ability of Apoptin to induce efficient apoptosis. More importantly, both the N- and C-terminal parts of Apoptin contain a domain conferring significant apoptotic activity, although both domains appear to be necessary for full potency.

Enforced Nuclear Localization of Apoptin in Normal Diploid Cells Does Not Cause Apoptosis—Based on the results mentioned above, nuclear localization appears to be a prerequisite for robust apoptosis induction by Apoptin in human tumor cells. Because Apoptin is predominantly found in the cytoplasm...
in untransformed cells and does not induce apoptosis (5), it was possible that Apoptin does not kill these cells solely because it is in the wrong compartment to do so. Therefore, we investigated whether enforced nuclear localization of Apoptin in normal diploid cells would result in apoptosis induction. To this end, we generated a construct (NLS-Apoptin) consisting of the Apoptin gene fused N-terminally to a heterologous NLS (Fig. 5). NLS-Apoptin was transiently expressed in normal human skin fibroblasts, called VH10 (25). In these cells, NLS-Apoptin was predominantly detected in the nucleus in a diffuse pattern. In contrast, wt Apoptin was found mainly in the cytoplasm as granular or thready structures (Fig. 5). However, despite its nuclear localization, NLS-Apoptin was unable to induce apoptosis in VH10 cells, just as wt Apoptin failed to do so (Fig. 5). We have shown before that the slight induction of apoptosis by wt Apoptin in normal cells is not significantly different from that induced by Desmin, a nonapoptotic cytoskeletal protein, and can be considered as background (5). The data presented here imply that in addition to nuclear localization, other events are necessary for Apoptin to induce cell death.

Apoptin-induced Apoptosis Does Not Require de Novo Gene Transcription and Translation—The strong correlation between nuclear localization and cell death activity of Apoptin suggests that Apoptin acts in the nucleus. Moreover, we have found that Apoptin directly interacts with DNA in vitro (17). Therefore, we set out to determine whether transcriptional activation of genes is involved in Apoptin-induced cell death. To evaluate the requirements for both de novo RNA and protein synthesis, we used one RNA synthesis inhibitor, actinomycin D, and two protein synthesis inhibitors, puromycin and emetine. After determining the subtoxic dosages for each inhibitor (10 \( \mu \)g/ml; data not shown), we assessed the effectiveness on transcription and translation processes. Saos-2 cells were incubated with each inhibitor for 3 h, microinjected with pEGFP-N1 plasmid, and then incubated for a further 6 h with the inhibitors. As determined by fluorescence microscopy, the expression of GFP was reduced to nondetectable levels in the presence of each inhibitor, whereas GFP was highly expressed in nontreated cells (Fig. 6A). These results indicate that at the given concentration and time point, all inhibitors were effective in blocking the processes of de novo gene transcription and translation.

Next, we examined the effect of RNA and protein synthesis inhibitors on the cell killing activity of Apoptin. Because the inhibitors would not allow expression of Apoptin from a transfected plasmid, we used instead a recombinant Apoptin protein fused on its N terminus to MBP, shown previously to exhibit
the same tumor-specific cell killing as wt Apoptin.3 Saos-2 cells were incubated with each inhibitor as described above and injected with either MBP-Apoptin protein or MBP as a negative control. Noninjected cells were analyzed as a control for possible apoptosis induction by inhibitor treatment. MBP-Apoptin induced almost equal levels of apoptosis 6 h after microinjection either in the presence or in the absence of each inhibitor (Fig. 6B), indicating that such inhibitors had no effect on the ability of Apoptin to kill. Treatment with subtoxic levels of cycloheximide (20 μg/ml), another inhibitor of protein synthesis, gave similar results (data not shown). These data suggest that de novo gene and protein expression are not required for the induction of apoptosis by Apoptin in tumor cells.

Apoptin Does Not Behave as a Transcriptional Repressor—Having ruled out the involvement of transcriptional activation in Apoptin-induced apoptosis, we next examined the possibility that Apoptin functions as a repressor of transcription. To this end, we fused full-length Apoptin N-terminally to the BD of Gal4 and determined its effects in U-2 OS osteosarcoma cells on a co-transfected luciferase reporter plasmid containing five Gal4-binding sites preceding the herpes simplex virus TK-promoter. This promoter is known to result in a relatively high basal level of luciferase activity and can therefore be used to study repression (26). A known enhancer of transcription, VP16, was shown to increase luciferase activity as expected (data not shown). Gal4-Apoptin did not significantly differ from the control plasmid expressing only the Gal4 BD, whereas Gal4-YY1, a known transcriptional repressor (26), was able to decrease the basal transcription levels 5-fold (Fig. 7). These data show that, under these conditions, Apoptin itself does not act as a transcriptional repressor. Taken together, our results are consistent with the hypothesis that Apoptin exerts its effects in the nucleus but in a manner independent of transcriptional activation and not involving direct transcriptional repression.

**DISCUSSION**

Nuclear Localization Strongly Correlates with the Cell Killing Activity of Apoptin—Previously, we have shown that Apoptin kills tumor and transformed cells, where it is mainly nuclear, but does not induce apoptosis in untransformed, normal cells, where it is found predominantly in the cytoplasm. This observation suggested that the underlying mechanism of tumor-specific killing by Apoptin involves nuclear localization of the protein (5). Here, we investigated the correlation between nuclear localization and cell killing activity by mutagenesis of Apoptin. Surprisingly, we found that both the N- and C-terminal halves of Apoptin have intrinsic apoptotic activity, albeit reduced compared with full-length Apoptin. This suggests that there are two independent apoptotic domains within the protein. However, using computer analysis, no homology between these domains could be found within their sequence or in their predicted secondary structure. Another possibility is the presence of one large apoptotic domain extending from the N terminus into the C terminus, of which the complete length is needed for full apoptotic activity.

Furthermore, we show here that nuclear localization of various Apoptin mutants was strongly correlated with apoptosis induction. GFP fusions of C-terminal parts of Apoptin that still permitted a profoundly nuclear localization also resulted in

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robust cell killing activity. Apoptin aa 1–69 fused to GFP, which localized to the cytoplasm, had only mild cell killing activity. Nevertheless, fusion of Apoptin aa 1–69 to a heterologous NLS resulted in visible translocation to the nucleus and markedly increased induction of apoptosis. Although these data do not rule out the possibility that fusion to the NLS also influences the structure of aa 1–69, thereby possibly enhancing the apoptotic function, they strongly suggest a requirement for nuclear localization. Furthermore, the mutant Ala086, which disrupts part of the bipartite NLS, showed an increased cytoplasmic localization and a markedly reduced apoptotic activity. Earlier, similar results were found when aa 111–121 of Apoptin were deleted (4). Thus, partially preventing translocation to the nucleus also diminishes the apoptotic activity of Apoptin in tumor cells, making it quite likely that nuclear localization is a cause rather than a result of apoptosis induction.

The data shown here also seem to indicate the possibility that Apoptin activates more than one pathway resulting in apoptosis, which may not be initiated all from within the nucleus. The fact that GFP-Apoptin(1–69) induced mild apoptosis suggests that Apoptin can also activate the apoptotic machinery from the cytoplasm. However, this fact may also be explained by small, undetectable quantities of GFP-Apoptin(1–69) being present and active in the nucleus or by untagged contaminants, which can occur when expressing fusion proteins, although double staining with an antibody against the Apoptin moiety did not result in a significantly different staining pattern (data not shown). Nevertheless, even though the nuclear pathway appears to be the most potent, a cytoplasmic pathway for Apoptin-induced cell death cannot be excluded. In support of this view, the tumor-specific cell killing protein Edors4 of adenovirus was recently shown to activate both a cytoplasmic and a nuclear apoptosis pathway (27).

**Apoptin Contains a Bipartite NLS in the C Terminus and Cytoplasmic Localization Activity in the N Terminus**—In this study, the minimal domain required for nuclear localization of Apoptin has been narrowed down to aa 80–121. This part was sufficient to redirect GFP to the nucleus. Earlier work has shown that deletion of NLS2 impaired nuclear localization (4). Here, we have demonstrated that mutation of aa 86–90 (KKRSC) to alanines similarly inhibited nuclear translocation. Taken together, these data confirm that the two putative NLSs, one stretching from aa 82 to 88 and the other stretching from aa 111 to 121, actually function as such. Moreover, both stretches are required to obtain complete nuclear localization, because deletion or mutation of either of the two NLSs resulted in a diminished nuclear translocation. These data imply that Apoptin contains a bipartite-type NLS. The classic example of a bipartite NLS is the one defined for the protein nucleoplasmmin, which consists of two basic domains separated by a spacer sequence of 10 amino acids. Other bipartite NLSs were found to have similarly short spacer sequences, e.g. interferon regulatory factor and hnRNP K (28–30). However, a novel variant was recently found in hypoxia-inducible factors that contain distinctly longer spacer sequences of 19–31 amino acids (31). Upon comparison of Apoptin with these sequences, we found that the Apoptin NLS is most similar to the hypoxia-inducible factor 2\(\alpha\) bipartite NLS (Fig. 2E), both in conserved residues...
Apoptin-induced cell death.


Apoptin protein binds to naked DNA and results in cell death. Recently, we determined that recombinant Apoptin co-localizes with heterochromatin, which is predominantly transcriptionally inactive. Nevertheless, here, Apoptin itself did not act as a transcriptional repressor in a reporter assay. However, the in vitro model system tested here is not entirely analogous to physiological conditions, alternatively, there remains a formal possibility that fusion of Apoptin to the Gal4 BD impairs its activity. Moreover, these experiments cannot eliminate the possibility of involvement of transcriptional repression via an indirect mechanism. For example, Apoptin may bind to co-factors involved in repression and tether them to the DNA, and such endogenous factors are in limited supply in an overexpression system. Indeed, we have recently found that Apoptin can bind to DEDAF, which has been shown to act as a transcriptional repressor and to be involved in apoptosis (34, 35).

Nuclear Localization Is Not the Sole Determinant of the Tumor Specificity of Apoptin—Previously we have shown that Apoptin does not induce apoptosis in nontransformed cells, where it is predominantly found in cytoplasmic structures (5). We have noticed, however, that variations in transfection methods and the condition of the cells can cause increased translocation of Apoptin to the nucleus of normal cells. We have not been able to pinpoint which factor(s) influence this outcome, although methods that prevent prolonged presence of ectopic DNA in the cytoplasm, e.g. microinjection of DNA constructs directly into the nucleus, result in the most predominantly cytoplasmic localization. Determination of the effect of DNA binding may also result in repression of transcription. Notably, we have recently shown by electron microscopy that Apoptin co-localizes with heterochromatin, which is predominantly transcriptionally inactive. Nevertheless, here, Apoptin itself did not act as a transcriptional repressor in a reporter assay. However, the in vitro model system tested here is not entirely analogous to physiological conditions, alternatively, there remains a formal possibility that fusion of Apoptin to the Gal4 BD impairs its activity. Moreover, these experiments cannot eliminate the possibility of involvement of transcriptional repression via an indirect mechanism. For example, Apoptin may bind to co-factors involved in repression and tether them to the DNA, and such endogenous factors are in limited supply in an overexpression system. Indeed, we have recently found that Apoptin can bind to DEDAF, which has been shown to act as a transcriptional repressor and to be involved in apoptosis (34, 35).

This study, we show that de novo gene transcription and translation are not required for Apoptin-induced apoptosis, implying that all downstream elements are already present in the cell. When we performed similar experiments in nontransformed VH10 fibroblasts, the transcription and translation inhibitors did not enable MBP-Apoptin to induce cell death in these normal cells, ruling out the possibility that tumor specificity is determined by the presence of a labile inhibitor in normal cells (data not shown).

DNA binding may also result in repression of transcription. Notably, we have recently shown by electron microscopy that Apoptin co-localizes with heterochromatin, which is predominantly transcriptionally inactive. Nevertheless, here, Apoptin itself did not act as a transcriptional repressor in a reporter assay. However, the in vitro model system tested here is not entirely analogous to physiological conditions, alternatively, there remains a formal possibility that fusion of Apoptin to the Gal 4 BD impairs its activity. Moreover, these experiments cannot eliminate the possibility of involvement of transcriptional repression via an indirect mechanism. For example, Apoptin may bind to co-factors involved in repression and tether them to the DNA, and such endogenous factors are in limited supply in an overexpression system. Indeed, we have recently found that Apoptin can bind to DEDAF, which has been shown to act as a transcriptional repressor and to be involved in apoptosis (34, 35).

FIG. 6. Transcription/translation inhibitors did not abrogate Apoptin-induced cell death. A. Saos-2 cells were treated with inhibitors as indicated, microinjected with plasmid expressing GFP, and co-injected with Dextran-rhodamine as a marker. The cells were analyzed for expression of GFP and presence of marker (Dex-Rhod) by microscopy. Images of representative cells are shown, and the scale bar corresponds to 10 μm. B. Saos-2 cells were treated with inhibitors as indicated, then injecting with recombinant MBP-Apoptin or MBP or not injected, and analyzed by immunofluorescence microscopy for presence of the proteins and nuclear morphology. The results are given as the means of two independent experiments, and the error bars represent the standard deviations (n > 30). Treatment without inhibitor was done once.

FIG. 7. Apoptin does not act as a repressor of transcription. U-2 OS cells were transfected with different amounts of plasmids encoding Apoptin or YY1 fused to the Gal4 BD or Gal4 BD alone as a control for basal activity and 5xGal4-TK-Luc as a reporter. A LacZ plasmid was co-transfected as a control for transfection efficiency. At day 2 post-transfection, the lysates were made and analyzed for β-galactosidase and luciferase activity. The values shown are normalized for β-galactosidase and represent three independent experiments, each performed in duplicate. The error bars represent the standard deviations.
activation of stress pathways on the localization of Apoptin in normal cells could shed light on the mechanisms influencing the localization of Apoptin. However, as reported here, nuclear localization does not enable Apoptin to induce cell death in normal cells, because fusion of a heterologous NLS to Apoptin forces it into the nucleus of non-transformed cells but does not result in induction of apoptosis. Consistent with these data, microinjection of Apoptin protein into the nucleus of normal diploid fibroblasts did not induce cell death either, and under the stressful transfection conditions mentioned above, nuclear Apoptin did not kill normal cells (data not shown). Therefore, it appears that besides nuclear localization, one or more additional events are required for Apoptin to become active. Indeed, our laboratory has recently shown that Apoptin is phosphorylated on its C terminus specifically in tumor cells. A gain-of-function point mutation that mimics constitutive phosphorylation enabled Apoptin to translocate to the nucleus of normal cells and induce apoptosis. However, a loss-of-function mutation did not completely abolish the cell killing activity of Apoptin, which is consistent with results found with N-terminal Apoptin variants. This suggests that the mechanism underlying tumor-specific cell killing involves more than phosphorylation alone. For this reason, it is conceivable that the two distinct apoptosis domains within Apoptin act by at least two different pathways.

Compartmentalization has been shown on many occasions to play a regulatory role in apoptosis pathways. For example, release of pro-apoptotic factors from the mitochondria (apoptosis-inducing factor, cytochrome c, and Smac) (37–39) or translocation of pro-apoptotic members of the Bcl-2 family from the cytosol to the outer mitochondrial membrane (Bax, and BAD) (40–42) both result in apoptosis induction. Based on the data reported here, we hypothesize that Apoptin is retained in the cytoplasm of non-transformed cells. In transformed cells, phosphorylation of Apoptin may enable it to migrate into the nucleus and induce apoptosis. Forcing Apoptin into the nucleus of non-transformed cells without the proper phosphorylation would not result in apoptosis induction. Therefore, the specificity for inducing cell death in transformed cells does not lie in the nuclear localization per se but involves phosphorylation and/or other modifications of Apoptin that in turn influence its localization. It is of great interest to determine further which factors or events enable Apoptin to become active in transformed cells, e.g. binding to downstream proteins or additional ways of modification, which may also tell us more about the nature of transformation.

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