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 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] (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-

[1] The abbreviations used are: CAV, chicken anemia virus; CMV, cytomegalovirus; DAPI, 2,4-diamidino-2-phenylindole; BD, DNA-binding domain; GFP, green fluorescent protein; MBP, maltose-binding protein; NLS, nuclear localization signal; TK, thymidine kinase; aa, amino acids; wt, wild type.

formed cells by fusing it to a heterologous NLS did not result in induction of apoptosis. Thus, nuclear localization appears to be important but not sufficient for the tumor-specific cell killing activity of Apoptin.

EXPERIMENTAL PROCEDURES

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 phGFP-S65T-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. phGFP-S65T expresses a variant of GFP from *Aequorea victoria*, which has been optimized for human codon usage, and serine 65 has been mutated to threonine to enhance the fluorescent signal (Clontech).

A DNA fragment encoding Apoptin was digested from pET16b-VP3[2] with *Nde*I/*Bam*HI. This fragment was then digested with *Bsr*I, 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 *Bsr*GI and *Not*I sites C-terminal of GFP, generating phGFP-VP3, phGFP-VP3 (1–69), and phGFP-VP3 (70–121). phGFP-VP3 (111–121) 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 *Bsr*GI and *Not*I sites of phGFP-S65T. PCR fragments of Apoptin aa 80–121, 90–121, and 100–121 with overhanging *Bsr*GI and *Not*I sites were cloned into phGFP-S65T to generate the plasmids phGFP-VP3 (80–121), phGFP-VP3 (90–121), and phGFP-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 codon and NLS, to the N terminus of an *Nde*I/*Bam*HI fragment of Apoptin. The resulting fragment was cloned into the *Bam*HI site of pCMV-neo-Bam. Similarly, the SV40 large T-NLS was fused to a *Nde*I/*Bsr*I fragment of Apoptin encoding aa 1–69, generating pCMV-NLS-VP3 (1–69). phGFP-NLS was constructed by fusing the same NLS to the C terminus of GFP. All of the plasmids were confirmed by sequencing.

The mutant Ala086 was constructed by replacing aa 86–90 of Apoptin with alanine residues. To this end, first, an Apoptin DNA was constructed containing additional unique restriction enzyme sites that allow for ease of cloning (sAPO). Then aa 86–90 of Apoptin were exchanged by 5 alanine residues using a linker substitution strategy. The Ala086 mutant and sAPO were sequenced and cloned in a modified expression plasmid vector pIRESneo (Clontech) under the control of the CMV promoter. pEGFP-N1 (Clontech) encodes enhanced GFP, which is optimized for human codon usage and contains chromophore mutations enhancing its fluorescence, under the control of the CMV promoter.

pGal4-YY1 encoding YY1 fused to the Gal4 DNA-binding domain (BD) was a kind gift from R. Johnstone and Y. Shi (15). The reporter plasmid 5xGal4-TK-Luc was a kind gift from E. Kalkhoven and contains the luciferase gene under the regulation of the herpes simplex virus TK promoter joined to 5xGal4-binding elements (16). pcDNA3.1/mycHis/LacZ (Invitrogen) encodes LacZ with a Myc and His tag attached to the C terminus, regulated by a CMV promoter. pFA-CMV (Stratagene) contains the Gal4 BD upstream of a multiple cloning site, under the regulation of a CMV promoter. A DNA fragment encoding Apoptin was cloned into the multiple cloning site of pFA-CMV and confirmed by sequencing (Baseclear). The resulting plasmid pFA-VP3 encodes a Gal4 BD fusion to Apoptin.

Expression and Purification of Proteins—The cloning, expression, and purification of recombinant MBP-Apoptin protein and the control protein MBP have been described (17). In short, the construct pMalT-BVP3 encodes MBP-Apoptin, an N-terminal fusion of MBP to Apoptin, separated by a flexible linker encoding 10 asparagine residues. The construct pMalTB encodes MBP alone. Both proteins were expressed in *Escherichia coli* and purified by standard methods.

Cell Culture, Transfection, and Microinjection Methods—The human osteosarcoma cell lines Saos-2 and U-2 OS and the human normal diploid skin fibroblast strain VH10 (up to passage 15) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin (Invitrogen).

Saos-2 cells were grown on glass coverslips and transfected with 3 μg of DNA/well of a 6-well dish by the CaPO$_4$ method as described (18). VH10 cells, which were grown in Permanox chamber slides (Invitrogen), were transfected with FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. 2 μg of DNA was transfected per chamber.

For microinjections Saos-2 cells were cultured on glass-bottomed microinjection dishes (MatTek Corporation) or in 35-mm dishes containing glass Cellocate coverslips (Eppendorf). The cells were microinjected in the nucleus with DNA (50 ng/μl in phosphate-buffered saline) or in the cytoplasm with protein (3 mg/ml) using an Eppendorf microinjector with the injection pressure condition of 55 hPa. The cells were co-injected with Dextran-rhodamine (molecular mass, 70 kDa; Molecular Probes) to be able to later identify injected cells.

Fluorescence Microscopy—Saos-2 cells were fixed with 1% formaldehyde in phosphate-buffered saline for 10 min, followed by 100% methanol for 5 min, and finally 80% acetone for 2 min. VH10 cells were fixed with 50% acetone, 50% methanol for 5 min. The slides were stored at −20 °C until further use. Immunocytochemical staining was performed as described (19). GFP and GFP fusion proteins were detected by their intrinsic fluorescent signal. Apoptin was detected either with the mouse monoclonal antibody CVI-CAV-111.3, which recognizes an epitope lying in the N terminus of Apoptin (5), or with the rabbit polyclonal antibody Rα-VP3-C (19). Desmin was detected with mouse monoclonal antibody 33 (Monosan). Secondary antibodies were either conjugated to fluorescein isothiocyanate or rhodamine (Jackson ImmunoResearch Laboratories). The chromatin was stained with 1 μg of 2,4-diamidino-2-phenylindole (DAPI)/ml. The cells were analyzed by fluorescence microscopy for expression of the transfected protein. Nuclear morphology of those cells expressing the transfected protein was analyzed by examining the DAPI staining, which indicates the apoptotic state. Namely, during apoptosis, the whole cell has shrunken, the chromatin is condensed, the nucleus is often fragmented, and finally DAPI staining is lost because of fragmentation of the DNA. The statistics were performed using Student's *t* test. Images of representative cells were made with either a standard photocamera or a digital video camera connected to a fluorescence microscope AX70 (Olympus) and AnalySIS software.

Transcription and Translation Inhibition—For the transcription/translation inhibition experiments, we used one RNA synthesis inhibitor, actinomycin D (Sigma), and two protein synthesis inhibitors, puromycin (Sigma) and emetine (Sigma). Numerous studies have shown that the inhibition of RNA and protein synthesis alone can directly trigger apoptosis in various cell types (20–22). Therefore, the cytotoxicity of each inhibitor used in our study was first analyzed by 24-h incubation at a dose range of 0, 5, 10, 20, 40, and 80 μg/ml concentration. On the basis of cell killing curves following 24 h of incubation, the subtoxic dosage of each inhibitor was then determined at 10 μg/ml for all three inhibitors. To determine the effective transcription and translation inhibition by each inhibitor, Saos-2 cells were injected in the nucleus with the plasmid pEGFP-N1 (50 ng/μl) expressing enhanced GFP, which was used as an indicator. To assess the effect of the inhibitors on Apoptin, Saos-2 cells were incubated for 3 h with each inhibitor before and for a further 6 h after microinjection in the cytoplasm with the recombinant proteins MBP-Apoptin or MBP (3 mg/ml). Dextran-rhodamine (1 mg/ml) was co-injected as a marker. The cells were fixed 6 h after microinjection as described above. The expression of GFP, the presence of MBP-Apoptin or MBP, and nuclear morphology were analyzed by fluorescence microscopy.

Luciferase Reporter Assay—U-2 OS cells were plated in 6-well plates and transfected with FuGENE 6 and with 1 μg/well 5xGal4-TK-Luc as the reporter plasmid, pcDNA3.1/mycHis/LacZ at 100 ng/well as an internal control, and Gal4 BD plasmids at 200 ng/well. Two days later, the cells were lysed in reporter lysis buffer (Promega), and the lysates were assayed for luciferase activity and for β-galactosidase activity according to the manufacturer's protocol (Promega).

RESULTS

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 aa, namely aa 82–88 (NLS1) and aa 111–121 (NLS2) (Fig. 1). Deletion of aa 111–121 has already been shown to result in decreased nuclear localization, suggesting that NLS2

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[2] M. H. M. Noteborn, unpublished data.

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 1            11           21           31
MNALQEDTPP  GPSTVFRPPT   SSRPLETPHC   REIRIGIAGI
41           51           61           71
TITLSLCGCA  NARAPTLRSA   TADNSESTGF   KNVPDLRTDQ
81           91           101          111          121
PKPPSKKRSC  DPSEYRVSEL   KESLITTTPS   RPRTAKRRIR   L
  NLS1                                   NLS2
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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. 2*A*). 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. 2*B*). 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. 2*A*) 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. 2*C*). 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 Ala086. Ala086 exhibited a partial but significant impairment of nuclear localization, together with the formation of aggregates in the cytoplasm (Fig. 2*D*). 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. 3*A*) 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. 3*A*). In contrast, GFP-Apoptin(70–121) displayed a more robust cell killing activity (Fig. 3*A*). 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. 3*A*). In contrast, GFP-Apoptin(80–121) caused a robust level of apoptosis (30%), although less than that induced by GFP-Apoptin (Fig. 3*A*). 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 Ala086, in which part of the bipartite NLS is disrupted. Ala086 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
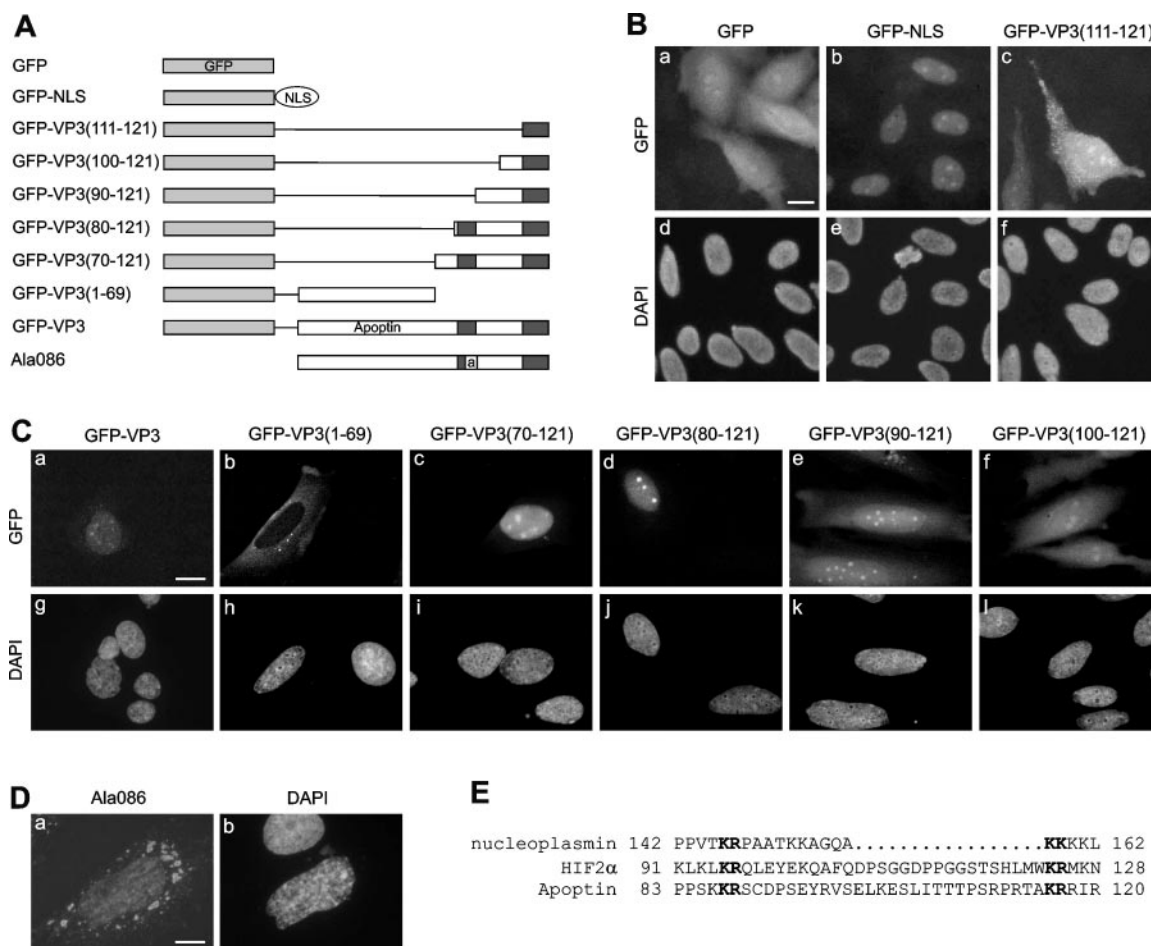
Fig. 2. **Localization of different Apoptin mutants.** *A*, schematic overview of plasmids. The GFP moiety is depicted as a *light gray bar*, Apoptin is a *white bar*, the putative NLSs within Apoptin are *dark gray bars*, and the alanine replacement is indicated with the *letter a*. The *numbers* indicate amino acid residues within the Apoptin moiety. *B*, Saos-2 cells fixed 2 days post-transfection, expressing GFP (*a* and *d*), GFP-NLS (*b* and *e*), and GFP-Apoptin(111–121) (*c* and *f*). GFP signal (*a–c*) or nuclear staining with DAPI (*d–f*) is shown. *C*, Saos-2 cells fixed 5 days post-transfection. The cells are expressing GFP fused to full-length Apoptin (*a* and *g*), or to Apoptin amino acids 1–69 (*b* and *h*), 70–121 (*c* and *i*), 80–121 (*d* and *j*), 90–121 (*e* and *k*), and 100–121 (*f* and *l*). GFP signal (*a–f*) and nuclear staining with DAPI (*g–l*) are shown. *D*, Saos-2 cells fixed at day 4 post-transfection, expressing Ala086. Staining for Ala086 with anti-Apoptin RαVP3-C (*a*) or nuclear staining with DAPI (*b*). All of the images were made of representative cells; the *scale bars* correspond to 10 μm. *E*, comparison of amino acid sequence of bipartite NLSs from *Xenopus* nucleoplasmin, human HIF2α, and Apoptin. The *numbers* indicate amino acid residues.

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 μg/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. 6*A*). 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
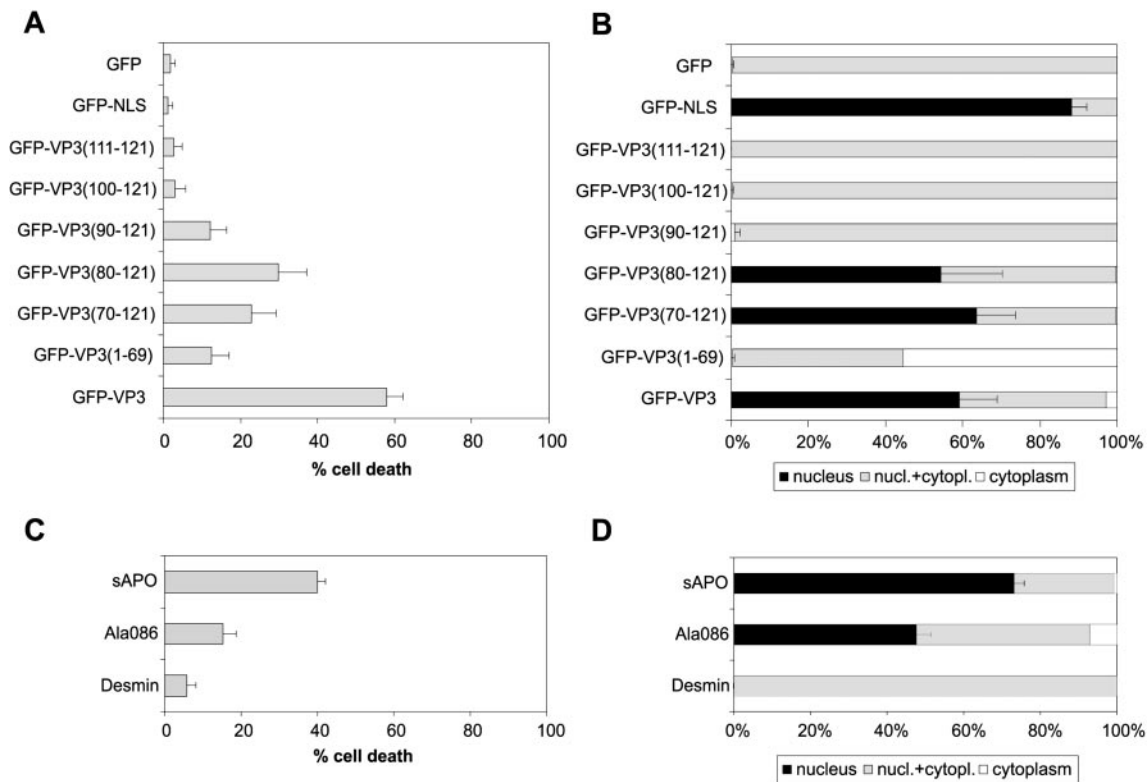
FIG. 3. **Apoptotic activity in correlation with the localization of several Apoptin mutants.** *A* and *B*, Saos-2 cells were transiently transfected with the indicated GFP fusions or GFP as a control, fixed 5 days later, and analyzed for protein expression and apoptosis induction by fluorescence microscopy. *C* and *D*, Saos-2 cells were transfected with Ala086, full-length Apoptin (sAPO), or Desmin as a negative control, fixed 4 days later, and analyzed by immunofluorescence microscopy. *A* and *C*, apoptosis induction as determined by nuclear morphology. *B* and *D*, localization in nonapoptotic cells. Given are the means of at least three (*A* and *B*) or two (*C* and *D*) independent experiments; in each experiment at least 100 positive cells were scored. The *error bars* represent the standard deviations.

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. 6*B*), 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

[3] Zhang, Y.-H., Leliveld, S. R., Kooistra, K., Molenaar, C., Rohn, J. R., Tanke, H. J., Abrahams, J. P., and Noteborn, M. H. M. (2003) *Exp. Cell Res.*, in press.
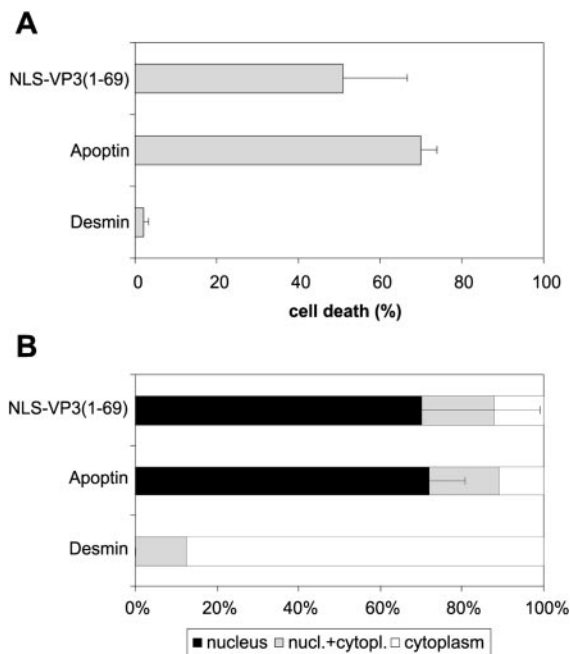
FIG. 4. **Apoptosis induction and nuclear localization of NLS-Apoptin(1–69).** Saos-2 cells were transiently transfected, fixed 5 days later, and analyzed by immunofluorescence microscopy. *A*, percentage cell death among cells expressing NLS-Apoptin(1–69), Apoptin, or Desmin (negative control), as determined by nuclear morphology. *B*, localization in nonapoptotic cells. The results are given as the means of three independent experiments, and the *error bars* represent the standard deviations. In each experiment at least 100 cells were scored.

FIG. 5. **NLS-Apoptin did not induce apoptosis in normal diploid fibroblasts.** VH10 cells fixed 4 days post-transfection were analyzed by immunofluorescence. *A*, apoptotic activity as determined by nuclear morphology. *B*, localization in nonapoptotic VH10 cells. Shown are the means of three independent experiments, in which at least 100 cells were scored. The *error bars* represent the standard deviations. *C*, images of representative cells expressing Apoptin (*a* and *b*) or NLS-Apoptin (*c* and *d*). The cells were stained with the antibody CVI-CAV-111.3 (*a* and *c*) or with DAPI (*b* and *d*). The *scale bar* corresponds to 10 μm.

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 E4orf4 of adenovirus was recently shown to activate both a cytoplasmic and a nuclear apoptosis pathway (27).

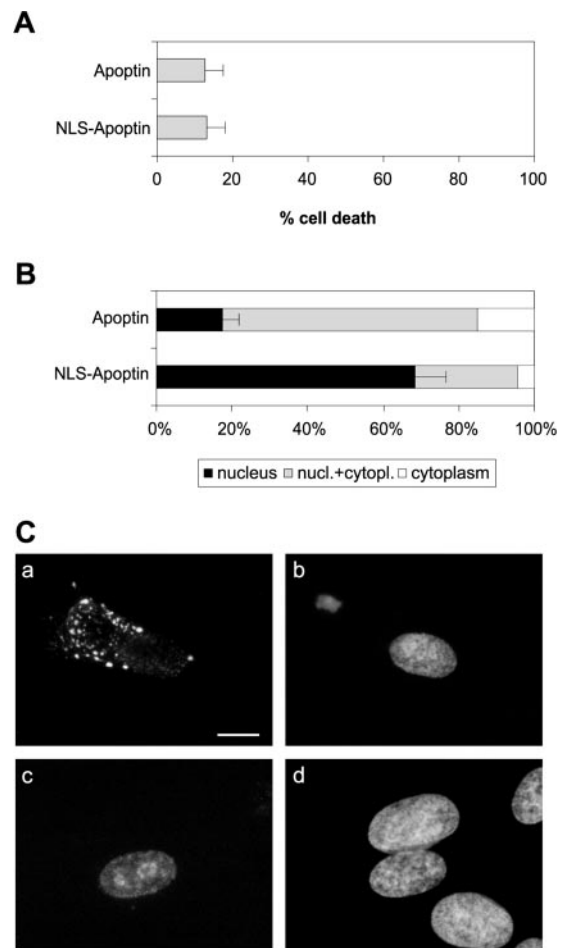*Apoptin Contains a Bipartite NLS in the C Terminus and Cytoplasmic Localization Activity in the N Terminus*—In this

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 nucleoplasmin, 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 2α bipartite NLS (Fig. 2*E*), both in conserved residues
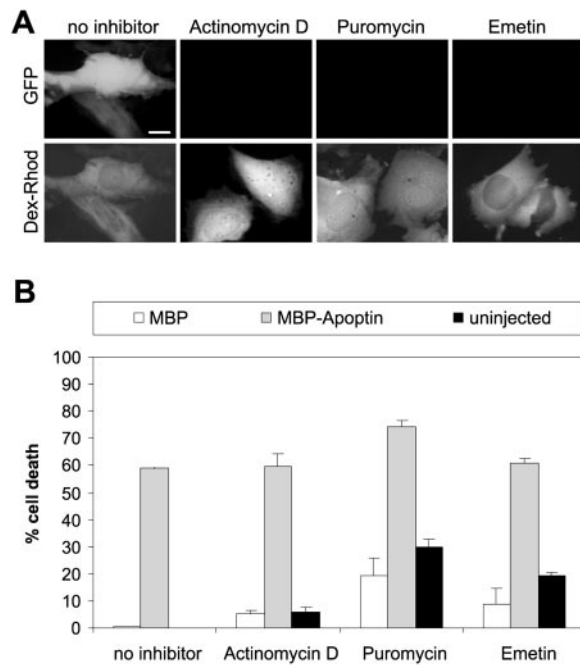
FIG. 6. **Transcription/translation inhibitors did not abrogate Apoptin-induced cell death.** *A*, Saos-2 cells were treated with inhibitors as indicated, microinjected with plasmid expressing GFP, and co-injected with Dextran-rhodamine as a marker. The cells were analyzed for expression of GFP and presence of marker (*Dex-Rhod*) by fluorescence 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 injected 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.

and spacing. Therefore, the Apoptin bipartite NLS may belong to the same category as those found in hypoxia-inducible factors.

Fusion with aa 1–69 of Apoptin redirected GFP predominantly to the cytoplasm. These data suggest that aa 1–69 of Apoptin contain either a nuclear export signal or a domain involved in cytoplasmic retention. The aa sequence of Apoptin in fact contains a stretch of alternating isoleucines and leucines resembling previously identified nuclear export signals of the proteins PKI and Rev (32, 33). However, fusion of this particular part of Apoptin, aa 33–46, to the C terminus of GFP, did not result in increased cytoplasmic localization (data not shown). It is possible that other residues are required as well for cytoplasmic localization. Conversely, it is also conceivable that aa 1–69 of Apoptin contain a domain involved in retention in the cytoplasm, perhaps through binding to another protein. We are currently investigating both possibilities.

Apoptin-induced Cell Death Neither Requires Transcriptional Activation nor Involves Direct Transcriptional Repression—The data mentioned above indicate that nuclear localization of Apoptin is important for its cell killing activity. It is still unknown how the presence of Apoptin in the nucleus would result in cell death. Recently, we determined that recombinant Apoptin protein binds to naked DNA *in vitro* in a non-sequence-dependent manner,[4] which, together with the importance of nuclear localization for its activity, suggests that Apoptin may play a role in the regulation of gene expression. However, in
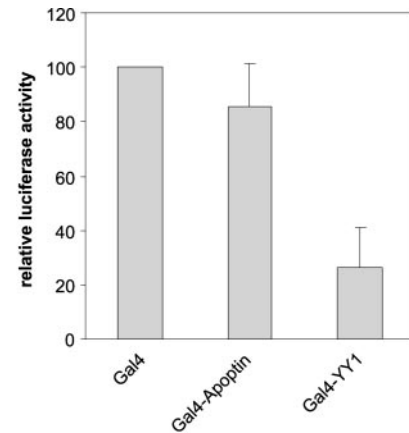

FIG. 7. **Apoptin does not act as a repressor of transcription.** U-2 OS cells were transfected with different amounts of plasmids encoding Apoptin or YY1 fused to the Gal4 BD or Gal4 BD alone as a control for basal activity and 5xGal4-TK-Luc as a reporter. A LacZ plasmid was co-transfected as a control 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.

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.[4] 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).[5]

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

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[5] A. A. A. M. Danen-van Oorschot, P. Voskamp, C. M. J. Seelen, M. H. A. M. van Miltenburg, M. W. Bolk, S.W. Tait, J. G. R. Boesen-de Cock, J. L. Rohn, J. Borst, and M. H. M. Noteborn, submitted for publication.

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 nontransformed 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,[3] 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 (36). 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 (tBid, 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 nontransformed cells. In transformed cells, phosphorylation of Apoptin may enable it to migrate into the nucleus and induce apoptosis (36). Forcing Apoptin into the nucleus of nontransformed 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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Importance of Nuclear Localization of Apoptin for Tumor-specific Induction of Apoptosis

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