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Recombinant Apoptin multimers kill tumor cells but are nontoxic and epitope-shielded in a normal-cell-specific fashion

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Abstract

Apoptin, a protein derived from chicken anemia virus, induces apoptosis in human transformed or tumor cells but not in normal cells. When produced in bacteria as a recombinant fusion with maltose-binding protein (MBP–Apoptin), Apoptin forms a distinct, stable multimeric complex that is remarkably homogeneous and uniform. Here, using cytoplasmic microinjection, we showed that recombinant MBP–Apoptin multimers retained the characteristics of the ectopically expressed wild-type Apoptin; namely, the complexes translocated to the nucleus of tumor cells and induced apoptosis, whereas they remained in the cytoplasm of normal, primary cells and exerted no apparent toxic effect. In normal cells, MBP–Apoptin formed increasingly large, organelle-sized globular bodies with time postinjection and eventually lost the ability to be detected by immunofluorescence analysis. Costaining with an acidotrophic marker indicated that these globular structures did not correspond to lysosomes. Immunoprecipitation studies showed that MBP–Apoptin remained fully antibody-accessible regardless of buffer stringency when microinjected into tumor cells. In contrast, MBP–Apoptin in normal cells was only recoverable under stringent lysis conditions, whereas under milder conditions they became fully shielded with time on two epitopes spanning the entire protein. Further biochemical analysis showed that the long-term fate of Apoptin protein aggregates in normal cells was their eventual elimination. Our results provide the first example of a tumor-specific apoptosis-inducing aggregate that is essentially sequestered by factors or conditions present in the cytoplasm of healthy, nontransformed cells. This characteristic should reveal more about the cellular interactions of this viral protein as well as further enhance its safety as a potential tumor-specific therapeutic agent.

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Introduction

Apoptin is a protein encoded by chicken anemia virus (CAV). Although CAV infection is restricted to avian-derived cells, Apoptin, when overexpressed alone, induces apoptosis in every human transformed or malignant cell line tested to date [1–4]. In contrast, numerous studies have established that Apoptin does not kill normal, healthy primary human cells *in vitro*, regardless of origin or cell type

[1,4]. This remarkable specificity may be exploitable for cancer therapy; indeed, *in vivo* studies in mice have demonstrated that Apoptin encoded by an adenovirus vector has a strong specific antitumor activity that can confer a significant survival benefit [5,6].

The mechanism of Apoptin's tumor-specific apoptosis activity remains to be fully elucidated, but one important factor is that the protein becomes specifically phosphorylated and thereby activated by a kinase activity found only in tumor or transformed cells [7]. Another striking distinction is that upon ectopic expression, Apoptin translocates to the nucleus of tumor cells, whereas in healthy, nontransformed cells, the majority of the protein is dispersed in the

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cytoplasm [1,4]. Although tumor cells have been examined extensively for their role in the specificity of Apoptin's mechanism, including the protein's late activities in the nucleus^{1,2} [5,8], little is known about how the normal cell cytoplasm responds to the continued presence of Apoptin and how that interaction might contribute to its specificity.

In order to analyze the early cytoplasmic events in the subcellular trafficking of Apoptin in further detail, as well as to explore the feasibility of delivering Apoptin protein directly as an antitumor agent, we generated a soluble, bacterially expressed recombinant Apoptin protein fused at its N-terminus with the bacterial maltose-binding protein (MBP–Apoptin) [8]. This recombinant protein was previously shown by a wide variety of techniques to exist as a stable, homogenous globular multimeric complex consisting of 30 to 40 monomeric subunits, which appears to be the active form of Apoptin *in vivo* [8]. In this report, we examined the biological behavior of this unique MBP–Apoptin multimer by introducing the protein into tumor versus normal cells by microinjection. After confirming that the recombinant Apoptin protein retained its remarkable potency and specificity for aberrant cells, we discovered that normal cells responded very differently to the recombinant Apoptin protein, inducing its higher-order aggregation, epitope shielding, and eventual disappearance in the cytoplasm. We hypothesize that this sequestration of the Apoptin protein helps to render it nonfunctional in normal cells, a feature that may reinforce the safety of Apoptin delivered as a therapeutic cancer treatment.

Materials and methods

Recombinant MBP–Apoptin protein and control protein MBP

The cloning, expression, purification and biophysical properties of these two soluble proteins have been described [8]. Briefly, the constructs pMalTBVP3 and pMalTB, encoding an N-terminally MBP-tagged recombinant Apoptin protein, MBP–Apoptin (55.8 kDa), or MBP (42.3 kDa) alone, respectively, were expressed in *Escherichia coli* and purified by standard methods. In pMalTBVP3, the MBP gene and the Apoptin gene (also known as VP3) [3] are separated by a flexible linker encoding 10 Asn residues.

Cells and cell culture

The human osteosarcoma cell line Saos-2 lacking functional p53 and human diploid skin fibroblasts derived from a healthy individual (VH10) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal

calf serum, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (Life Technologies). Human bone-marrow-derived mesenchymal stem cells (hMSC, Cat. No. PT-2501, LOT No. 9F1938, BioWhittaker Europe SPRL, Belgium) were cultured in a T-25 flask with their accompanying medium MSCGM Bulletkit (PT-3001, BioWhittaker) and used from passage 2 to passage 5 as recommended by the manufacturer. Primary human hepatocytes (hNheps, Cat. No. CC-2591, LOT No. 9F0740, BioWhittaker) were seeded on an extracellular matrix (ECM, Sigma)-coated dish, cultured in their accompanying medium HCM (HCM Bulletkit CC-3199+CC-4182, BioWhittaker), and used at passage 1.

Microinjection

Cell microinjection was performed with the aid of an inverted microscope (Axiovert 135 TV, Zeiss, Germany) equipped with a programmable microinjector (IM 300, Narishige Co., Japan) and a joystick hydraulic micromanipulator (MMO-202, Narishige Co., Japan). Microinjection needles with a $0.5 \pm 0.2 \mu\text{m}$ diameter tip (Sterile Femtotips II, Eppendorf) were loaded with protein or DNA samples by using Eppendorf microloaders (Eppendorf). The concentration of protein samples used was generally 3 mg/ml in PBS. When DNA was used for nuclear microinjection, the samples contained 50 ng/ μl of DNA. To identify and trace injected cells, the injection solution was supplemented with 1 mg/ml dextran-conjugated tetramethylrhodamine (Rhodex; 70,000 MW, lysine fixable; Molecular Probes). Prior to microinjection, all injection samples were microcentrifuged at 16,000g (IEC, Micromax RF, Model 230, USA) at 4°C for 15 min to eliminate any precipitates that might block the microinjection needles. For microinjection, cells were seeded in 35-mm tissue culture dishes with a glass-bottomed microwell (P35G-1.5-14-C, No. 1.5, uncoated, MatTek Corporation, Ashland, MA) at 30–40% confluence 24 h prior to microinjection. During microinjection, cells were incubated in RPMI 1640 medium (without phenol red, containing 25 mM Hepes, pH 7.2, 5% fetal calf serum, penicillin, and streptomycin: Life Technologies). The temperature of the medium was kept at a constant 37°C using an objective heater (Bioptechs, Butler, PA) and a heated ring surrounding the culture chamber. Protein or DNA delivery into the cytoplasm or nucleus of cells was carried out under an injection pressure of 40–50 hPa and an injection time of 0.2–0.5 s. Immediately after microinjection, cells were given fresh culture medium.

Prior to the beginning of this study, we compared the microinjection of MBP–Apoptin to the transfection protocols established in our laboratory (data not shown). The visualization of Apoptin expression by immunofluorescence confirmed that microinjection led to a roughly equivalent amount and distribution of protein among the various cell types. Furthermore, the intensity of the protein signal by immunofluorescence was not markedly different from that seen following transfection of Apoptin DNA constructs.

¹ Danen-Van Oorschot et al., submitted for publication.

² Danen-Van Oorschot et al., manuscript in preparation.

Finally, transfection studies with an ectopically expressed MBP–Apoptin DNA construct did not reveal any major differences in either cytoplasmic morphology or apoptosis activity from those observed with the wild-type gene, suggesting that the MBP tag does not unduly affect Apoptin's behavior in this context. Taken together, these pilot observations confirmed that further studies using microinjected MBP–Apoptin protein would be relevant.

Indirect immunofluorescence assay

At various time points after microinjection of approximately 100 cells per dish, cells were fixed with 1% formaldehyde (freshly made in PBS) for 10 min, cold methanol for 5 min, and then cold 80% acetone (in H₂O) for 2 min. To detect the presence and cellular localization of recombinant Apoptin protein MBP–Apoptin in injected cells, we used the Apoptin-specific rabbit antibody R α VP3C, which recognizes an epitope residing between amino acids 76 and 90 in the C-terminus of Apoptin [9]. A rabbit antibody raised against a peptide mapping within the C-terminal domain of MBP (R α MBP, MBP-probe [C-18]; Santa Cruz Biotechnology, Inc.), or a monoclonal mouse antibody specific for MBP (M α MBP, ZYMED Laboratories, Inc), was used to detect not only MBP–Apoptin but also the control protein MBP. In certain experiments, we used the mouse monoclonal against human FADD (BD Transduction Laboratories, Belgium) to detect cells transfected with the construct pCDNA3-HA-FADD as a positive control for apoptosis induction. The appropriate fluorescein–isothiocyanate (FITC)-labeled goat antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa.) were used as secondary antibody. The nuclear morphology indicative of the apoptotic state was determined after DAPI (2,4-diamidino-2-phenylindole) staining. The stained cells were analyzed by fluorescence microscopy (Olympus, PROVIS AX). Images were captured by digital image analysis equipment (Sony DXC-950P, 3CCD color video camera) and processed with analySIS software (3.00, Soft Imaging System).

LysoTracker staining

Cells were microinjected with protein as described above. Twenty-four hours later, they were incubated at 37°C with the fixable acidotropic probe LysoTracker Red DND-99 (50nM; Molecular Probes) for 30 min and washed with PBS once before fixation with 2% formaldehyde for 30 min at room temperature [10]. For further immunocytochemical staining, the LysoTracker-labeled cells were permeabilized and blocked for 30 min in 5% normal goat serum plus 0.05% Tween 20 in PBS before antibodies were added. The rest of the immunofluorescence procedure was as described above.

Immunoprecipitation and Western blot analysis

Equal numbers of cells were plated and grown to 30–40% confluency in 35-mm dishes. In each dish, 500 cells were cytoplasmically injected with MBP–Apoptin protein as described above. At various intervals following microinjection, cells were washed with cold PBS and lysed for 30 min on ice in 50 μ l of mild lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100) or 50 μ l of stringent RIPA lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP40, and 1% sodium deoxycholate). As Apoptin multimers do not dissociate under denaturing conditions (8 M urea, 7 M GuHCl) (data not shown), it is very unlikely that detectable amounts of multimeric Apoptin would dissolve either in mild or RIPA buffer. Both lysis buffers were supplemented with the following protease or phosphatase inhibitors: 20 mM β -glycerol phosphate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM NaF, 200 μ g/ml trypsin inhibitor, 18 μ g/ml Na₃VO₄, 1 μ g/ml leupeptine, and 1 μ g/ml pepstatin. Cell debris was removed by microcentrifugation at 16,000g at 4°C for 10 min. The supernatant of the lysate from each clarified dish was divided into two equal aliquots (25 μ l each). Each aliquot was selected either for immunoprecipitation (IP) and Western blotting assay or for direct Western blotting analysis without IP (Non-IP), using the appropriate antibodies. For IP, supernatant aliquots were incubated overnight at 4°C on a rotator in a volume of 400 μ l lysis buffer containing 40 μ l of 10% protein A–Sepharose beads (Sigma–Aldrich, Zwijndrecht, The Netherlands), which were first coupled to either R α VP3C or M α MBP. The beads were washed three times in lysis buffer at 4°C and left with 25 μ l supernatant of lysis buffer. All IP and Non-IP aliquots were added to 4 \times denaturing Laemmli sample buffer to a final volume of 35 μ l and boiled for 4 min. Each aliquot was loaded in its entirety and fractionated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto PVDF (Immobilon) membranes. PVDF membranes were incubated with R α VP3C or R α MBP and subsequently probed with horseradish peroxidase-conjugated protein A (ProtA-HRP, Amersham) for IP samples or horseradish peroxidase-conjugated goat antibody against rabbit IgG (G α R-HRP, Amersham) for Non-IP samples. Detection was achieved with enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham).

Results

MBP–Apoptin protein retains its apoptotic potency in tumor cells

When Apoptin is ectopically expressed in mammalian cells, it may become posttranscriptionally or posttransla-

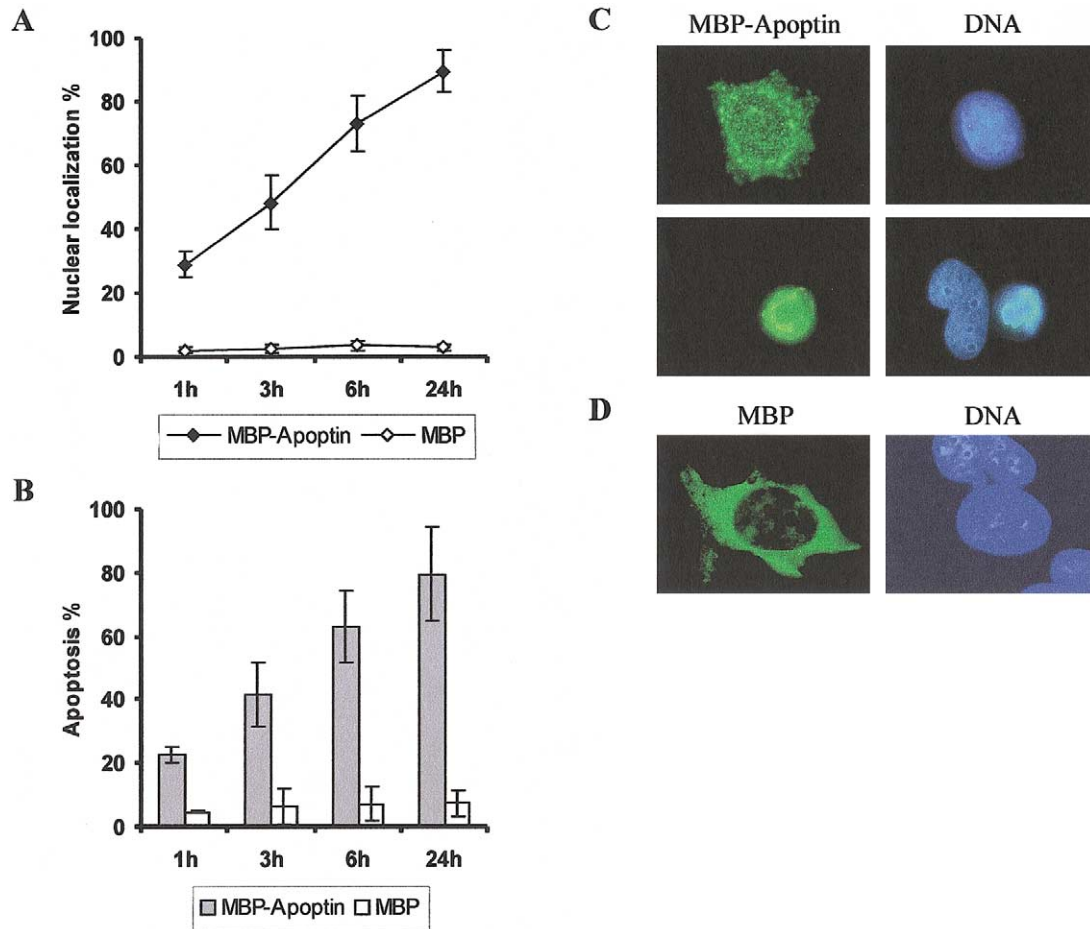


Fig. 1. MBP-Apoptin induces apoptosis in Saos-2 human tumor cells. MBP-Apoptin protein, or MBP as a negative control, was injected into the cytoplasm of Saos-2 osteosarcoma cells. To determine the presence and localization of microinjected protein, an immunofluorescence assay was performed at the indicated time points after microinjection. Apoptosis was scored by determining the nuclear morphology after DAPI staining of DNA. (A) Percentage of injected cells containing nuclear localized protein. (B) Percentage of apoptosis induction. The mean values of three independent experiments are given, with the standard deviations indicated as bars. (C) Immunofluorescence staining of MBP-Apoptin with Apoptin antibody α VP3C, showing MBP-Apoptin's translocation to the nucleus even 1 h postinjection (top left panel) and subsequent apoptosis induction 24 h later (bottom left panel) as determined with DAPI staining of the nuclear DNA (bottom right panel). (D) Immunofluorescence staining of MBP with antibody α MBP, as a control, showing primarily cytoplasmic distribution of MBP and no significant apoptosis induction (24 h), as shown in A and B.

tionally modified in a way that could be important for its function. Moreover, the presence of the N-terminal MBP tag could influence the protein's behavior. Therefore, it was important to determine whether recombinant MBP-Apoptin protein was still able to induce tumor-specific apoptosis. To this end, MBP-Apoptin was microinjected into the cytoplasm of Saos-2 cells, a human osteosarcoma cell line, and analyzed at various times both early and late postinjection (1, 3, 6, and 24 h). Fixed cells were examined by immunofluorescence microscopy for the presence of MBP-Apoptin and for the nuclear changes associated with apoptosis after staining DNA with DAPI. As shown in Fig. 1A, MBP-Apoptin protein translocated to the nucleus of Saos-2 cells with time postinjection, as reported before for the expression of the wild-type Apoptin gene after transfection [4]. Although a fair portion of the injected protein also remained

in the cytoplasm as well as accumulated around the perinuclear area, it was clear from examining multiple focal planes that there was a large amount of MBP-Apoptin within the nucleus of the injected cells as well, even as early as 1 h postinjection (Fig. 1C, top left panel). During the time course (Fig. 1A), 29% of injected Saos-2 cells contained nuclear localized MBP-Apoptin protein at the 1-h time point, which increased to 90% at the 24-h time point. Along with the nuclear translocation of MBP-Apoptin protein, the injected Saos-2 cells exhibited a high degree of apoptosis, an outcome that increased with time postinjection, reaching 80% after 24 h (Figs. 1B and C, bottom panels). In contrast, the negative control protein, MBP, did not induce significant levels of apoptosis at any time point and remained in the cytoplasm throughout the course of the experiment (Figs. 1A, B, and D).

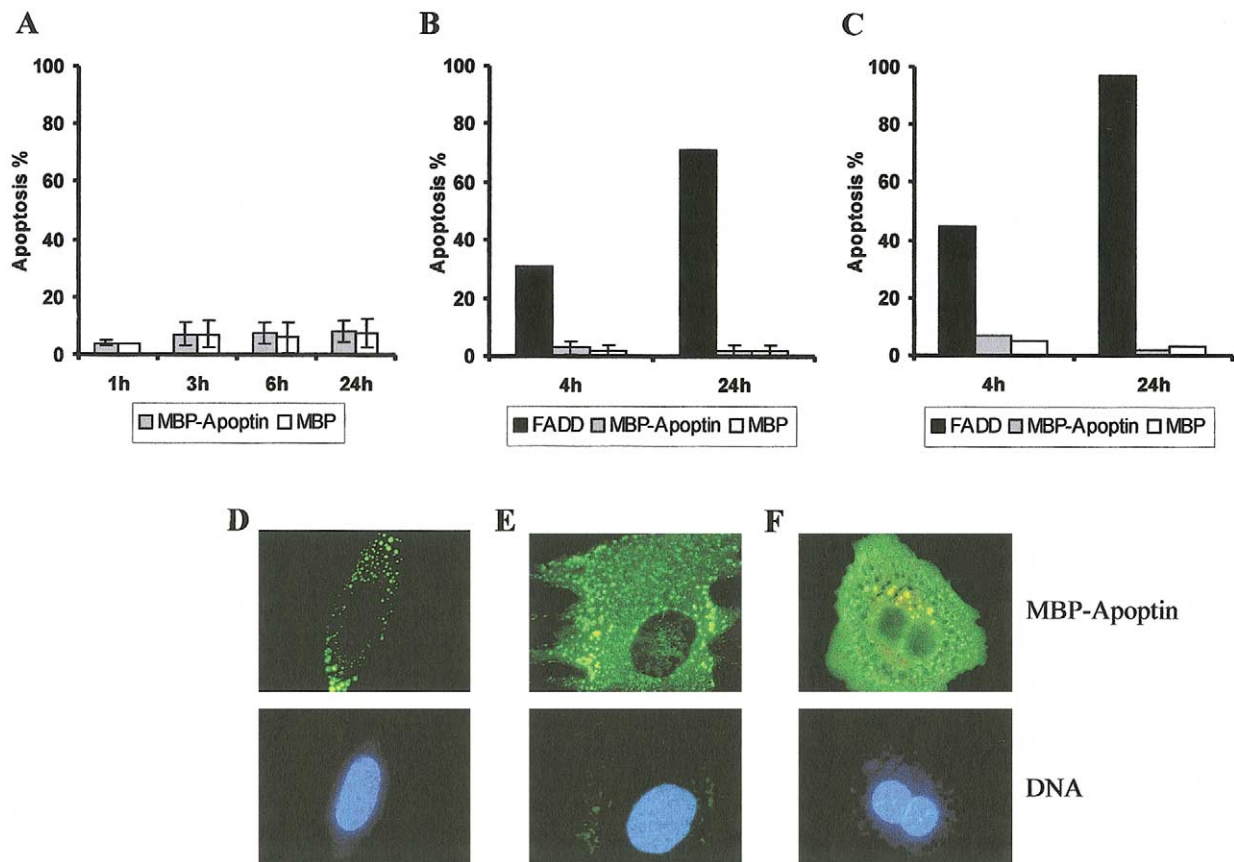


Fig. 2. MBP-Apoptin does not induce apoptosis in human primary, nontransformed cell types. (A) Percentage of apoptosis induction in VH10 diploid skin fibroblasts. Experiment was performed as described in Fig. 1. The mean values of three independent experiments are given with the standard deviations indicated as bars. Both B and C show the percentage of apoptosis induction in two human primary cell types: mesenchymal stem cells (hMSC) and hepatocytes (hNheps), respectively. For B and C, a parallel culture with a DNA construct encoding the death-inducing positive control, FADD, was injected into the nucleus, and immunofluorescence staining was carried out at 4 and 24 h postinjection. Microinjection of proteins was carried out twice in hMSC and once in hNheps. (D) VH10, (E) hMSC, and (F) hNheps show the respective immunofluorescence staining of MBP-Apoptin with an antibody against Apoptin (top panels) and nuclear staining with DAPI (bottom panels), 3 h postinjection in VH10 and 4 h in hMSC and hNheps.

MBP-Apoptin protein maintains its tumor specificity, remaining not toxic in normal cells

To confirm that neither the N-terminal fusion partner nor the lack of posttranscriptional or translational modifications that could occur during synthesis from mRNA would alter the nontoxic nature of Apoptin in normal cells, in a parallel set of injections we microinjected MBP-Apoptin protein into the cytoplasm of normal human diploid foreskin fibroblast cells (VH10). It has been shown that VH10 cells are resistant to apoptosis induced by the wild-type Apoptin gene [4]. Here, MBP-Apoptin behaved similarly to the negative control protein, MBP, in that it did not induce above-background levels of apoptosis during the same time course (Fig. 2A), suggesting that MBP-Apoptin maintains its tumor-specific apoptosis-inducing behavior *in vivo*.

Using a similar approach, we tested the apoptosis-inducing ability of MBP-Apoptin vs MBP in two primary cell types known to be particularly sensitive to chemotherapeutic agents: human primary mesenchymal stem cells (hMSC)

and human primary hepatocytes (hNheps). As a positive control to confirm that the cells were competent to undergo apoptosis, we also microinjected a plasmid encoding the death-effector FADD [11] into the nucleus of these cells in the same experiments (VH10 cells also susceptible to FADD [data not shown]). As shown in Figs. 2B and C, respectively, both cell types, although exquisitely sensitive to FADD-induced apoptosis, were not killed significantly by MBP-Apoptin, further supporting the idea that the MBP-Apoptin protein multimer is a tumor-specific apoptosis effector. In contrast to Saos-2 tumor cells, MBP-Apoptin protein remained compartmentalized primarily in the cytoplasm of the injected normal cells VH10 (Fig. 2D), hMSC (Fig. 2E), and hNheps (Fig. 2F), as did the negative control protein MBP (data not shown).

Parallel nuclear microinjection of the DNA plasmid pCMV-VP3 encoding the wild-type Apoptin gene in the same tumor and normal cells above confirmed that MBP-Apoptin protein mimicked the tumor specificity and normal cell nontoxicity of the wild-type Apoptin gene (data not

shown). Taking together with Fig. 1, these results confirm that microinjected MBP–Apoptin possesses the potency, tumor specificity, and nuclear trafficking characteristics of the wild-type version of Apoptin expressed from a gene, making it a suitable model system for further cell biological studies.

Normal-cell-specific aggregation of MBP–Apoptin

As mentioned above, after cytoplasmic microinjection and visualization with indirect immunofluorescence, MBP–Apoptin protein was consistently localized in the cytoplasm of normal VH10 cells, where it was distributed as fine particles at early time points (3 h, Fig. 3A). However, when probed with the Apoptin antibody R α VP3C at a later time point (6 h), these particles were found to have coalesced into increasingly larger aggregates that appeared to have excluded the inert Rho–Dex marker substance, manifested as a black hole in the red fluorescence field (Fig. 3A, 6 h, indicated by arrows). This aggregation and exclusion behavior was not observed with the control protein MBP (data not shown). At the 24-h time point, although the black holes visible in the rhodamine channel persisted in cells that had been microinjected with MBP–Apoptin, these deposits had in most cases lost their reactivity with the Apoptin antibody (Fig. 3A, 24 h, arrows). Specifically, of cells coinjected with Rho–Dex and MBP–Apoptin, 50 and 80% of Rho–Dex-positive cells failed to react with the Apoptin antibody R α VP3C at late time points, 6 and 24 h, after microinjection, respectively (data not shown). This failure could be due to dense deposits of Apoptin that excluded Rho–Dex and were not accessible to, or recognized by, antibodies specific for the Apoptin protein. Alternatively, these “holes” could be regions where a deposit of Apoptin protein had caused the exclusion of Rho–Dex material in the past, and when the protein eventually disappeared (by, for example, degradation) the Rho–Dex material did not return to reclaim the space.

To rule out that these normal-cell-specific aggregate structures were due merely to cell type peculiarities, we repeated the analysis in hMSC cells. As shown in Fig. 3B, similar aggregate structures were observed, and the majority of coinjected, Rho–Dex-positive cells again failed to react with the Apoptin antibody R α VP3C, 61 and 86% at 6 and 24 h, respectively (data not shown), again manifesting round and vesicle-like structures of excluded Rho–Dex material (indicated with arrows) similar to those seen in VH10 cells.

To determine whether the entire MBP–Apoptin had disappeared, or only the Apoptin portion, we stained similarly microinjected VH10 cells and hMSC cells with R α MBP, an antibody recognizing the MBP tag. In VH10 cells, MBP–Apoptin, as revealed by R α MBP, formed aggregates as seen with the Apoptin antibody. In contrast to the data presented in Figs. 3A and B, however, nearly all the cells were

double-positive even at later time points (6 and 24 h), with the MBP-positive bodies occasionally colocalizing with the black areas of Rho–Dex exclusion (data not shown). This result suggests that at least the MBP portion was still present and/or accessible in VH10 cells. However, in the second normal cell type, hMSC, 42% of Rho–Dex-traced cells were not detectable by R α MBP at 24 h (data not shown), indicating that inaccessibility or disappearance might develop with more rapid kinetics in different cell types and that the R α MBP epitope might be involved in the phenomenon as well as epitope(s) on Apoptin in this case. At the late time point of 24 h, some MBP-stained aggregate bodies became marginalized around the plasma membrane of hMSC cells (Fig. 3C, indicated with arrows). This marginalization of MBP-stained material was also observed in hNheps cells (data not shown).

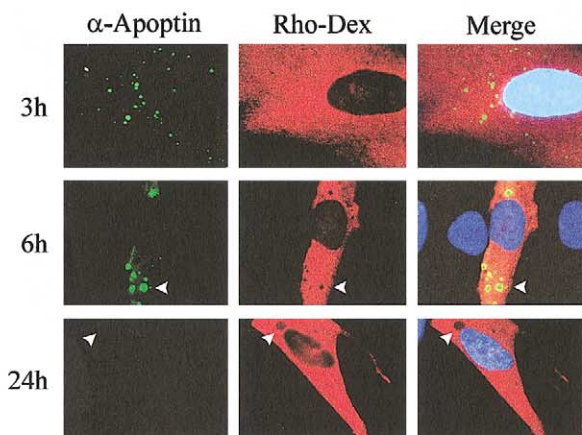
MBP–Apoptin aggregate structures in normal cells do not colocalize with acidic organelles

Because the MBP–Apoptin structures that accumulated in normal cells tended to have a round and vesicle-like appearance and because Apoptin’s loss of immunoreactivity could be due to degradation of the Apoptin portion of the recombinant protein, we wanted to determine whether these structures colocalized with acidic organelles such as lysosomes. Therefore, we performed a fluorescent double-staining of MBP–Apoptin in VH10 cells with LysoTracker Red, a fluorescent acidotropic probe for labeling and tracing acidic organelles in live cells. As shown in Fig. 4, Apoptin-containing aggregate structures, as detected with the Apoptin antibody, did not colocalize significantly with LysoTracker-stained organelles, suggesting that they are not contained within acidic organelles such as lysosomes. We obtained similar results with cells transfected with the plasmid pCMV-VP3 encoding nontagged Apoptin (data not shown). To rule out the possibility that the lysosomal compartment had been disrupted during fixation and staining, we used LysoTracker staining on cells microinjected with green fluorescein–5-maleimide-labeled MBP–Apoptin for direct observation in live VH10 cells and hMSC cells, as well as on VH10 cells transfected with GFP-tagged Apoptin. Both of these alternative approaches gave similar results (data not shown) and, together, suggest that the eventual disappearance of MBP–Apoptin protein is not caused by lysosomal degradation.

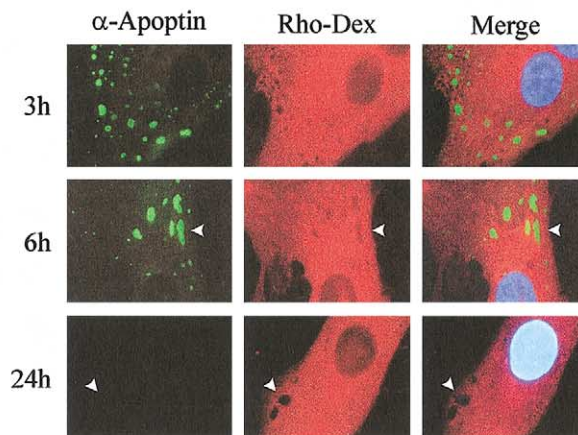
Normal-cell-specific epitope shielding of MBP–Apoptin

The apparent disappearance of Apoptin antibody reactivity in the cytoplasm of normal cells could be caused by epitope shielding, degradation, or a combination of both. In favor of the first possibility, we had already noticed that wild-type Apoptin transfected into normal cells could be

A. VH10



B. hMSC



C. hMSC, 24h

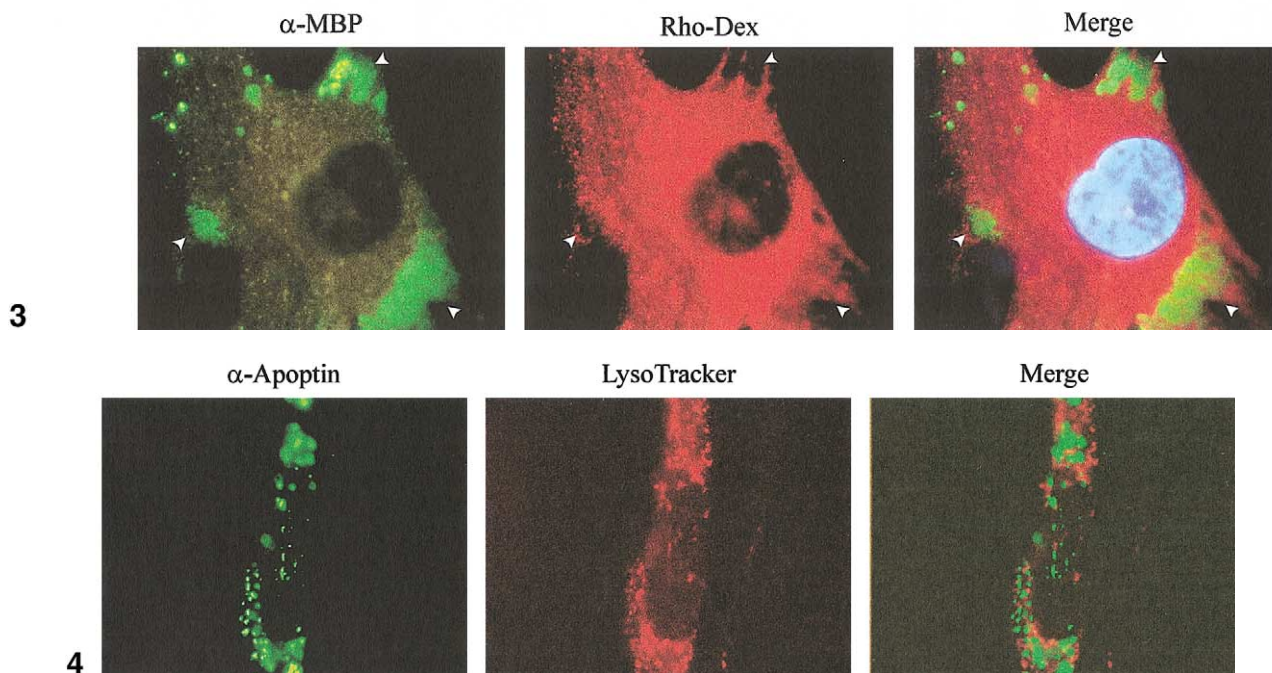


Fig. 3. MBP–Apoptin aggregates in the cytoplasm of normal cells. Immunofluorescence staining of injected cells was performed as described for Fig. 1. (A) In VH10 cells stained with the Apoptin antibody, MBP–Apoptin was visible as fine particles at an early time point (3 h) and aggregated gradually (6 h). With increasing time postinjection (24 h), the MBP–Apoptin signal disappeared, often accompanied with black areas in the Rho–Dex material (indicated with arrows). (B) In hMSC cells stained with the Apoptin antibody, MBP–Apoptin exhibited a similar development of aggregation and disappearance (indicated with arrows). (C) In hMSC cells, using the MBP antibody R α MBP, at the late time of 24 h, aggregate bodies formed by MBP–Apoptin became clustered and marginalized around the plasma membrane (indicated with arrows).

Fig. 4. MBP–Apoptin does not colocalize with lysosomes in normal cells. VH10 cells were incubated with LysoTracker Red to pre-stain lysosomal bodies 24 h after cytoplasmic microinjection with MBP–Apoptin, followed by fixation and probing with R α VP3–C antibody. The left panel (green) indicates the antibody staining, the middle panel (red) depicts the LysoTracker staining, and the right panel shows the merged image of α -Apoptin and LysoTracker staining.

readily immunoprecipitated with stringent RIPA buffer, but not at all with mild buffer.³ To address the relative contributions of epitope shielding versus degradation further, we introduced MBP–Apoptin into normal and tumor cells by microinjection and

then, after lysing the cells, traced the fate of the protein using denaturing SDS–PAGE. If epitope shielding was the explanation for Apoptin’s apparent disappearance in our previous experiments, then antibody detection should be recovered by immunoprecipitation with stringent buffers. In parallel, degradation could be assessed by inspecting the size or presence of recovered Apoptin after direct loading of lysates onto the gels.

³ Rohn, unpublished observations.

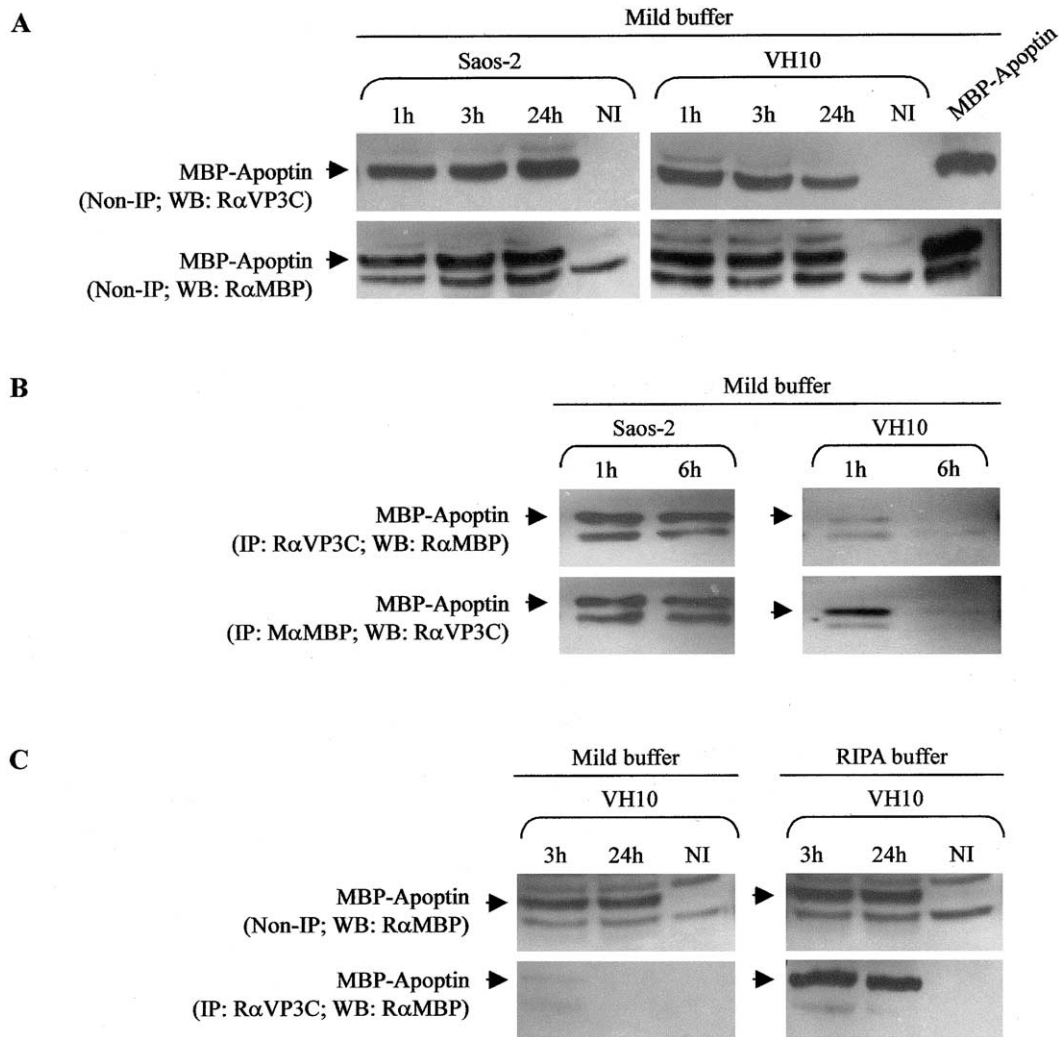


Fig. 5. MBP-Apoptin becomes epitope-shielded specifically in normal cells. Cells were microinjected with MBP-Apoptin and then lysed with either mild lysis buffer or more stringent RIPA buffer. Each lysate supernatant was divided into two equal aliquots. The aliquots were selected for either first immunoprecipitation (IP) or direct SDS-PAGE fractionation and Western blot analysis with antibody staining as indicated. In all cases, noninjected lysate (NI) was included as a control (not shown for B). For all three panels, arrows distinguish the full-length MBP-Apoptin recombinant protein from the cellular background signal (in A, bottom, and in C, top) or the comigrating truncated recombinant protein that coimmunoprecipitates with the protein complex (in B, and in C, bottom). (A) Both Saos-2 tumor cell and VH10 normal cell mild lysates were directly Western blotted without immunoprecipitation (Non-IP) and detected with R α VP3 (top) and R α MBP (bottom) antibodies at the indicated times after injection. The last lane shows 50 ng of purified MBP-Apoptin directly loaded onto the gel. (B) IP in the presence of mild buffer from tumor cells and normal cells using R α VP3C and M α MBP, and Western-detected with R α MBP (top) and R α VP3C (bottom), respectively. (C) IP with R α VP3 and Western detection with R α MBP (bottom) from normal cell lysates using stringent RIPA buffer versus mild buffer, compared to non-IP samples (top).

First, to assess the possibility of MBP-Apoptin degradation, MBP-Apoptin protein was microinjected into the cytoplasm of both VH10 and Saos-2 cells, and lysates were made using mild buffer at various times postinjection. The supernatant of each clarified lysate was split into two equal aliquots, resuspended in denaturing sample buffer, resolved by SDS-PAGE, and subjected to Western blot analysis using the antibodies against Apoptin and MBP. Noninjected cell lysate was analyzed in parallel as a negative control. Fifty nanograms of purified MBP-Apoptin was loaded directly onto the gel to indicate the original state of the input protein. The recombinant MBP-Apoptin multimer exists as a combination of full-length fusion protein and several less-

abundant and C-terminally truncated products that are stably incorporated into and copurified with the MBP-Apoptin [8] and that are variously detected depending on which antibody epitopes are still present in a given species. Here, full-length MBP-Apoptin protein could be detected (upper band) as well as one of the truncated products (lower band) when using R α MBP antibody in Western blot analysis but not when using R α VP3C (Fig. 5A, last lane). As shown in Fig. 5A, the microinjected MBP-Apoptin (indicated by an arrow) was detectable at roughly equal intensities at each time point (1, 3, and 24 h) in both cell types by both R α VP3C and R α MBP antibody, indicating that, under the denaturing conditions used, both the Apoptin and MBP

epitopes were present. Furthermore, the proteins migrated at the expected size, and no breakdown products were visible. On the bottom left and right panels, the lower band apparent in these lanes is an irrelevant cellular protein that cross-reacts with the MBP antibody, as is apparent from its presence in the noninjected control lanes of this experiment, though it happens to comigrate with the truncated MBP–Apoptin. These cellular background signals are also seen in Fig. 5C (top panels). These results indicate that the entire recombinant protein was not detectably degraded in either VH10 or Saos-2 cells, suggesting that its disappearance in immunofluorescence assays at similar time points was due to antibody inaccessibility.

To compare the antibody reactivity of MBP–Apoptin in tumor versus normal cells, we performed a similar experiment as described above, except that we first immunoprecipitated the proteins before fractionating them on gels. We chose the time points of 1 and 6 h for our analysis, as at 6 h 50% of Rho–Dex-traced VH10 cells were already unable to be detected with R α VP3C. The microinjected MBP–Apoptin was immunoprecipitated with either R α VP3C or M α MBP in the presence of mild buffer (Fig. 5B). Due to its stable incorporation into the multimer along with full-length fusion proteins, the truncated product was nevertheless pulled down under nondisrupting native conditions with the rest of the full-length multimer as expected; consequently, the lower band is detectable on Western blot after this indirect immunoprecipitation with these antibodies. Our experiment showed that, whereas the R α VP3C antibody could readily immunoprecipitate MBP–Apoptin (both the full-length and truncated forms) from lysates of tumor cells, it could not efficiently pull down either MBP–Apoptin species from normal cells lysed even 1 h after microinjection. Furthermore, MBP–Apoptin could not be immunoprecipitated at all from normal cells lysed 6 h after microinjection. Parallel analyses with Laemmli-treated samples confirmed that, as in Fig. 5A, the MBP–Apoptin protein, though not detectably immunoprecipitated, was indeed present in the lysate (data not shown). This result indicates that the C-terminal epitope of Apoptin was shielded under native conditions in normal cells but not in tumor cells, just as was seen in intact cells by immunofluorescence (Fig. 3A, 24 h). But in contrast to the immunofluorescence data obtained from VH10 cells with R α MBP, the MBP epitope also manifested significant normal-cell-specific shielding under the conditions of mild buffer immunoprecipitation (Fig. 5B, 6 h), although the shielding was not as severe as that for the VP3-C epitope. These data suggest that in normal VH10 cells the entire recombinant protein achieves a state of antibody inaccessibility that cannot be resolved under mild buffer conditions, with the Apoptin epitope being affected more severely than the MBP epitope, whereas the semistringent conditions of the immunofluorescence protocol frees the MBP epitope, but not that of VP3-C.

To analyze the situation in more detail in normal cells, we microinjected VH10 cells with MBP–Apoptin and lysed

them at 3 and 24 h postinjection with mild lysis buffer versus the strongly denaturing RIPA lysis buffer. Each lysate supernatant was divided into two equal aliquots. One aliquot was immunoprecipitated with R α VP3C, while the other aliquot was mixed directly with sample buffer for SDS–PAGE and Western blotting without immunoprecipitation. Noninjected cell lysates were made with both lysis buffers as controls. As seen in Fig. 5C, MBP–Apoptin from the nonimmunoprecipitated lysate made with either mild buffer or RIPA buffer was equally detected by R α MBP antibody at both early (3) and late (24 h) time points. Consistent with the data reported above, the Apoptin-specific antibody R α VP3C did not strongly immunoprecipitate MBP–Apoptin from the lysate made with mild lysis buffer, allowing only very weak detection of MBP–Apoptin at the early time point (3 h) and a complete lack of signal at the late time point (24 h). In contrast, the same antibody R α VP3C efficiently immunoprecipitated MBP–Apoptin from the lysate made with RIPA lysis buffer at both time points.

Taken together, these results strongly suggest that the failure to detect MBP–Apoptin in normal cells is predominantly due to global epitope shielding and that this shielding increases with time postinjection. A high stringency environment, such as that provided by RIPA or Laemmli buffer, disrupts this increasing inaccessibility, allowing both epitopes to bind their respective antibodies. Semistringent conditions, such as those resulting from immunofluorescence, reveal partial shielding of the protein (manifested as a free MBP epitope and a shielded C-terminal Apoptin epitope), while mild buffer conditions cannot facilitate antibody binding to either end of the protein once it has become occluded in the cytoplasm of a normal cell. Such variable accessibility of the protein in response to different conditions of stringency did not occur detectably in tumor cells, indicating that tumor cells provide a very different environment to the Apoptin protein.

MBP–Apoptin is eventually eliminated in normal cells

To determine the long-term fate of MBP–Apoptin protein following its epitope shielding in normal cells, we microinjected VH10 cells with MBP–Apoptin plus Rho–Dex, MBP plus Rho–Dex, or Rho–Dex alone. By fluorescent microscopy, both MBP and Rho–Dex were still detectable even after 5 days, clearly indicating that the cells did not die, whereas MBP–Apoptin disappeared within 2 days, as assessed by the antibody R α VP3C (data not shown). In stringent RIPA lysates, similar to those described above, R α VP3C could still detect MBP–Apoptin 1 day postinjection using the immunoblot assay, but the protein was only faintly detectable at day 2 and had almost entirely vanished at the 3-day time point after injection (Fig. 6). The unvarying intensity of staining of two cellular proteins, actin and cytochrome c, confirmed equal loading of cell lysates. Taken together, these data suggest that MBP–Apoptin is

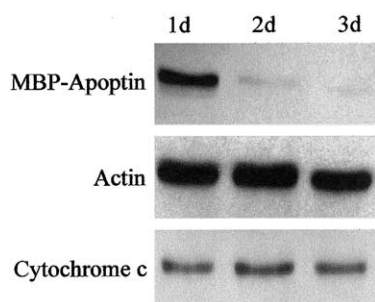


Fig. 6. MBP–Apoptin is eventually eliminated in normal cells. VH10 cells were injected with MBP–Apoptin and subjected to biochemical analyses similar to those in Fig. 5A, except that the time course was prolonged up to day 3 postinjection. As an equal loading control, blots were reprobbed with antibodies against cellular actin and cytochrome c.

unstable in normal cells, and epitope shielding occurs prior to the eventual disappearance of MBP–Apoptin.

Discussion

The remarkable tumor specificity of Apoptin together with its effectiveness against xenografted human tumors in preclinical murine studies with adenovirus delivery [5,6] support the concept of Apoptin as a promising new agent for antitumor therapy. While it has been known for many years that the tumor cell environment induces Apoptin to translocate to the nucleus and ultimately mediate apoptotic cell death [1,4], this report provides the first evidence that Apoptin can invoke a specific biochemical response in normal, nontransformed cells as well.

In this study, the recombinant Apoptin protein MBP–Apoptin mirrored the biological activity of untagged, ectopically expressed Apoptin with regard to intracellular trafficking and death induction. When microinjected into the cytoplasm of tumor cells, a large proportion of the MBP–Apoptin quickly migrated into the nucleus and induced apoptosis. Because microinjection of the control protein MBP failed to kill the cells, such activity could be ascribed to the Apoptin portion of the recombinant protein. In contrast to the situation in tumor cells, however, MBP–Apoptin remained distributed primarily in the cytoplasm of normal, nontransformed cells and did not induce apoptosis, even in particularly chemosensitive human primary cells (mesenchymal stem cells and hepatocytes). This study proves that a recombinant Apoptin protein maintains its tumor-specific killing and localization properties, indicating that a mammalian-specific folding environment is not required for these functions. Further, this work suggests that Apoptin delivered as protein therapy, for example, via liposomes or after addition of a heterologous transduction domain, might one day be a feasible alternative to conventional gene therapies with this agent.

During the course of evaluating the activity of MBP–Apoptin in normal cells, we discovered that Apoptin under-

went a series of unexpected events in the cytoplasm of normal cells. MBP–Apoptin was distributed early as fine particles and accumulated into increasingly larger bodies, often marginalized to the plasma membrane, and eventually disappeared. In parallel to these visual changes in intact cells, biochemical analyses revealed that MBP–Apoptin physically altered with time, ultimately becoming completely antibody-inaccessible under native conditions on both a C-terminal Apoptin epitope as well as an MBP epitope, with the shielding of the former appearing to be more severe or rapid than that of the latter. This epitope shielding was totally absent in tumor cells, suggesting that Apoptin exhibits a normal-cell-specific alteration in conformation and/or accessibility. This same phenomenon was also seen in immunoprecipitation studies with tumor versus normal cells transfected with the wild-type Apoptin gene,³ although the vesicle-like structures were not as strongly evident by immunofluorescence, perhaps due to the differences in delivery between a single bolus of protein versus gradual accumulation and continuous ectopic expression from a gene.

The fact that MBP–Apoptin undergoes epitope shielding following microinjection into normal cells could be caused by several mechanisms, such as covalent modification or engaging with binding partners. Indeed, Apoptin is known to bind several different cellular proteins.^{2,4} Furthermore, biophysical studies of the MBP–Apoptin protein *in vitro* have shown that it self-assembles to form multimers of approximately 30 to 40 monomeric subunits and that this multimer may very well be the active form of Apoptin *in vivo*, in conjunction with other cellular proteins [8]. This tendency of Apoptin to multimerize may underlie the eventual occurrence of visible aggregates in normal cells and the inaccessibility of Apoptin to antibodies under native conditions. Because such aggregates and epitope shielding are only seen in normal cells, tumor cells seem to provide an environment preventing the cytoplasmic accumulation of Apoptin. One possibility is that normal cells contain one or more active molecules or pathways mediating Apoptin accumulation. Another is that tumor cells would also cause Apoptin to aggregate in the cytoplasm given the opportunity, but Apoptin escapes such a fate by translocating to the nucleus in a tumor-specific way. We think this second possibility is not as likely, because residual cytoplasmic MBP–Apoptin in tumor cells is readily detectable in immunofluorescence analyses. Further experiments will be required to determine the molecular causes and consequence of normal cell-specific shielding of the function of Apoptin.

Our study suggests that MBP–Apoptin protein aggregation in the cytoplasm of normal cells is a gradual event, starting with fine particles at early time points that coalesce into large structures later, at a time point coinciding with epitope shielding. The failure of LysoTracker staining to

⁴ Danen-Van Oorschot et al., unpublished observations.

colocalize with Apoptin protein aggregates suggests that they are not contained within lysosomes or other acidic organelles and that the long-term fate of Apoptin protein, being eventually eliminated from normal cells, is mediated by some other pathway. Biophysical studies have shown that untagged recombinant Apoptin protein can be highly susceptible to proteolysis, at least *in vitro* (data not shown). Preliminary evidence suggests that Apoptin is ubiquitinated *in vivo* and thereby degraded by the proteasome pathway.⁵ Indeed, Apoptin protein expression in normal cells derived from mice transgenic for Apoptin can be significantly enhanced by treatment with various proteasome inhibitors, suggesting that Apoptin degradation is a very prominent feature in the cytoplasmic compartments of such cells.⁵ In addition to its degradation, the marginalization of MBP–Apoptin (Fig. 3C) seen at late time points may be a prelude to expulsion or secretion of the aggregate bodies through the plasma membrane. Further studies on the pathways and mechanisms by which Apoptin is selectively neutralized in normal cells are underway.

Protein aggregation is known to be associated with disease. For example, aggregation of proteins such as prions, synuclein- α , and amyloid- β are all associated with toxic effects in human tissues [12,13]. Some aggregation-prone proteins have been directly implicated in apoptosis induction [14,15] and, recently, it has been reported that protein misfolding may be a universal cause of cell death and that the type of aggregate formed can be a major determinant of cytotoxicity [16,17]. Although Apoptin may share some characteristics with such self-assembling toxic and/or apoptosis-inducing aggregates, it differs in at least one important aspect: the death it induces is tumor-specific. Moreover, our current results show that an aggregate can manifest yet another level of specificity: a neutralization or sequestration in normal, healthy cells, which may help to render Apoptin nonfunctional. Such a feature enhances further the attractiveness of Apoptin as a safe, effective candidate for cancer therapy.

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References

- [1] M. Noteborn, Apoptin as an anti-cancer agent, in: N. La Thangue, L. Bandara (Eds.), *Targets for Cancer chemotherapy: transcription factors and other nuclear proteins*. Cancer Drug Discovery and Development, Humana Press, Totowa, NY, 2002, pp. 275–288.
- [2] M.H. Noteborn, D. Todd, C.A. Verschueren, H.W. de Gauw, W.L. Curran, S. Veldkamp, A.J. Douglas, M.S. McNulty, A.J. van der Eb, G. Koch, A single chicken anemia virus protein induces apoptosis, *J. Virol.* 68 (1994) 346–351.
- [3] M. Noteborn, G. Koch, Chicken anaemia virus infection: molecular basis of pathogenicity, *Avian Pathol.* 24 (1995) 11–31.
- [4] A.A. Danen-van Oorschot, D. Fischer, J. Grimbergen, B. Klein, S.-M. Zhuang, J. Falkenburg, C. Backendorf, P. Quax, A. van der Eb, M. Noteborn, Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells, *Proc. Natl. Acad. Sci. USA* 94 (1997) 5843–5847.
- [5] A. Pietersen, M. van der Eb, H. Rademaker, D. van den Wollenberg, M. Rabelink, P. Kuppen, J. van Dierendonck, H. van Ormondt, D. Masman, A. van der Eb, R. Hoeben, M. Noteborn, Specific tumor-cell killing with adenovirus vectors containing the apoptin gene, *Gene Ther.* 6 (1999) 882–892.
- [6] M. Van der Eb, A. Pietersen, F. Speetjes, P. Kuppen, C. van de Velde, M. Noteborn, R. Hoeben, Gene therapy with Apoptin induces regression of xenografted human hepatomas, *Cancer Gene Ther.* 9 (2002) 53–61.
- [7] J. Rohn, Y. Zhang, R. Aalbers, N. Otto, J. den Hertog, N. Henriquez, J. van de Velde, P. Kuppen, D. Mumberg, P. Donner, M. Noteborn, A tumor-specific kinase activity regulates the viral death protein Apoptin, *J. Biol. Chem.* 277 (2002) 50820–50827.
- [8] R. Leliveld, Y. Zhang, J. Rohn, M. Noteborn, P. Abrahams, Apoptin induces tumor-specific apoptosis as a globular multimer. *J. Biol. Chem. J. Biol. Chem.* epub ahead of print, 19 Dec 2002.
- [9] A. Danen-van Oorschot, A. Van der Eb, M. Noteborn, The chicken anaemia virus-derived protein Apoptin requires activation of caspases for induction of apoptosis in human tumour cells, *J. Virol.* 74 (2000) 7072–7078.
- [10] C. Bucci, P. Thomsen, P. Nicoziani, J. McCarthy, B. van Deurs, A key to lysosome biogenesis, *Mol. Biol. Cell* 11 (2000) 467–480.
- [11] A. Chinnaiyan, K. O'Rourke, M. Tewari, V. Dixit, FADD, a novel death domain-containing protein, *Cell* 81 (1995) 505–512.
- [12] S. Lovestone, D. McLoughlin, Protein aggregates and dementia: is there a common toxicity? *J. Neurol. Neurosurg. Psych.* 72 (2002) 152–161.
- [13] M. Khan, R. Falk, Amyloidosis, *Postgrad. Med. J.* 77 (2001) 686–693.
- [14] S. Li, S. Lam, A. Cheng, X. Li, Intracellular huntingtin increases the expression of caspase-1 and induces apoptosis, *Hum. Mol. Genet.* 9 (2000) 2859–2867.
- [15] K. Andersson, A. Olofsson, E. Nielsen, S. Svehag, E. Lundgren, Only amyloidogenic intermediates of transthyretin induce apoptosis, *Biochem. Biophys. Res. Commun.* 294 (2002) 309–314.
- [16] M. Buccianti, E. Giannoni, F. Chiti, F. Baroni, L. Formigli, J. Zurdo, N. Taddei, G. Ramponi, C. Dobson, M. Stefani, Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases, *Nature* 416 (2002) 507–511.
- [17] K. Dahlgren, A. Manelli Jr., W. Stine, L. Baker, G. Krafft, M. LaDu, Oligomeric and fibrillar species of amyloid- β peptides differentially affect neuronal viability, *J. Biol. Chem.* 277 (2002) 32046–32053.

⁵ Pietersen et al., unpublished observations.