A Tumor-specific Kinase Activity Regulates the Viral Death Protein Apoptin*

Received for publication, August 21, 2002, and in revised form, October 3, 2002
Published, JBC Papers in Press, October 21, 2002, DOI 10.1074/jbc.M208557200

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Apoptin, a chicken anemia virus-encoded protein, is thought to be activated by a general tumor-specific pathway, because it induces apoptosis in a large number of human tumor or transformed cells but not in their normal, healthy counterparts. Here, we show that Apoptin is phosphorylated robustly both in vitro and in vivo in tumor cells but negligibly in normal cells, and we map the site to threonine 108. A gain-of-function point mutation (T108E) conferred upon Apoptin the ability to accumulate in the nucleus and kill normal cells, implying that phosphorylation is a key regulator of the tumor-specific properties of Apoptin. An activity that could phosphorylate Apoptin on threonine 108 was found specifically in tumor and transformed cells from a variety of tissue origins, suggesting that activation of this kinase is generally associated with the cancerous or precancerous state. Moreover, analyses of human tissue samples confirm that Apoptin kinase activity is detectable in primary malignancies but not in tissue derived from healthy individuals. Taken together, our results support a model whereby the dysregulation of the cellular pathway leading to the phosphorylation of Apoptin contributes to human tumorigenesis.

To maximize the potential of a relatively small genome, viruses have often evolved mechanisms to exploit host cell signal transduction pathways. Such versatility is typified by oncogenic agents like human papillomavirus and SV40, which have evolved convergently to express proteins that bind to the cellular effectors p53 and pRb. This virus-host interaction results in deregulation of the cell cycle, thus facilitating viral replication (1, 2). Given the diverse strategies employed by the viruses that infect higher organisms, it is therefore no surprise that studies into the mechanisms by which the host machinery is subverted during infection have unearthed a wealth of information about a wide variety of cellular processes.

Chicken anemia virus (CAV),1 an endemic and agriculturally important global pathogen, induces apoptosis and thereby serves depletion of cortical thymocytes and erythroblastoid cells in the bone marrow of newborn chickens, often with fatal consequences (3–6). CAV encodes three proteins, one of which is Apoptin (also known as VP3 (7)). Apoptin is a small protein of 121 amino acids. Although it has no known cellular endogenous counterpart or homologous regions, it does contain two putative nuclear localization signals and a hydrophobic stretch reminiscent of a nuclear export signal (6). In transformed chicken cells in vitro, the ectopic expression of Apoptin is sufficient to recapitulate the apoptosis seen during virus infection (8).

Subsequent overexpression studies in human cells led to an unexpected finding; namely, in human tumor or transformed cells, Apoptin translocated rapidly to the nucleus and induced apoptosis, whereas in primary, untransformed cells, it remained dispersed predominantly in the cytoplasm and did not kill the cell (9). This intriguing correlation ultimately held true in a wide variety of cell types, with no known exceptions to date (>70 cell types tested; see Ref. 10). Moreover, Apoptin has also demonstrated long term survival benefits with negligible toxicity when delivered with an adenovirus vector in mice using a xenografted human hepatoma model (11, 12).

Tumor formation requires the acquisition of a discrete collection of genetic lesions, including disruptions in the processes of cell cycle progression, apoptosis, and telomere maintenance (13), and it is likely that more pathways remain to be elucidated in this complex process (14). We have therefore been investigating the mechanism of action of Apoptin with the goal of uncovering novel pathways and processes involved in human neoplasia. Apoptin was shown to induce apoptosis independently of functional p53 (15), and its activity could be enhanced rather than inhibited by the anti-apoptotic gene Bcl-2 (16). More recently, it was demonstrated that Apoptin relies on downstream effector caspase activation to induce apoptosis and stimulates cytochrome c release from the mitochondria. These results indicate that Apoptin execution likely feeds into the classical apoptosis pathway (17). However, the upstream activation requirements of Apoptin have remained unknown, and up until this point, none of the mechanistic information reported has explained the tumor specificity of Apoptin.

One logical regulation of the tumor-specific action of Apoptin could lie in its provocative differential nuclear trafficking behavior. However, whereas nuclear localization in tumor cells seems to be necessary for the apoptotic activity of Apoptin, it is not sufficient, as forcing Apoptin into the nucleus of normal

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1 The abbreviations used are: CAV, chicken anemia virus; pCMV, cytomegalovirus promoter; GFP, green fluorescent protein; GST, glutathione S-transferase; MALDI, matrix-assisted laser desorption/ionization; MBP, maltose-binding protein; HPLC, high performance liquid chromatography.

2 M. Noteborn, unpublished observations.
cells does not result in apoptosis (18, 19). Therefore, we pos-
tulated that a further tumor-specific activation step was re-
quired. Because phosphorylation is known to regulate the func-
tion of many proteins (20), and because Apoptin is rich in ser-
ines and threonines, we sought to determine whether the be-
havior of Apoptin could be explained by such modification.
Here, we show that Apoptin is regulated by a kinase activity
present in sAPO cell lines from the tumor cell lines of origins
as well as in clinical tumor samples, but negligibly in healthy cells
and tissues. These studies reveal the existence of a common
tumor-specific pathway that may be involved in the compi-
cated process of tumorogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—All cells were cultured at 37 °C in a humidified 10% CO2
culturater in Dulbecco’s modified Eagle’s medium containing 10%
fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin
(Invitrogen) except human bone-marrow-derived mesenchymal stem
cells (BioWhittaker Europe SPRL, Belgium) which were rec-
ommended in their accompanying medium MSCGM Bulletkit and
used between passages 2 and 4, and keratinocyte-based cultures, which
were grown at CO2 levels of 7.5% in Dulbecco’s modified Eagle’s medi-
um/Ham’s F-12 (3:1) supplemented with 5% bovine calf serum, 10−6 M
isoproterenol, 10−4 M hydrocortisone, and 10−7 M insulin and used between
passage 2. All diploid neonatal foreskin-derived fibroblasts were used
below passage 14.

Constructs—Wild-type Apoptin (also known as VP3) was overex-
pressed either using pCMV-VP3 (9) (see Fig. 3E) or αAPO, a construct in
pRRESneo (Clontech) expressing a variant of Apoptin (Arg→Lys
mutation at position 116) whose expression and behavior are indistin-
guishable from that of pCMV-VP3. All in-frame green fluorescent pro-
tein (GFP)-fused deletion mutants of Apoptin were constructed with
standard linker and restriction enzyme strategies using the vector
pGEX-T vector (Amersham Biosciences), providing an in-frame glutath-
ione S transferase tag on the N terminus of the protein (when green fluo-
cent protein is used; a gift from Bob van de Water), and MBP-probe (against
malto-β-glucosidase protein; Santa Cruz Biotechnology, Inc.). For the p53
controls shown in Fig. 2, the empty vector-transfected samples were
immunoprecipitated with an irrelevant antibody (VP3-C) so as not to
drown out endogenous p53 from VH10 cells as a better comparison for
ectopic p53 expression in the adjacent lanes.

In Vitro Kinase Assay and Analysis of Human Tissue Sections—
Exponentially growing cell cultures, or sections (ranging from 5 to 20
μm) cut from blocks of fresh tissue snap-frozen in isopentane, were
lyzed by repeated freeze-thawing in kinase buffer (20 mM HEPes, pH
7.4, 20 mM MgCl2, 150 mM NaCl, and standard inhibitors of proteases
and phosphatases, the same as used for radioimmunoprecipitation
assay buffer lysis (see above), as well as the phosphatase inhibitor
cocktails 1 and 2 (Sigma)). Rectal cancer tissues were chosen based on
previous TNM3-based classification. Flanking sections were re-anal-
ized by standard hematoxylin and eosin staining, and the tumor cell
index was calculated by multiplying the approximate percentage of
tumor material in the section by the approximate percentage of tumor
cells in the involved area. Equal amounts (10–20 μg, depending on the
experiment) of protein from clarified supernatants were added to equal
amounts of recombinant Apoptin substrate (0.5–3.0 μg, depending on the
experiment) in the presence of 50 μM ATP, 0.15 μM of γ-[32P]ATP
(when radioactive label was used), and 2 mM dithiothreitol. In some
experiments, control reactions were performed without ATP and in the pres-
ence of EDTA to inhibit kinase activity. Phosphorylated proteins were
subjected to Western blot analysis before autoradiography (for radioac-
tive ATP) or immunopurophipping with purified α108-P, α-MBP, or anti-
Apoptin 111.3 antibodies. The preservation of general kinase activity in
normal cell material was generally confirmed by analyzing the sample
supernatants for the presence of radiolabeled cellular phosphoproteins.

In Vivo Phosphorylation Assay—Transfected cultures were pre-
treated in phosphate-free Dulbecco’s modified Eagle’s medium supple-
mented with dialyzed serum (Sigma) for 10 min before metabolic label-
ing with 1 μCi/ml [32P]orthophosphate for 4 h. After lysis in cold radiomi-
nume precipitation assay buffer, proteins were immunoprecipita-
ted with the antibodies VP3-C, α-GFP, or DO-1 as appropriate. Pur-
ified immunoprecipitates were resolved by SDS-PAGE and Western
blot, and the polyclinidine difluoride membranes were subjected to
autoradiography and then immunostained with 111.3 or anti-GFP for
precise overlaying of the radioactive signal with the antibody signal. All
films were exposed along with fluorescent marks to facilitate subse-
quent orientation for band identification (see below).

Phosphoamino Acid and Peptide Mapping—Radioactive bands cor-
responding to phosphorylated Apoptin or negative controls were excised
from polyclinidine difluoride, digested with Tpck-trypsin, and sub-
jected to phosphoamino acid analysis using the method of Hunter and
co-workers (22). Briefly, thin-layer electrophoresis was performed with
1.5 kV for 20 min in pH 1.9 buffer in the first dimension; the plates were
rotated 90° and run at 1.3 kV for 16 min in pH 3.5 buffer in the second
dimension. The phosphoamino acid standards (Sigma) were visualized
by ninhydrin staining, and the plates were subjected to phosphorimag-
ing analysis to detect the radioactive spots. Samples split off from the
appropriate lanes were subjected to parallel tryptic mapping, also using the
method of Hunter and co-workers (22); thin-layer electrophoresis was
performed in the first dimension at 1 kV for 25 min in pH 1.9 buffer and
Phosphochrom liquid chromatography in the same orientation for the
second dimension. Dried plates were subjected to phosphorimag-
ing analysis.

Spectroscopy—In vitro phosphorylated GST-Apoptin protein was
purified from lysates by standard glutathione-Sepharose purifica-
tion, carbamylated, and precipitated with trichloroacetic acid, and
following resuspension was digested with Tpck-treated trypsin. The
peptides were eluted, and the radioactive fractions were identified
using reverse-phase high performance liquid chromatography (HPLC).
The fractions containing radiolabeled phosphate additions were then

2 TNM classification system (31) is as follows: T, the extent of the primary tumor; N, condition of the regional lymph nodes; M, the
absence or presence of distant metastases.

1 A. Danen-van Oorschot, Y.-H. Zhang, S. R. Leliveld, J. Rohn, M.
Seelen, M. Bolk, A. van Zon, S. Erkeland, J. P. Abrahams, D. Mumberg,
and M. Noteborn, manuscript in preparation.

2 S. R. Leliveld, Y.-H. Zhang, J. Rohn, M. Noteborn, and J. P.
Abrahams, submitted for publication.
FIG. 1. Apoptin is phosphorylated by Saos-2 tumor cell lysates but negligibly by normal human menescymal stem cell (hMSC) lysates in vitro. Left, autoradiograph of in vitro kinase assay samples resolved by SDS-PAGE and transferred to nitrocellulose (M-Ap, MBP-Apoptin substrate; MBP, control substrate). Arrows indicate the migration of the two substrates. Right, the same membrane immunostained with anti-MBP (α-MBP).

subjected to matrix-assisted laser desorption/ionization (MALDI) analysis on a Voyager-DE STR (Applied Biosystems). Phosphorylated peptides were analyzed by electron spray ionization collision-induced dissociation on a QSTAR (Applied Biosystems) to assign the phosphorylated modification to a particular amino acid residue.

Antisera Production—Production and purification of the α108-P phosphospecific antibody were performed by EuroGentec (Belgium) using standard methods. Briefly, the phosphopeptide SLITT(tyrosine phosphopeptide) (corresponding to Apoptin residues 103–115) was incoated into rabbits, and resultant sera were tested for specific reactivity to the phosphorylated peptide by enzyme-linked immunosorbent assay and column-purified against the phosphorylated peptide. Next, those recovered antibodies were passed over a non-phosphopeptide column. The flow-through of the second column was designated α108-P, and its phosphospecificity was confirmed by competitive enzyme-linked immunosorbent assay tests using pre-incubations with various phosphorylated or nonphosphorylated peptides as appropriate. As a control, antibody that bound to the second column, which presumably represented species recognizing the peptide regardless of phosphorylation state, was able to detect tumor- or normal cell-derived Apoptin equally well.

Immunofluorescence and Apoptosis Assays—DNA constructs (50 ng/μl) were introduced into the nuclei of normal VH10 diploid fibroblasts growing on glass-bottomed plates (MatTek) using standard nuclear microinjection techniques. Cells were fixed sequentially with 1% formaldehyde (10 min), cold methanol (5 min), and cold 80% acetone (2 min) and then stained and analyzed by indirect immunofluorescence for Apoptin (using antibody 111.3) and 2,4-diamidino-2-phenylindole staining for the morphological appearance of apoptosis essentially as described (9). Microinjected DNAs were mixed with dextran-conjugated tetramethylrhodamine (1 ng/ml; Molecular Probes) to help trace the injected cells. At least 100 Apoptin-positive cells were scored per experiment, and several independent experiments were performed. For visualizing phosphorylated Apoptin in intact tumor cells, Saos-2 cells growing on glass coverslips in six-well plates were transfected with 2 μg of pCMV-VP3 DNA using Fugene (Roche Molecular Biochemicals), fixed with fresh methanol:acetone (1:1) (5 min at room temperature), and stained and analyzed as above, except cells were incubated simultaneously with the antibodies 111.3 to detect all Apoptin regardless of phosphorylation state and with α108-P to detect phosphorylated on Thr-108.

RESULTS

Apoptin Is Phosphorylated in Vitro Specifically by Tumor or Transformed Cell Lysates—To determine whether tumor cell lysates contained an activity that could phosphorylate Apoptin, we developed a radioactive in vitro kinase assay using a bacterially produced recombinant Apoptin protein possessing an N-terminal MBP tag as a substrate. As shown in Fig. 1, the Apoptin portion of this substrate was phosphorylated robustly by a lysate derived from the human osteosarcoma cell line Saos-2 but negligibly by one from primary human mesenchymal stem cells. These results were extended with additional tumor or transformed cell lines (including human Jurkat T lymphoma, U2OS osteosarcoma, HT29 colon carcinoma, LCL Epstein-Barr transformed B lymphoma, and COS-1 SV40-transformed monkey kidney fibroblasts) versus normal cells (two independent sources of human neonatal foreskin fibroblasts, including VH10 (23), as well as low passage mouse embryo fibroblasts) (data not shown). Similar results were obtained with different E. coli-produced recombinant fusion proteins, including Apoptin bearing a C-terminal hexahistidine tag (Apoptin-His) and an N-terminal glutathione S-transferase tag (GST-Apoptin) (data not shown). These results indicate that an activity enriched in tumor or transformed cells, but not in healthy primary cells, can phosphorylate Apoptin.

Apoptin Is Phosphorylated in Vivo Specifically in Tumor or Transformed Cells—To determine whether Apoptin becomes phosphorylated differentially in live cells, we transfected Apoptin into various tumor cells versus normal VH10 fibroblasts and assessed the incorporation of radiolabel using immunoprecipitation and SDS-PAGE after metabolic 32Porthophosphate labeling. As shown in Fig. 2A, Saos-2 osteosarcoma cells did indeed harbor phosphorylated Apoptin, whereas in low passage VH10 cells there was no detectable radiolabel on a comparable amount of Apoptin protein. As a control, expression and phosphorylation of ectopically expressed p53 were detectable in both cells types (Fig. 2B). In vivo phosphorylation of Apoptin also occurred in U2OS cells and H1299 lung carcinoma cells (Fig. 2A). This specific difference in phosphorylation state was not trivially because of cell type, as an SV40-transformed version of VH10 fibroblasts (VHSV), but not its untransformed primary counterpart, was able to phosphorylate Apoptin in vivo (Fig. 2C). A similar result was seen for the SV40-transformed keratinocyte line SVK14 versus a non-transformed passage one breast-derived keratinocyte culture (data not shown). These results suggest that Apoptin becomes modified specifically in a tumor or transformed environment.

Apoptin Is Phosphorylated on Threonine 108—Because phosphorylation of Apoptin correlated with the transformed or tumorigenic state of the cell both in vitro and in vivo, we set out to map the modified residue to aid further exploration into the possible functional significance. Phosphoamino acid analysis of in vivo labeled protein derived from pCMV-VP3-transfected Saos-2 tumor cells versus vector-transfected controls revealed that specific phosphorylation occurred strongly on threonine(s) (Fig. 3A). A similar result was obtained using U2OS cells (data not shown). We also saw a fainter phosphoserine signal, but this activity occurred in vector-transfected samples, as well, and was therefore likely derived from a closely migrating cel-
Fig. 3. Apoptin is phosphorylated on threonine(s) within residues 106–108. A, phosphoamino acid analysis of in vivo-ortholabelled Saos-2 cells transfected with Apoptin (Ap; left) or the empty vector (Vec; middle). The position of the standards is depicted in the right panel; X marks the origin. B, tryptic peptide map of parallel samples from A: Apoptin (Ap; left) and empty vector (Vec; right). X marks the origin. C, in vivo phosphorylation of deletion mutants of Apoptin with an N-terminal GFP (G) tag in Saos-2 cells; left is the autoradiograph, and right is the same membrane immunostained with anti-GFP. D, in vivo phosphorylation of alanine mutants of Apoptin (see E for mutant nomenclature). These mutants habitually migrate as doublets; the position of the lower, wild-type-sized species is noted with arrows. The top panel shows the autoradiograph, and the bottom panel shows the immunostaining with anti-Apoptin 111.3. E, the amino acid sequence of Apoptin (pCMV-VP3), with the positions of the relevant predicted tryptic cleavage sites (upward arrows) and alanine mutations noted underneath. Thr-108 is underlined.

lular contaminant. Parallel analyses using tryptic phosphopeptide mapping in Saos-2 cells demonstrated that only a single tryptic fragment was radiolabeled, indicating that the complexity of Apoptin phosphorylation was fairly low (Fig. 3B).

To narrow the region of phosphorylation further, we transfected Saos-2 cells with a series of N-terminal GFP-fused deletion mutant constructs of Apoptin and assessed the phosphorylation status of their expressed products using the in vivo orthophosphate labeling assay. As shown in Fig. 3C, whereas the N-terminal half of Apoptin (GFP-1-69) was not phosphorylated over the background of the assay (GFP alone), the C-terminal half (GFP-70–121) was as strongly phosphorylated as the full-length fusion protein (GFP-1–121). The analysis of smaller truncation mutants showed that the minimal domain required for phosphorylation resided between residues 100 and 121 (GFP-100–121), although including the region from 80 to 99 (GFP-80–121) resulted in more robust modification.

The sequence between amino acids 100 and 121 contains only four threonine residues, a triple Thr run (106–108) on one predicted tryptic fragment and a lone Thr (position 114) on a separate fragment (Fig. 3E). To distinguish between these two loci, as well as to assess the contribution to robust phosphorylation of other regions between 80 and 121, we tested a series of five-alanine scanning mutants in the ortholabeling assay. As shown in Fig. 3D, abolishing the triple Thr run with a stretch of alanines from 106 to 110 (mutant Ala-106) completely eliminated phosphorylation of Apoptin in Saos-2 cells in vivo, whereas replacing the sequence including the lone Thr by five alanines at 111–115 (mutant Ala-111) had no effect. Taken together with the phosphoamino acid analysis and tryptic phosphopeptide mapping data, this result suggests that phosphorylation of wild-type Apoptin occurs in the triple Thr region (106–108).

Our experiments also demonstrated that replacement of region 91–95 with alanine residues (mutant Ala-91) reduced the phosphorylation efficiency reproducibly (Fig. 3D). This result is consistent with the reduced phosphorylation seen with GFP-100–121 compared with GFP-80–121. Most likely the domain including 91 through 95 is required for efficient phosphorylation at the triple Thr region, perhaps contributing directly to the kinase docking site or indirectly to conformation at the phosphorylation site, but does not itself contain a modified residue. Five alanine replacements elsewhere between 86 and 121 had no effect on phosphorylation (data not shown; Ala-81 to Ala-85 could not be assessed, because these mutations abolish the immunoprecipitation epitope).

To identify the phosphorylation site(s) conclusively, recombinant GST-Apoptin protein was phosphorylated by Saos-2 lysates in the radioactive in vitro kinase assay. After purification, the phosphorylated substrate was digested with trypsin, and the radiolabeled fragments were identified using reverse-phase high performance liquid chromatography. Afterward, these radioactive peptides were analyzed by mass spectrometry. This experiment showed that Apoptin was phosphorylated solely on threonine 108 (Fig. 4).

Thr-108 Is Phosphorylated in Vivo—To confirm that Thr-108 was indeed phosphorylated in living cells, we raised and purified a rabbit polyclonal antibody (α108-P) against a short peptide phosphorylated at position 108 and determined its reactivity against ectopically expressed Apoptin in various tumor cells versus normal cells using Western blot analysis (a representative example using Saos-2 versus VH10 cells is shown in Fig. 5A). As expected, this antibody detected tumor cell-derived Apoptin strongly but the normal cell-derived protein only faintly, thus confirming that Thr-108 is phosphorylated in vivo preferentially in tumor cells.

To confirm that Apoptin was phosphorylated on Thr-108 in intact cells, the α108-P antibody was also used to detect Apoptin with immunofluorescence microscopy. In Saos-2 cells, nearly all of the pre-apoptotic cells (>90%) that stained positively for Apoptin protein with the global Apoptin antibody 111.3 were simultaneously positive with the phosphospecific antibody α108-P (Fig. 5B shows a representative example of Apoptin localized in the nucleus, 2 days post-transfection). We observed similar results in HeLa and U2OS tumor cells (data not shown). The staining pattern of the two antibodies mostly overlapped in both cytoplasm and nucleus, except that α108-P...
staining seemed enriched in the nucleus but absent from the characteristic round, subnuclear bodies that Apoptin tends to form in tumor cells (see Fig. 5B, especially noticeable in the merged image). Moreover, reactivity with α108-P was not observed frequently in end-stage apoptotic cells (data not shown), perhaps because the energy levels of dying cells (which are known to have compromised mitochondrial function (17)) are not sufficient to maintain the phosphorylated epitope, or because Apoptin is dephosphorylated actively at late time points. This immunofluorescence experiment shows that phosphorylation of transfected Apoptin on Thr-108 occurs in virtually every pre-apoptotic tumor cell, the majority of which will eventually undergo apoptosis, and strengthens the correlation between the phosphorylation of Apoptin and the induction of apoptosis.

To confirm further that Thr-108 phosphorylation can be mediated by tumor cells other than Saos-2, we screened various cell types using a non-radioactive in vitro kinase assay with the α108-P antibody and Apoptin-His recombinant protein as a substrate and verified that the Thr-108 modification was mediated by lysates of transformed or tumor cell lines derived from diverse tissue types but not by primary normal cell material. Fig. 5C shows one representative normal cell type, primary human breast keratinocytes (left panel), versus various tumor cell types (middle panel; right panel is the Saos-2 control for reference) as follows: U2OS osteosarcoma, Jurkat T lymphoma, MDA-MB-231 breast carcinoma, DU145 prostate cancer, CC-LP and CC-SW cholangiocarcinoma, and A549 lung carcinoma. Taken together, our various results with the antibody α108-P indicate that phosphorylation on Apoptin at position Thr-108 in vivo is associated with the tumor specificity of Apoptin and that this activity can be found in a variety of cultured human tumor cell types both in vivo and in vitro.

Phosphorylation on Thr-108 Activates Apoptin—Our data thus far showed a correlation between the phosphorylation of Apoptin on Thr-108 and a transformed or tumorigenic environment. Because the ability of Apoptin to kill human cells is also associated with such an environment, we hypothesized a link between this modification and Apoptin function. To assess this issue, we mutated Thr-108 to a glutamic acid (T108E) to mimic constitutive phosphorylation and expressed the construct in normal cells. The substitution of this negatively charged amino acid at position 108 conferred a dramatic gain-of-function activity in human diploid VH10 fibroblasts, imparting the ability of T108E not only to induce apoptosis (Fig. 6, A–F) but also to translocate to the nucleus (Fig. 6D). In the same experiment, both wild-type Apoptin and an Apoptin construct containing Thr-108 mutated to an alanine residue (T108A) remained primarily in the cytoplasm and did not induce apoptosis (Fig. 6, A–C). The T108E gain-of-function was also seen in a batch of independently derived foreskin fibroblasts and in primary human mesenchymal stem cells of bone marrow origin (data not shown). Comparisons between the ability of wild-type Apoptin to kill tumor cells and the gain-of-function mutant to kill normal cells showed largely similar kinetics of apoptosis induction (data not shown). These data strongly suggest that phosphorylation of Thr-108 is sufficient to allow the characteristic tumor-specific trafficking and apoptotic behavior of Apoptin. Therefore, whereas the machinery needed for Apoptin to execute apoptosis is apparently present in both normal and tumor cells, the activation of Apoptin by the upstream kinase responsible is likely to be specific for the tumor or transformed cell environment.

Apoptin Kinase Activity Is Detectable in Human Tumor Tissue—Our cumulative evidence demonstrated Apoptin kinase activity in a variety of tumor or transformed cell lines but not in normal cells. To exclude the possibility that such activation resulted from adaptation of tumor cells to in vitro culture conditions, we determined whether Apoptin kinase activity was present in human clinical samples. To this end, we first used the in vitro kinase assay with the α108-P antibody to test a panel of tissues derived from healthy individuals, including colon, ovary, cervix, uterus and tonsil, all of which were negative for Apoptin kinase activity (Fig. 7A). A similar lack of Apoptin kinase activity was observed in various primary rat tissues (including liver, spleen, colon, skin, and stomach; data
not shown). Next, we performed the analysis in human stage 3 rectal tumors removed by total mesorectal excision. Flanking sections on either side of the material used in the kinase assay were analyzed by histocytochemistry to confirm the percentage of tumor cells by morphology (Fig. 7C shows a section from one of the two sides). Tumor sections (<5 μm) were compared with morphologically normal material immediately adjacent to the excised tumor, and Fig. 7B shows three representative patient samples. We detected strong Apoptin kinase activity in two of the three tumors (patients 1 and 2). In contrast, very weak (patient 1) or no (patient 2) activity was observed in the proximal normal material. Although the tumor material from patient 3, which contained a relatively low number of tumor cells, appeared to be negative at first sight, one could observe slightly above-background activity on a longer exposure (Fig. 7B, lower inset). We further detected Apoptin kinase activity in the tumor material of six of eight additional stage 3 rectal tumor patients, as well as in two primary tumors obtained from another hos-
pital (colon and esophageal carcinoma; data not shown). The demonstration of Apoptin kinase activity in clinical tumor samples further supports the idea that such activity may be generally associated with human neoplasia.

**DISCUSSION**

In this report, we provide the first evidence favoring the hypothesis that the tumor-specific activities of Apoptin result from direct activation by a cancer-associated cellular pathway. Phosphorylation on residue Thr-108 appears to be sufficient to confer upon Apoptin the ability to behave in normal cells as it does in tumor cells. Because previous studies suggest that nuclear localization is necessary but not sufficient for full activity of Apoptin (18, 19), our results with the T108E mutant indicate that modification at this residue may act as a bifunctional switch inducing both accumulation in the nucleus and the ability to induce subsequent apoptosis. However, precisely how phosphorylation at Thr-108 facilitates the downstream execution activities of Apoptin remains to be elucidated. Preliminary experiments with various alanine mutants of full-length Apoptin that could not be phosphorylated showed a significant impairment in the ability to induce apoptosis in Saos-2 tumor cells, although apoptosis was not abolished completely (data not shown). Further studies will be needed to understand the contribution of phosphorylation to the overall ability of Apoptin to kill tumor cells.

In chicken cells, Apoptin is expressed during CAV infection and appears to be essential for replication (6). In our hands, the phosphospecific a108-P antibody detected robust phosphorylation of Apoptin in Marek’s virus-transformed MDCC-MB2-1 chicken T cells infected with CAV in culture (data not shown). Although the unfortunate lack of a non-transformed in vitro model system for CAV infection makes it difficult to assess this question under more natural conditions, these results do suggest that phosphorylation on Thr-108 can occur during the CAV replication cycle. Because phosphorylation regulates the activity of Apoptin, it is possible that the virus has evolved to exploit a natural host cell signal transduction pathway to facilitate its own replication cycle. Susceptible chicken cells may possess active kinase naturally or may be induced to do so as a result of CAV infection. Alternatively, CAV infection is often associated with the co-infection of transforming avian viruses such as Marek’s disease (3), which may up-regulate the kinase of Apoptin during cellular transformation, thereby offering an opportunistic advantage to CAV. Interestingly, it was shown recently that VP2, one of the other proteins of CAV, can function as a dual specificity protein phosphatase in vitro (24). Although this work did not address in vivo substrates, it is consistent with the idea that phosphorylation pathways in general may be important for the replication cycle of CAV.

In addition to revealing functional aspects about the regulation of the activity of Apoptin, our results provide evidence for the existence of a previously unrecognized cellular pathway correlated with human cancer. Here, we show that the entity responsible for phosphorylation of Apoptin is active in tumor or transformed cells but is very low or absent in healthy, untransformed cells. This phenomenon could in principle result from either a tumor-specific kinase activity or a normal-specific phosphatase activity. However, as the in vitro assay is performed in the presence of a mixture of diverse phosphatase inhibitors, which do not liberate kinase activity in normal cells, we believe that the former possibility is more likely. Although some cancer markers are restricted to tumor type, e.g. bcr-Abl or c-Kit kinase in chronic myeloid leukemia and gastrointestinal stromal tumors (25) or the Her-2/neu receptor tyrosine kinase in breast cancer (26), our results in transformed or tumor cell lines from tissue types of endodermal, ectodermal, and mesodermal origin suggest that Apoptin kinase represents a more general activity.

In this report, we describe the detection of Apoptin kinase activity in very small amounts of rectal tumor tissue and its absence in the tissues from healthy individuals. The low level of activity seen in morphologically normal tissue adjacent to positive tumor sections (Fig. 7B, patient 1) may be because of nonspecific background or because of an activation of the kinase in normal cells induced by the nearby tumor cell population (27). Alternatively, the phenomenon could reflect a genetic lesion related to the kinase in the surrounding pool of normal cells prior to the onset of frank malignancy. Such a lesion could offer a growth advantage to a “field” of pre-malignant cells, some of which could progress subsequently, via the accumulation of additional hits, to morphologically apparent cancer cells as has been suggested for, e.g. breast carcinoma (28). Further studies into the prevalence of Apoptin kinase activity at various stages of tumor progression should provide insight into these questions.

Because of the remarkably high number of diverse human transformed or tumor cell types tested that are susceptible to Apoptin-induced apoptosis, we speculate that these cells must share a common lesion that is highly selected, and possibly essential, in tumor development or maintenance. Apoptin kinase is a strong candidate member of such a pathway. The kinase may be dysregulated quite early in the development of a transformed phenotype, as Apoptin can be rendered active soon after transient transfection of normal cells with SV40 large T antigen (29). Moreover, Apoptin can be activated swiftly in otherwise unsusceptible cells from cancer-prone patients after a dose of UV irradiation (30). The elucidation of both upstream and downstream effectors in this pathway should further improve our understanding of the complex process of malignant transformation and thereby may lead to the development of novel therapeutic approaches for its treatment.

**Acknowledgments**—We thank M. Seelen, M. Eikenboom, G. Ensink, P. Voskamp, L. van Dijk, K. Kooistra, A. Wegg, M. Lerch, and H. Groening for expert technical assistance, I. Bahar for mass spectrometric analysis, R. Leliveld for the gift of MBP, MBP-Apoptin, and Apoptin-His 

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J. Biol. Chem. 2002, 277:50820-50827.
doi: 10.1074/jbc.M208557200 originally published online October 21, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208557200

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