THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 52, Issue of December 27, pp. 50820–50827, 2002
Printed in U.S.A.

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

Jennifer L. Rohn‡, Ying-Hui Zhang‡, Remco I. J. M. Aalbers‡§, Norbert Otto¶, Jeroen den Hertog‖, Niek V. Henriquez‡, Cornelis J. H. van de Velde§, Peter J. K. Kuppen§, Dominik Mumberg¶, Peter Donner¶, and Mathieu H. M. Noteborn‡‡‡**

*From ‡Leadd B.V., 2300 RA Leiden, the Netherlands, §Department of Surgery, Leiden University Medical Center, 2300 RC Leiden, the Netherlands, ¶Research Laboratories of Schering AG, D-13342 Berlin, Germany, ‖Hubrecht Laboratory, Netherlands Institute for Developmental Biology, 3584 CT Utrecht, the Netherlands, and **Department of Molecular Cell Biology, Leiden University Medical Center, 2300 RA Leiden, the Netherlands*

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 severe 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).[2] 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

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed. Tel.: 31-71-527-8736; Fax: 31-71-527-1736; E-mail: noteborn@leadd.nl.

[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).[3] Therefore, we postulated that a further tumor-specific activation step was required. Because phosphorylation is known to regulate the function of many proteins (20), and because Apoptin is rich in serines and threonines, we sought to determine whether the behavior of Apoptin could be explained by such modification. Here, we show that Apoptin is regulated by a kinase activity present in cancer cell lines from a variety of tissue 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 complicated process of tumorigenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—All cells were cultured at 37 °C in a humidified 10% CO_2 incubator 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 grown as recommended in their accompanying medium MSCGM Bulletkit and used between passages 2 and 4, and keratinocyte-based cultures, which were grown at CO_2 levels of 7.5% in Dulbecco's modified Eagle's medium/Ham's F-12 (3:1) supplemented with 5% bovine calf serum, 10^{-6} M isoproleronol, 10^{-6} M hydrocortisone, and 10^{-7} M insulin and used below passage 2. All diploid neonatal foreskin-derived fibroblasts were used below passage 14.

Constructs—Wild-type Apoptin (also known as VP3) was overexpressed either using pCMV-VP3 (9) (see Fig. 3*E*) or sAPO, a construct in pIRESneo (Clontech) expressing a variant of Apoptin (Arg → Lys mutation at position 116) whose expression and behavior are indistinguishable from that of pCMV-VP3. All in-frame green fluorescent protein (GFP)-fused deletion mutants of Apoptin were constructed with standard linker and restriction enzyme strategies using the vector phGFPS65T (Clontech) as a backbone and pCMV-VP3 as the source of various inserts; in these constructs, GFP was positioned at the N terminus, followed by a four-amino acid tether sequence and then the relevant portions of Apoptin (refer to Fig. 3*E* for the amino acid numbering system used). All alanine and glutamic acid substitution mutants of Apoptin were constructed in the sAPO background using a standard linker substitution strategy. In experiments using these mutants, sAPO was employed as a positive control, and pCMV-VP3 was used in all other cases. Use of the constructs encoding desmin and p53 for transfection assays has been described (17). All new constructs were confirmed by standard automated nucleotide sequence analysis. Precise details of the construction and content of all constructs used are available on request.

Recombinant Proteins—MBP-Apoptin, MBP, and Apoptin bearing a C-terminal hexahistidine tag (Apoptin-His) proteins were a kind gift from Rutger Leliveld and have been described.[4] Briefly, MBP-Apoptin was expressed and purified by standard methods in *Escherichia coli* from a construct comprised of a modified vector pMal-c2 (New England Biolabs) encoding an N-terminal maltose-binding protein, a ten-Asn linker, and a thrombin cleavage site, followed by Apoptin based on pCMV-VP3 in the same reading frame. Apoptin-His was cloned and purified by standard methods in *E. coli* from a construct comprised of the vector pET22b (Novagen), which provides a six-histidine tag and a stop codon C-terminal to Apoptin based on pCMV-VP3. GST-Apoptin was cloned using a standard restriction enzyme strategy using the pGEX-T vector (Amersham Biosciences), providing an in-frame glutathione *S*-transferase tag on the N terminus, and sAPO as the Apoptin insert in-frame downstream. Precise details of the construction, expression, and purification of all proteins used are available on request.

Protein Detection—Cell cultures were lysed with ice-cold radioimmune precipitation assay buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, supplemented with trypsin inhibitor, pepstatin, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, β-glycerol phosphate, sodium orthovanadate, and sodium fluoride, all at standard concentrations), and supernatants were clarified by centrifugation. Immunoprecipitations, when required, were performed with protein A-agarose-coupled antibodies; this procedure, along with SDS-PAGE, Western blotting onto polyvinylidene difluoride membranes (Immobilon), immunostaining, and enhanced chemiluminescence detection, was performed using standard methodology (21). Antibodies used were VP3-C (a polyclonal rabbit serum against the C terminus of Apoptin (17)), α108-P (see below), 111.3 (a mouse monoclonal antibody against the N terminus of Apoptin (9)), DO-1 (against p53; Santa Cruz Biotechnology, Inc.), anti-GFP (against green fluorescent protein; a gift from Bob van de Water), and MBP-probe (against maltose-binding 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 pull down 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 lysed by repeated freeze-thawing in kinase buffer (20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 150 mM NaCl, and standard inhibitors of proteases and phosphatases, the same as used for radioimmune precipitation assay buffer lysis (see above), as well as the phosphatase inhibitor cocktails 1 and 2 (Sigma)). Rectal cancer tissues were chosen based on previous TNM[5]-based classification. Flanking sections were re-analyzed 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 μCi of [γ-^{32}P]ATP (when radioactive label was used), and 2 mM dithiothreitol. In some cases control reactions were performed without ATP and in the presence of EDTA to inhibit kinase activity. Phosphorylated proteins were subjected to Western blot analysis before autoradiography (for radioactive ATP) or immunoprobing 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 pretreated in phosphate-free Dulbecco's modified Eagle's medium supplemented with dialyzed serum (Sigma) for 10 min before metabolic labeling with 1 mCi/ml [^{32}P]orthophosphate for 4 h. After lysis in cold radioimmune precipitation assay buffer, proteins were immunoprecipitated with the antibodies VP3-C, α-GFP, or DO-1 as appropriate. Purified immunoprecipitates were resolved by SDS-PAGE and Western blot, and the polyvinylidene 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 subsequent orientation for band isolation (see below).

Phosphoamino Acid and Peptide Mapping—Radioactive bands corresponding to phosphorylated Apoptin or negative controls were excised from polyvinylidene difluoride, digested with Tpck-trypsin, and subjected 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 phosphorimaging analysis to detect the radioactive spots. Samples split off from the above assay 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 PhosphoChromo liquid chromatography in the same orientation for the second dimension. Dried plates were subjected to phosphorimaging analysis.

Mass Spectroscopy—In vitro phosphorylated GST-Apoptin protein was purified from lysates by standard glutathione-Sepharose purification, carboxymethylated, and precipitated with trichloroacetic acid, and following resuspension it 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

[3] 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.

[4] S. R. Leliveld, Y.-H. Zhang, J. Rohn, M. Noteborn, and J. P. Abrahams, submitted for publication.

[5] 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.

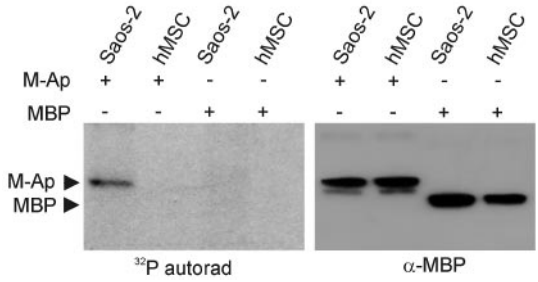

FIG. 1. **Apoptin is phosphorylated by Saos-2 tumor cell lysates but negligibly by normal human mesenchymal stem cell (*hMSC*) lysates *in vitro*.** *Left*, autoradiograph of *in vitro* kinase assay samples resolved by SDS-PAGE and Western-blot (*M-Ap*, MBP-Apoptin substrate; *MBP*, control substrate). *Arrows* indicate the migration of the two substrates. *Right*, the same membrane immunostained with anti-MBP (*α-MBP*).

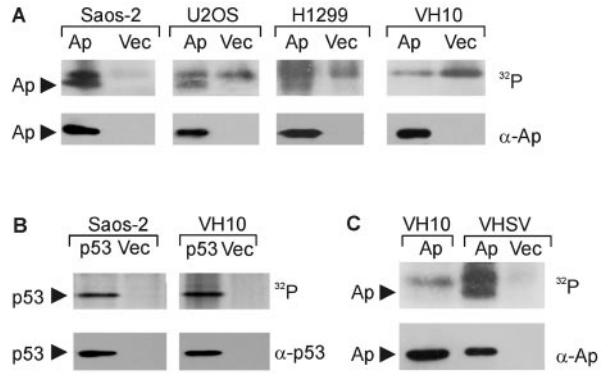

FIG. 2. **Apoptin is phosphorylated in transformed or tumor cells specifically *in vivo*.** The *in vivo* [^{32}P]orthophosphate labeling assay was performed on the indicated cell types transfected with Apoptin (*Ap*), the empty vector (*Vec*), or p53. In all cases, the *top panels* show the autoradiograph (*^{32}P*), and the *bottom panels* show the same membrane immunostained with Apoptin (*α-Ap*) or p53 (*α-p53*) antibodies. The *lower-most* migrating *band* corresponds to Apoptin protein (indicated with *arrows*), whereas the *top band* is an irrelevant cellular phosphoprotein that fails to hybridize to the Apoptin antibody.

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(pT)PSRPRTA (corresponding to Apoptin residues 103–115) was inoculated 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 mg/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 Apoptin 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 [^{32}P]orthophosphate labeling. As shown in Fig. 2*A*, 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 cell types (Fig. 2*B*). *In vivo* phosphorylation of Apoptin also occurred in U2OS cells and H1299 lung carcinoma cells (Fig. 2*A*). 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. 2*C*). 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. 3*A*). 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-

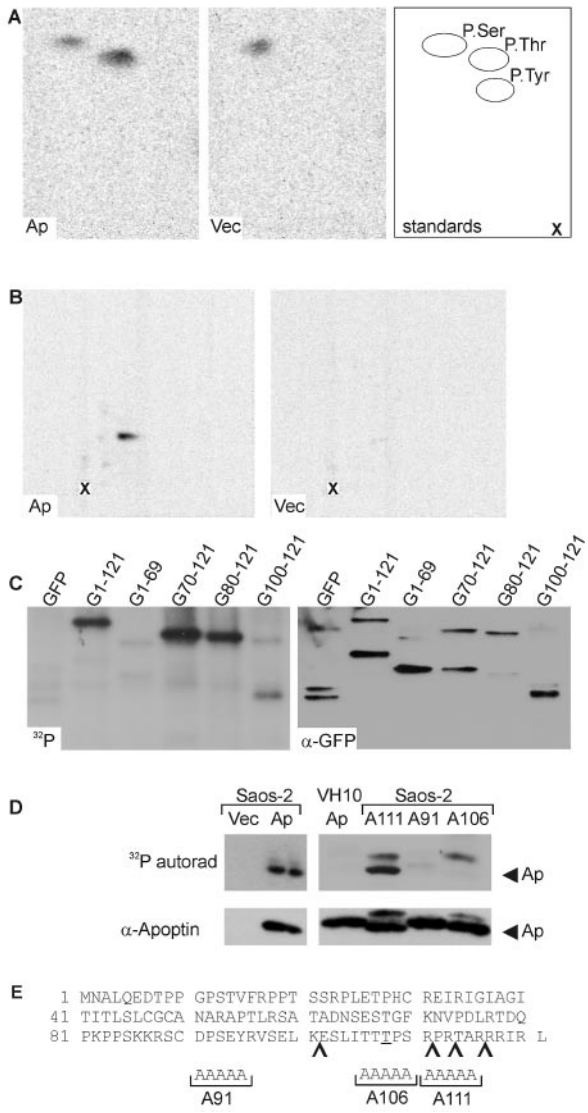

FIG. 3. **Apoptin is phosphorylated on threonine(s) within residues 106–108.** *A*, phosphoamino acid analysis of *in vivo*-ortholabeled Saos-2 cells transfected with Apoptin (*Ap*; *left*) or the empty vector (*Vec*; *middle*). The position of the standards is depicted in the *right panel*; *X* marks 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 showed that only a single tryptic fragment was radiolabeled, indicating that the complexity of Apoptin phosphorylation was fairly low (Fig. 3*B*).

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. 3*C*, 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. 3*E*). 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. 3*D*, 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. 3*D*). 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. 5*A*). 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. 5*B* 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

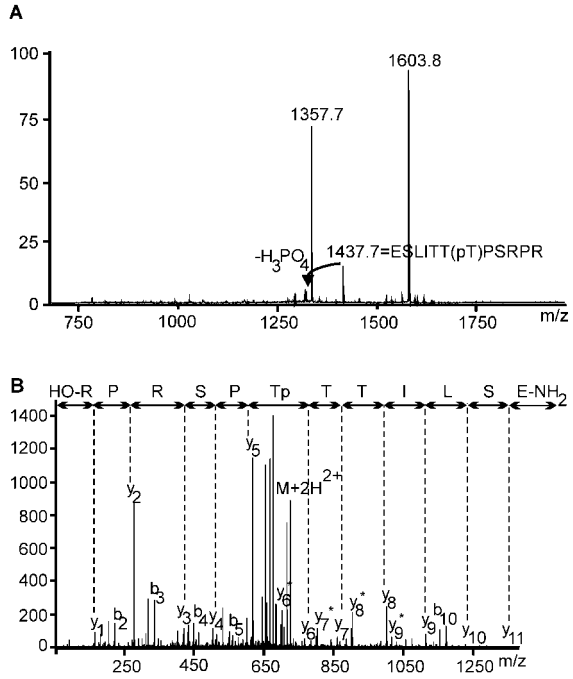

FIG. 4. **Apoptin is phosphorylated on threonine 108 *in vitro*.** Recombinant GST-Apoptin was *in vitro* phosphorylated with Saos-2 lysates and subjected to mass spectrometric analysis. *A*, MALDI spectrum of the radiolabeled HPLC fraction of the tryptic digest of GST-Apoptin. *B*, electron spray ionization collision-induced dissociation spectrum of the $(M+2H)^{2+}$ ion of the phosphorylated peptide ESLIT-T(pT)PSRPR. The *asterisk* (*) signifies loss of H_3PO_4.

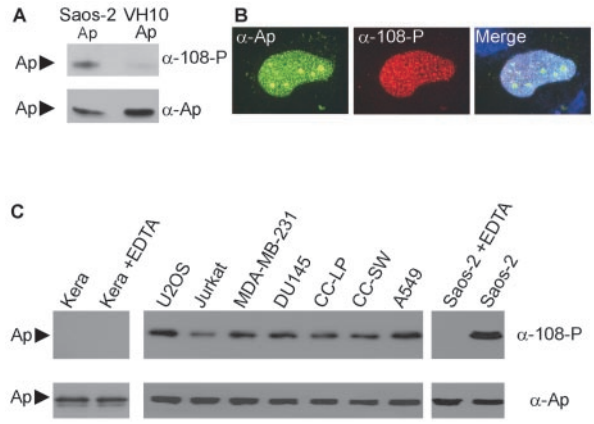

FIG. 5. **A Thr-108-phosphospecific antibody recognizes Apoptin *in vivo* and *in vitro*.** *A*, Western blot analyses of Saos-2 tumor cells *versus* VH10 fibroblasts transfected with Apoptin (*Ap*), in which the *top panel* is stained with α108-P, and the *bottom panel* is stained with Apoptin antibody (α-*Ap*). *Arrows* indicate the protein. *B*, immunofluorescence image of a Saos-2 tumor cell transfected with Apoptin and stained simultaneously with α-Ap (*green*), α108-P (*red*), and 2,4-diamidino-2-phenylindole (*blue*; the *right panel* shows a merge of all three filters). *C*, α108-P-based *in vitro* kinase assay (Western blot, labeled as in *A*) of samples from normal keratinocytes (*kera*) with or without EDTA to inhibit the kinase, compared with various tumor cell lines and Saos-2 as a positive control.

staining seemed enriched in the nucleus but absent from the characteristic round, subnuclear bodies that Apoptin tends to form in tumor cells (see Fig. 5*B*, 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. 5*C* 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*, *E*, and *F*) but also to translocate to the nucleus (Fig. 6*D*). 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. 7*A*). A similar lack of Apoptin kinase activity was observed in various primary rat tissues (including liver, spleen, colon, skin, and stomach; data

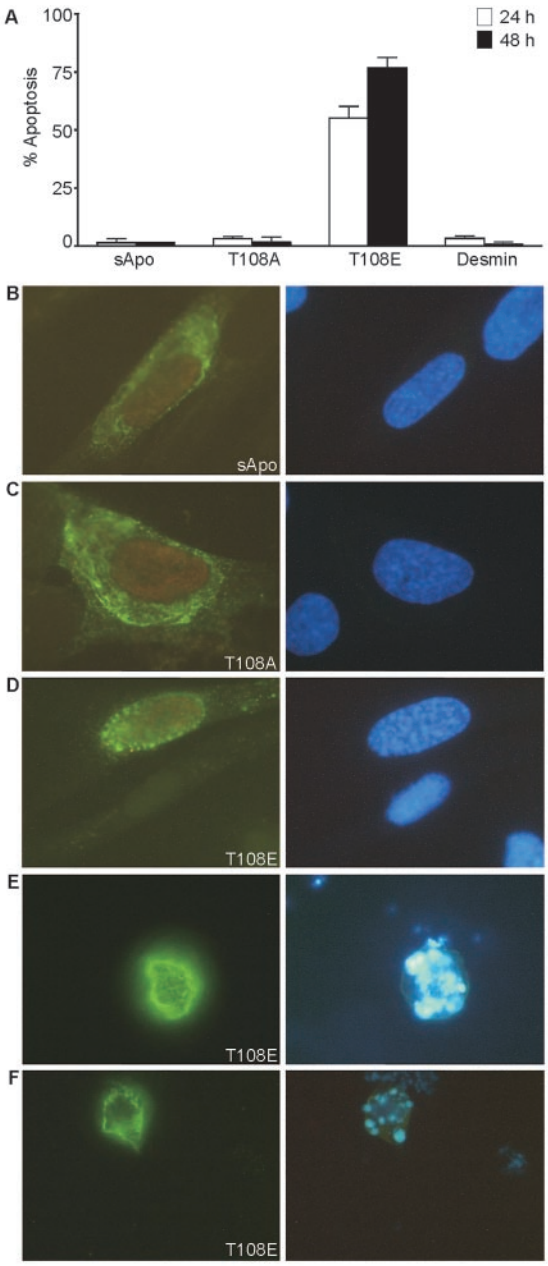

FIG. 6. **Phosphorylation of threonine 108 activates Apoptin.** An Apoptin construct with a Thr to Glu mutation at 108 (*T108E*) was expressed in human neonatal foreskin VH10 fibroblasts and compared with wild-type Apoptin (s*APO*), a Thr to Ala mutation at Thr-108 (*T108A*), and the negative control Desmin. Cells were fixed at the indicated time points and assessed for apoptotic activity (*A*) and subcellular localization and morphological characteristics (*B–F*; 48-h *time point*). For all images in *B–F*, the *left panel* shows Apoptin antibody staining in *green*, whereas the *right panel* shows the 2,4-diamidino-2-phenylindole-stained nucleus in *blue*.

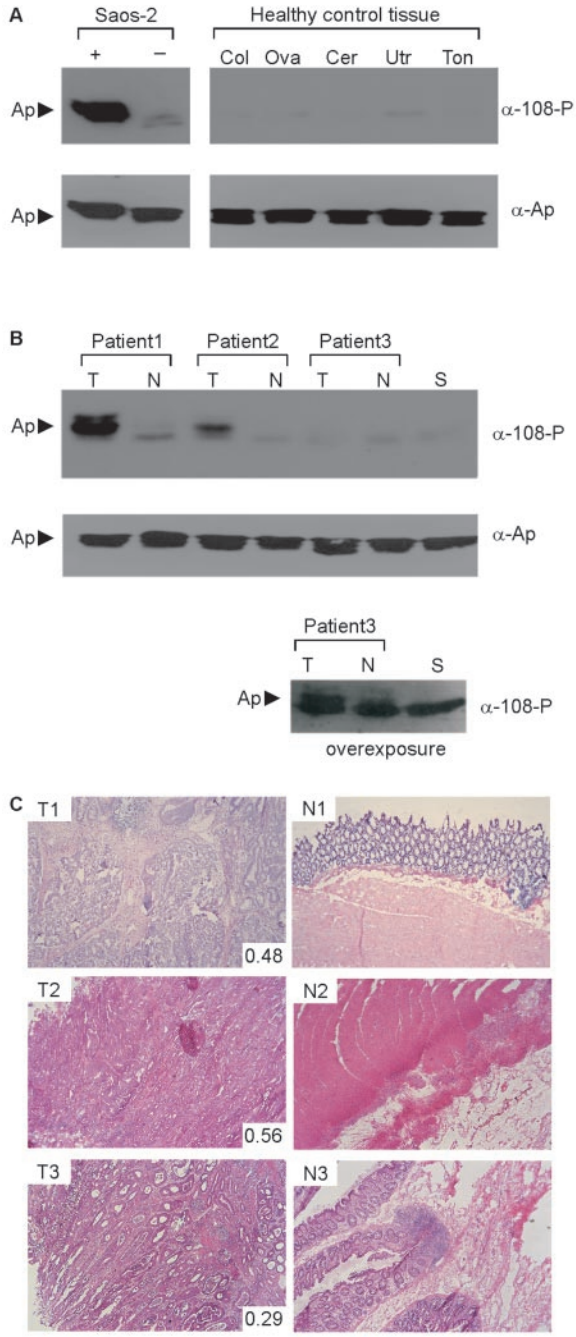

FIG. 7. **Apoptin is phosphorylated on Thr-108 in primary tumor tissues.** Western blot of *in vitro* kinase assay samples with α108-P (*top*) and Apoptin antibody (α-*Ap*; *bottom*) from healthy humans (*A*) or rectal cancer patients (*B*). The plus sign (+) in the Saos-2 controls indicates a normal reaction, and the minus sign (−) indicates a reaction without ATP and with EDTA. The *S* in *panel B* indicates the substrate protein loaded directly onto the gel. *T* indicates tumor, and *N* indicates the proximal morphologically normal tissue. An overexposure of samples from the third patient is shown as a *lower inset under panel B*. *C*, the corresponding hematoxylin- and eosin-stained serial sections of the patient samples shown in *B*, with the estimated tumor cell index given in the *lower right-hand corner*.

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. 7*C* 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. 7*B* 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. 7*B*, *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),[3] 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).[6] In our hands, the phosphospecific α108-P antibody detected robust phosphorylation of Apoptin in Marek's virus-transformed MDCC-MSB-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. 7*B*, *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. Bahr for mass spectrometric analysis, R. Leliveld for the gift of MBP, MBP-Apoptin, and Apoptin-His purified proteins, J. Kempenaar, N. Annels, M. Ponec, L. Mullenders, J. Rommelaere, J. P. Medema, and A. Jochemsen for providing cell lines, EuroGentec Belgium for antisera generation, P. Verrijzer, N. Divecha, and D. Weinkove for critical review of the work, and members of our laboratories, especially A. Danen-van Oorschot, for helpful discussions.

REFERENCES

1. Tommasino, M., and Crawford, L. (1995) *Bioessays* **17,** 509–518
2. Brodsky, J. L., and Pipas, J. M. (1998) *J. Virol.* **72,** 5329–5334
3. Yuasa, N., Taniguchi, T., Noguchi, T., and Yoshida, I. (1980) *Avian Dis.* **24,** 202–209
4. Gelderblom, H., Kling, S., Lurz, R., Tischer, I., and von Bulow, V. (1989) *Arch. Virol.* **109,** 115–120
5. Jeurrisen, S. H., Wagennaar, F., Polz, J. M., van der Eb, A. J., and Noteborn, M. H. M. (1992) *J. Virol.* **66,** 7383–7388
6. Noteborn, M. H. M., and Koch, G. (1995) *Avian Pathol.* **24,** 11–31
7. Noteborn, H. M. H., de Boer, G. F., van Roozelaar, D. J., Karreman, C., Kranenburg, O., Vos, J. G., Jeurissen, S. H., Hoeben, R. C., Zantema, A., Koch, G., van Ormondt, H., and van der Eb, A. J. (1991) *J. Virol.* **65,** 3131–3139
8. Noteborn, M. H. M., Todd, D., Verschueren, C. A., de Gauw, H. W., Curran, W. L., Veldkamp, S., Douglas, A. J., McNulty, M. S., van der Eb, A. J., and Koch, G. (1994) *J. Virol.* **68,** 346–351
9. Danen-van Oorschot, A. A. A. M., Fischer, D. F., Grimbergen, J. M., Klein, B., Zhuang, S., Falkenburg, J. H., Backendorf, C., Quax, P. H., van der Eb, A. J., and Noteborn, M. H. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **11,** 5843–5847
10. Noteborn, M. H. M. (2002) in *Targets for Cancer Chemotherapy: Transcription Factors and other Nuclear Proteins. Cancer Drug Discovery and Development* (La Thangue, N., and Bandara, L., eds) Humana Press, Totowa, NJ
11. Pietersen, A. M., van der Eb, M. M., Rademaker, H. J., van den Wollenberg, D. J., Rabelink, M. J., Kuppen, P. J., van Dierendonck, J. H., van Ormondt, H., Masman, D., van de Velde, C. J., van der Eb, A. J., Hoeben, R. C., and Noteborn, M. H. M. (1999) *Gene Ther.* **6,** 882–892
12. Van der Eb, M. M., Pietersen, A. M., Speetjens, F. M., Kuppen, P. J., van de

[6] M. Noteborn, unpublished observations.

Velde, C. J., Noteborn, M. H. M., and Hoeben, R. C. (2002) *Cancer Gene Ther.* **9,** 53–61
13. Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100,** 57–70
14. Hahn, W. C., Dessain, S. K., Brooks, M. W., King, J. E., Elenbaas, B., Sabatini, D. M., DeCaprio, J. A., and Weinberg, R. A. (2002) *Mol. Cell. Biol.* **22,** 2111–2123
15. Zhuang, S.-M., Shvarts, A., van Ormondt, H., Jochemsen, A. G., van der Eb, A. J., and Noteborn, M. H. M. (1995) *Cancer Res.* **55,** 486–489
16. Danen-Van Oorschot, A. A. A. M., Zhang, Y.-H., Erkeland, S. J., Fischer, D. F., van der Eb, A. J., and Noteborn, M. H. M. (1999) *Leukemia* **13,** Suppl. 1, S75-S77
17. Danen-van Oorschot, A. A. A. M., van der Eb, A. J., and Noteborn, M. H. M. (2000) *J. Virol.* **74,** 7072–7078
18. Zhuang, S.-M., Landegent, J. E., Verschueren, C. A., Falkenburg, J. H., van Ormondt, H., van der Eb, A. J., and Noteborn, M. H. M. (1995) *Leukemia* **9,** Suppl. 1, S118–120
19. Danen-van Oorschot, A. A. A. M. (2001) *Apoptin, a Viral Protein That Induces Tumor-specific Apoptosis.* Ph.D. thesis, Ponsen and Looijen BV, Wageningen, The Netherlands
20. Cohen, P. (2000) *Trends Biochem. Sci.* **25,** 596–601
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149
23. Klein, B., Pastink, A., Odijk, H., Westerveld, A., and van der Eb, A. J. (1990) *Exp. Cell Res.* **191,** 256–262
24. Peters, M. A., Jackson, D. C., Crabb, B. S., and Browning, G. F (2002) *J. Biol. Chem.* **277,** 39566–39573
25. Drukker, B. J. (2002) *Cancer Cell* **1,** 31–36
26. Sawyers, C. L. (2002) *Curr. Opin. Genet. Dev.* **12,** 111–115
27. Bissel, M. J., and Radusky, D. (2001) *Nat. Rev. Cancer* **1,** 46–54
28. Guoren, D., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. (1996) *Science* **274,** 2057–2059
29. Noteborn, M. H. M., Zhang, Y.-H., and van der Eb, A. J. (1998) *Mutat. Res.* **400,** 447–455
30. Zhang, Y.-H., Abrahams, P. J., van der Eb, A. J., and Noteborn, M. H. M. (1999) *Cancer Res.* **59,** 3010–3015
31. Sobin, L. H., and Wittekind, C. (1992) *TNM Classification of Malignant Tumours*, Springer-Verlag, Berlin, Germany

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

Jennifer L. Rohn, Ying-Hui Zhang, Remco I. J. M. Aalbers, Norbert Otto, Jeroen den Hertog, Niek V. Henriquez, Cornelis J. H. van de Velde, Peter J. K. Kuppen, Dominik Mumberg, Peter Donner and Mathieu H. M. Noteborn

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