Apoptin : oncogenic transformation & tumor-selective apoptosis

Zimmerman, R.M.E.

Chapter 6

PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective cell killing

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Abbreviations: apoptin, apoptosis inducing protein; AKIP1, A-kinase interacting protein; BCA3, breast cancer-associated gene 3; DAPI, 2,4-diamidino-2-phenylindole; NLS, nuclear localization signal; PKA, protein kinase A; PP2A, protein phosphatase 2A; Rb, Retinoblastoma protein; RNAi, RNA interference; RSV, Rous sarcoma virus; SV40 LT, SV40 large T antigen; SV40 ST, SV40 small t antigen

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Abstract

Apoptin harbors tumor-selective characteristics, making it a potentially safe and effective anticancer agent. Apoptin becomes phosphorylated and induces apoptosis in a large panel of human tumor but not normal cell lines. Here, we explored minimal cellular factors required for the activation of apoptin. Flag-apoptin was introduced into normal fibroblasts together with the transforming SV40 LT and ST antigens. We found that expression of nuclearly located SV40 ST in normal cells was sufficient to induce phosphorylation of apoptin. Mutational analysis showed that C103S mutation within the protein phosphatase 2A (PP2A)-binding domain of ST counteracted this effect. Knock-down of the ST-interacting PP2A B56γ subunit in normal fibroblasts mimicked the effect of nuclear ST expression, resulting in induction of apoptin phosphorylation. The same effect was observed upon down-regulation of the PP2A B56δ subunit, which is targeted by PKA. Apoptin interacts with the PKA-associating protein BCA3 (AKIP1), and inhibition of PKA in tumor cells by treatment with H89 increased the phosphorylation of apoptin, whereas the PKA activator cAMP partially reduced it. We therefore conclude that inactivation of PP2A, particularly of the B56γ and B56δ subunits, is a crucial step in triggering apoptin-induced tumor-selective cell death.

Introduction

Tumor formation occurs due to a complicated set of processes roughly based on enhanced survival and limited cell death activities (Wenner, 2010). Remarkably, a set of viral and cellular proteins has been found to selectively induce cell death in tumor cells (Bruno, et al., 2009). Among these proteins is the avian virus protein apoptin, which has been shown to induce p53-independent apoptosis in a broad spectrum of human transformed cells (Backendorf, et al., 2008). Recent preclinical studies demonstrated the therapeutic potential of apoptin as a safe and efficient anticancer agent (Grimm and Noteborn, 2010). Therefore, it is of interest to study the mechanisms underlying apoptin-induced apoptosis, particularly the 'switch' responsible for its activation during oncogenic transformation.

SV40 T antigens are known to be involved in oncogenic transformation through interference with many cellular processes (Pipas, 2009). Distinct domains on LT that bind and inactivate tumor suppressors p53 and Rb, have long been known to play crucial roles in tumor formation (Eichhorn, et al., 2009). The SV40 ST protein enforces transformation of normal cells via negative effects on the protein phosphatase 2A (Sablina, et al., 2010).

This feature is in accordance with the reduced PP2A levels found in various human tumor cell types (Sablina and Hahn, 2008), and accumulating evidence supporting major tumor suppressive roles for PP2A (Westermarck and Hahn, 2008). Another major cellular regulator implicated in carcinogenesis is protein kinase A (PKA) (Naviglio, et al., 2009). Its targets include PP2A (Usui, et al., 1998), and for instance, the PKA interacting protein Breast Cancer-associated gene 3 (BCA3) is known to be highly expressed in breast and prostate cancer cells compared to the normal surrounding tissue (Kitching, et al., 2003).

In this study, we investigated the minimal steps leading to the activation of apoptin upon malignant transformation. We previously showed that

transient expression of the SV40 large and small T antigens in normal human fibroblasts results in activation of apoptin, displaying all three of its characteristic features, namely phosphorylation, nuclear localization, and apoptosis (Zhang, et al., 2004). Here, we analyzed which domains of the SV40 large and/or small T antigens were responsible for this activity. In parallel, BCA3 was identified as an apoptin-interacting protein, and the effects of PKA on apoptin phosphorylation were examined. Analysis of both transformation-related pathways revealed that inactivation of PP2A is crucial for the activation of apoptin.

Materials and Methods

Cells and Cell culture

Human diploid foreskin F9 fibroblasts, isolated from neonatal foreskin, were obtained in the late 1980's from Dr. M. Ponec (Dept. Dermatology, Leiden University Medical Center). Cells were batch-frozen after careful morphological inspection. At subsequent passages cells were regularly screened for their typical fibroblast-like morphological appearance. All cells used were below passage 15 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands). The human Saos-2 osteosarcoma and the HeLa cervical carcinoma cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in the same medium as mentioned above. Cultures were regularly tested to ensure the absence of *Mycoplasma* infection. Cell morphology was regularly monitored to control the absence of cross-contamination. The sensitivity to apoptin is characteristic of the various cell types used (Danen-van Oorschot, et al., 1997) and is regularly assessed (see below).

DNA plasmids

The DNA sequence encoding apoptin was synthesized by BaseClear (Leiden, the Netherlands) according to the apoptin sequence published by Noteborn et al. (1991), and cloned into the mammalian expression vector pcDNA3.1(+)

(Invitrogen). The oligonucleotide fragment encoding the Flag-tag (Invitrogen) was inserted to create the pcDNA-Flag-apoptin plasmid encoding apoptin fused with a Flag-tag at its N-terminus.

Plasmid pRSV-TN136 encoding the first 136 N-terminal amino acids of SV40 LT, including the region coding for SV40 ST, was a kind gift from Dr. J.M. Pipas (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA). From pRSV-TN136, we generated the pcDNA-LT136/ST plasmid, which encodes the LT136 (The N-terminally truncated LT fragment containing the first 1-136 aa) and full length ST. pcDNA-LT136, encoding LT136 only, was constructed by introducing an intron deletion disabling ST expression (Yamashita, et al., 1990). pcDNA expression vectors encoding only ST sequences were derived from pRSV-ST. pcDNA-ST encodes for ST; pcDNA-NLS-ST contains a ST fused to a nuclear localization signal (NLS-ST) and pcDNA-NLS-ST(C103S) encodes the N-terminal NLS-ST fusion protein containing the C103S mutation within the PP2A binding site (Gjoerup, et al., 2001).

The sequences encoding the N-terminal 136 aa of LT together with either full-length ST or the C103S ST-mutant were cloned into pEXPR-IBA105 vector to generate pEXPR-IBA105-LT136/ST and pEXPR-IBA105-LT136/STC103S respectively. These plasmids expressed Strep-tagged LT136/ST or LT136/ST C103S proteins enabling interaction studies with PP2A (see below) (Schmidt and Skerra, 2007). pCEP-4HA-B56γ, encoding 4HA-tagged B56γ, was a kind gift from Dr. M. Mumby (University of Texas, USA).

Apoptin-interacting partners were obtained by yeast two-hybrid screening and verified by immunoprecipitation assays in mammalian cells, as previously described by Danen-Van Oorschot et al. (Danen-van Oorschot, et al., 2004)**.** Positive clones from the yeast two-hybrid screen were digested with Xho-I to generate cDNA fragments, and subcloned into pMT2SM-myc to provide the fragments with an in-frame N-terminal myc-tag. The cDNA

fragment encoding BCA3, including the myc-tag, was subsequently cloned into pcDNA.

Transfection Methods

We used transfection reagent DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (Danen-van Oorschot, et al., 1997) or AMAXA nucleofection technology in conjunction with cell type specific NucleofectorTM solution (Lonza, Cologne, Germany) (Rohn, et al., 2002) for DNA delivery into cells. When co-transfection or triple-transfection was performed, the ratio of each plasmid was 1:1 or 1:1:1 (in micrograms). In addition to the analysis by Western blot, the cells were seeded on several glass cover slips to allow parallel analysis at the single-cell level by immunofluorescence assay.

Western Blot analysis

Cells were lysed directly in Laemmli buffer (2% SDS, 10% Glycerol, 60mM Tris-Cl [pH6.8], 2% β-mercaptoethanol, 0.002% bromophenol blue). Cell lysates were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). Blots were then incubated with antibodies against phosphorylated-apoptin (α-108-P; Zhang, et al., 2004), Flag-apoptin (α-Flag M2, Sigma-Aldrich), SV40 LT (PAb416, Pab419, Calbiochem), SV40 ST (Pab280, Calbiochem), PP2A Aα (C-20, Santa Cruz), PP2A B56γ (α-B56γ, kind gift from Dr. Marc Mumby, Health Science Center, University of Texas, Texas, USA), PP2A B56δ (α-B56δ, Santa Cruz Biotechnology), myc-tagged BCA3 (α-myc, BD Biosciences), and actin (α-actin, Santa Cruz). Horseradish peroxidase-conjugated goat antibody against rabbit or mouse immunoglobulin G, or rabbit antibody against goat immunoglobulin G (Sigma-Aldrich) was used as secondary antibody for signal detection by enhanced chemiluminescence.

Protein interaction assays

Detection of a possible interaction of ST or ST-C103S mutant protein with PP2A in human HeLa cells was performed as follows. Twenty-four hours after DNA transfection, cells were washed twice with phosphate buffered saline and harvested in ice-cold mild lysis buffer (50mM Tris [pH 7.5], 5mM EDTA, 250mM NaCl, 0.1% Triton X-100, 5 mM NaF, 1mM Na_3VO_4, 20mM beta-glycerolphosphate, and Protease Inhibitor Cocktail (Roche), followed by incubation on ice for 30 min. The supernatant of the lysates was prepared by centrifugation at 13,000 × g and 4°C for 30 min. Strep-tagged proteins and their interacting proteins were captured using the One-strep kit (IBA, Germany) according to the manufacturer's protocol, and resolved on sodium dodecyl sulfate-polyacrylamide gel, followed by Western blotting analysis with appropriate antibodies.

RNA interference assay

For human PP2A B56γ, the target sequence was: 5'-GATGAACCAACGTTAGAAG-3'; for PP2A B56δ two sequences were targeted (1): 5' GTGTGTCTCTAGCCCCCAT 3' (Arnold, et al., 2006) and (2) 5' GACCATTTTGCATCGCATC 3' (van Kanegan and Strack 2009) (data not shown). The pSUPER vector was designated for shRNA plasmid constructions (Boutros and Ahringer, 2008). The amplification and purification of plasmids were performed as specified by manufacturer's instruction (GeneService, Cambridge, UK). Cells transfected with shRNA plasmids were lysed at 24-48 h after transfection, and then analyzed by Western blot assay as described above.

Immunofluorescence assay

Cells were grown on glass cover slips. At indicated time points after transfection, cover slips were first washed once with phosphate buffered saline, and subsequently fixed with methanol/acetone (50%/50%) for 5-10 min at room temperature. After air-drying, the slides were used for immunocytochemical staining or stored at -20°C for further analysis. Immunocytochemical staining was carried out as described by Danen-Van

Oorschot et al. (2004). The following antibodies were used: α-108-P, a rabbit polyclonal antibody recognizing phosphorylated apoptin at T108 and α-Flag, a mouse monoclonal antibody recognizing Flag-apoptin. SV40 proteins were visualized with PAb416, a mouse monoclonal antibody recognizing the epitope residing in amino acids 83-128 of LT and non-reactive with ST or PAb280, a mouse monoclonal antibody against the C terminus of ST. The fluorescein isothiocyanate (FITC) or rhodamine conjugated goat antibodies (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Nuclei were stained with 2,4-Diamidino-2-phenylindole (DAPI) and apoptosis was assessed according to characteristic morphological changes (Danen-van Oorschot et al., 1997).

PKA inhibition and stimulation

Saos-2 cells were transfected with pcDNA-Flag-apoptin and incubated 24 hours post-transfection with 10μM H89 (Millipore) for 1 hour (PKA inhibition), or 30 minutes with H89 followed by incubation with 1mM cAMP (Sigma) for another 30 minutes (PKA stimulation). Cells were then lysed in Laemmli buffer and the cell lysates analyzed for apoptin phosphorylation by Western blotting.

Results

Transient expression of N-terminal SV40 LT136/ST activates apoptin in normal human fibroblasts

Apoptin activity can be induced in normal cells by transient expression of transforming SV40 antigens (Zhang, et al., 2004). Here, we examined the minimal SV40 domains responsible for this effect. Expression of the SV40 LT136/ST DNA sequence results, due to alternative splicing, in 2 proteins: LT136, comprising the N-terminal 136 a.a. of LT, and the entire ST protein, consisting of 174 amino acids (Figure 6.1A) (Srinivasan, et al., 1997). LT136 contains a DNA J domain, an Rb-binding site and a nuclear localization signal (NLS). ST shares its DNA J domain with LT136, but has a unique C-terminal domain encompassing a PP2A binding site (Pipas, 2009).

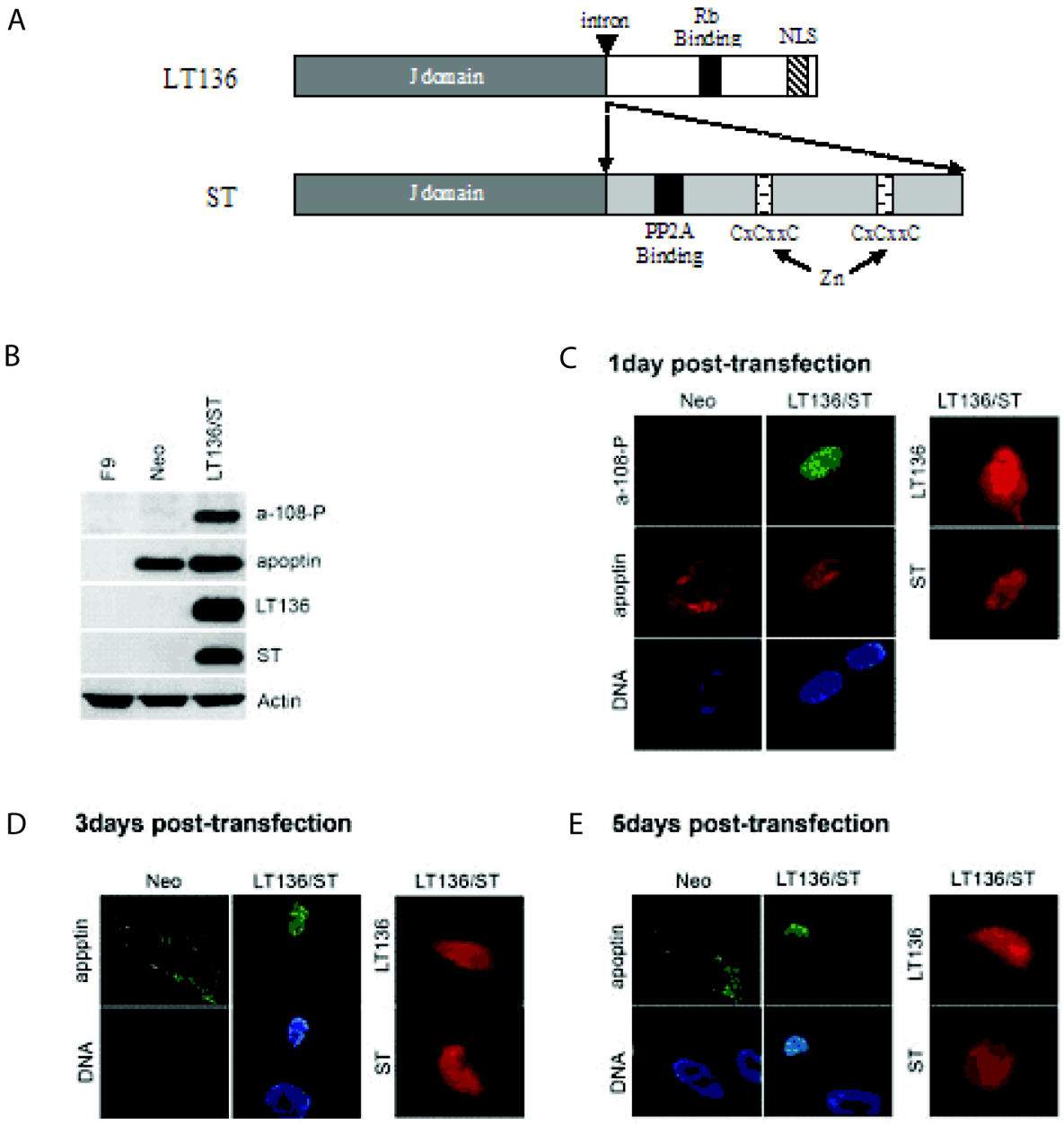

Figure 6.1 Transient expression of N-terminal determinants of SV40 T antigens activate apoptin. **A.** Schematic representation of the domains in SV40 LT136/ST. The J domain (a.a. 1-82) is identical in both LT136 and ST. The Rb binding domain and nuclear localization signal (NLS) in LT136 are also shown. ST is expressed by differential splicing and has a unique C-terminus, which contains the PP2A binding domain. **B.** Human Fibroblasts (F9) were co-transfected with plasmids pcDNA-Flag-apoptin and pcDNA-LT136/ST (LT136/ST) or pcDNA-neo (neo) by AMAXA nucleofector transfection. Twenty-four hours post-transfection, cells were lysed for Western blot analysis with the indicated antibodies. Mock-transfected F9 cells were used as control. Antibody α-108-P specifically recognizes phospho-apoptin at its Thr108. Flag-apoptin, LT and ST show the respective total protein amounts in the transfected cells. Actin was used as loading control. **C-E.** Cells were fixed for immunofluorescence analysis at each given time point after transfection and then stained with the indicated antibodies by indirect immunofluorescence assay. Scale bar = 20 μm.

In normal human foreskin fibroblasts, co-transfection of the SV40 LT136 and ST proteins with Flag-tagged apoptin resulted in the latter's phosphorylation (Figure 6.1B). Immunofluorescence analysis of F9 cells expressing all three proteins showed that Flag-apoptin became nuclear already 1 day after transfection. Nuclear Flag-apoptin was also shown to be phosphorylated (Figure 6.1C), and 3 and 5 days post transfection, apoptin was shown to induce apoptosis (Figure 6.1D, E). In contrast, F9 fibroblasts expressing apoptin alone contained mainly cytoplasmic Flag-apoptin, which was, as expected, not phosphorylated (Alvisi, et al., 2006; Rohn, et al., 2004) and did not induce apoptosis (Figure 6.1C-E).

Our results clearly reveal that transient expression of LT136 and ST proteins in human F9 fibroblasts results in tumor-selective activation of apoptin, providing us with the possibility to explore which domains and respective cellular targets of LT136/ST were responsible for this activity.

Nuclearly localized ST triggers apoptin activation

Next, we examined whether expression of either LT136 or ST protein alone was sufficient to activate apoptin phosphorylation. F9 fibroblasts were co-transfected with plasmids encoding Flag-tagged apoptin and either one of the following: a) pcDNA-LT136, encoding LT136, b) pcDNA-ST, encoding ST, c) pcDNA-LT136/ST, where both LT136 and ST are produced via alternative splicing, or d) pcDNA-neo, as a negative control. In each experiment, activation of apoptin was assayed by its phosphorylation at position T108, assessed by means of Western blotting. Expression of LT136 alone did not trigger apoptin phosphorylation, whereas expression of ST clearly induced apoptin phosphorylation, albeit at a low level (Figure 6.2). Co-transfection of Flag-tagged apoptin with ST fused to an artificial nuclear location signal (NLS-ST) increased the level of apoptin phosphorylation significantly (Figure 6.3B).

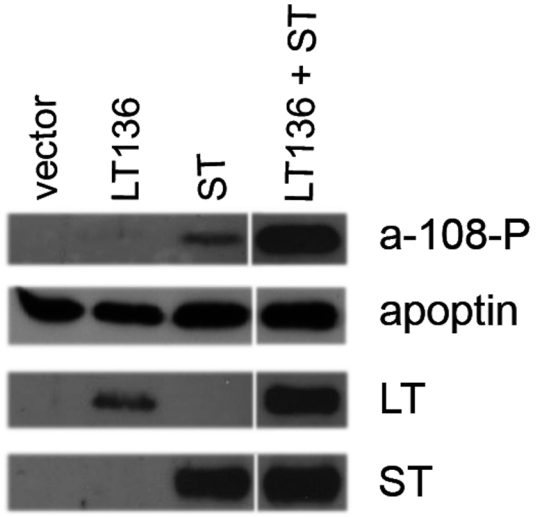

Figure 6.2 Nuclear targeting of SV40 ST activates apoptin. In normal human fibroblasts, apoptin was co-transfected with vector only (Neo), LT136, ST, or both LT136 and ST. Twenty-four hours post-transfection, cells were lysed for Western blot analysis with the antibodies indicated.

C103S mutation within PP2A-binding domain of ST disables activation of apoptin

Besides its J domain, shared with LT136, ST contains a unique site for the binding and inactivation of PP2A. This domain has been shown to contribute to cellular transformation (Pipas, 2009). A single amino-acid mutation C103S within the ST protein drastically diminishes the interaction of ST with PP2A (Figure 6.3A) and its transforming capacity (Fahrbach, et al., 2008). Therefore, we studied the effect of the C103S mutation within the PP2A binding site on the activation of apoptin by (nuclear) ST in normal human cells.

F9 cells were analyzed for phosphorylation of Flag-apoptin upon co-expression with ST, NLS-ST or NLS-ST(C103S) protein. Figure 6.3B shows that expression of NLS-ST clearly induced apoptin phosphorylation. Introduction of the NLS-ST(C103S) mutation completely abolished this induction, although apoptin protein was expressed at a similar level. These results suggest that ST interaction with PP2A is crucial to apoptin activation, and that inactivation of PP2A by ST might be sufficient to activate apoptin.

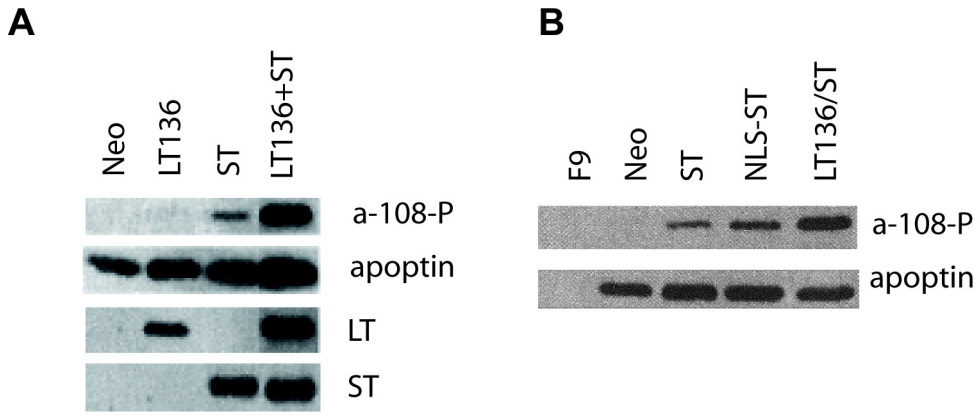

Figure 6.3 Apoptin activation via inhibition of PP2A by ST. **A.** PP2A binding to ST is abolished by C103S mutation. LT136 and ST with or without the C103S mutation (LT136/ST or LT136/ST(C103S) were fused with a Strep-tag at their N-terminus. Cell lysates were prepared at 24h post-transfection for protein-protein interaction assays as indicated in Material and Methods. The final elutions were analyzed by Western Blot with antibodies against PP2Aα subunit, LT and ST, respectively. Actin was taken as equal loading control. The first lane (input control) indicated total amount of endogenous proteins in direct cell lysates. **B.** pcDNA-Flag-apoptin was co-transfected with plasmids encoding the indicated proteins or vector DNA into F9 primary fibroblasts. Western blot assays were performed with the indicated antibodies at 24h post-transfection.

Knock-down of PP2A B56γ via RNAi activates apoptin phosphorylation in normal human fibroblasts

Two independent studies reported that ST interaction with PP2A resulted in the inhibition of the B56γ regulatory subunit, resulting in cellular transformation (Chen, et al., 2007; Cho, et al., 2007). Therefore, we examined whether down-regulation of B56γ via RNAi could trigger phosphorylation of apoptin in normal cells. Our shRNA sequence was verified to reduce ectopic expression of B56γ (Figure 6.4A). Normal F9 fibroblasts co-expressing both apoptin and shRNA directed against B56γ mRNA manifested a clear level of phosphorylated apoptin in comparison to the cells transfected with apoptin and the RNAi control vector (Figure 6.4C). Our data thus indicate that inhibition of the PP2A B56γ subunit is a crucial and sufficient step for apoptin phosphorylation.

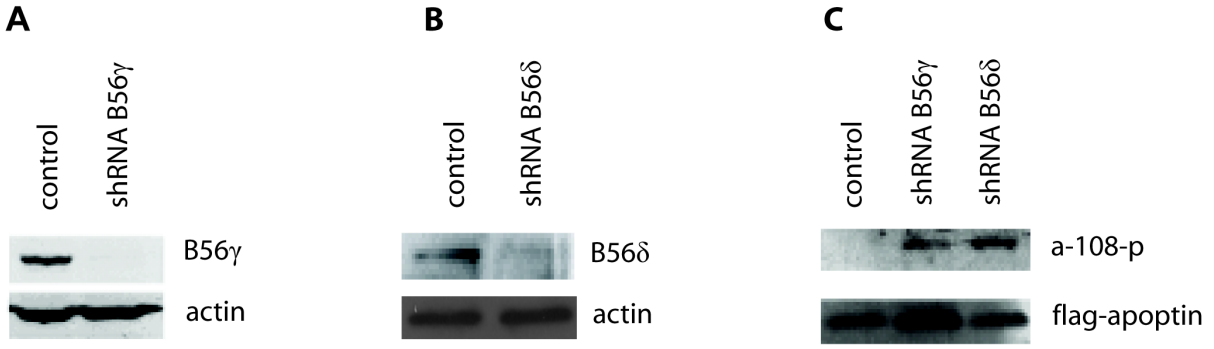

Figure 6.4 Knock-down of PP2A B56γ and B56δ subunits triggers apoptin phosphorylation in normal cells. **A.** Down-regulation of PP2A B56γ subunit by shRNA. HeLa cells were co-transfected with pCEP-4HA-B56γ expressing 4HA-tagged B56γ, and either shB56γ or control pSuper vector and lysed 48h post-transfection, followed by western blotting analysis with the indicated antibodies. **B.** Down-regulation of PP2A B56δ subunit by shRNA. F9 cells were transfected with pSuper vector encoding shRNA directed against PP2A B56δ or pSuper vector control; 24h post-transfection, cell lysates were prepared and subsequently analyzed by Western blot. **C.** F9 cells were co-transfected with Flag-apoptin and either pSuper vector encoding shRNA directed against PP2A B56γ, δ or control; 24-48h post-transfection, cell lysates were prepared and subsequently analyzed by Western blot.

Over-expression of PKA-interacting protein BCA3 stimulates apoptin activity in tumor cells

Analogous to the enhancing effect of ST on apoptin phosphorylation in normal cells, we observed an enhancement of apoptin phosphorylation in tumor cells by BCA3. BCA3 was identified as an apoptin-interacting protein by means of a yeast two-hybrid assay, and interacts with apoptin in a human cellular background (Figure 6.5A). Co-expression of BCA3 and Flag-apoptin in human Saos-2 tumor cells resulted in a significant increase in the apoptosis activity of apoptin (Figure 6.5B). In fact, as early as 6 hours after transfection, phosphorylated apoptin could readily be detected in Saos-2 cells expressing both apoptin and BCA3, whereas in cells expressing apoptin alone (control), apoptin phosphorylation was not yet visible at this early time-point (Figure 6.5C).

As BCA3 (9) has been shown to interact with the catalytic subunit of PKA (Sastri, et al., 2005), the involvement of PKA in apoptin phosphorylation was investigated. Treatment of Saos-2 cells with H89, a known PKA inhibitor (Lochner and Moolman, 2006), enhanced apoptin phosphorylation. In

contrast, addition of the PKA activator cAMP (Bulun and Simpson, 2008) diminished apoptin phosphorylation (Figure 6.5D). These results suggest that interference with PKA activity favors activation of apoptin. Taking into account the fact that PP2A has been shown to be a target of PKA (Ahn, et al., 2007), this provides another link pointing to the involvement of PP2A in the activation of apoptin.

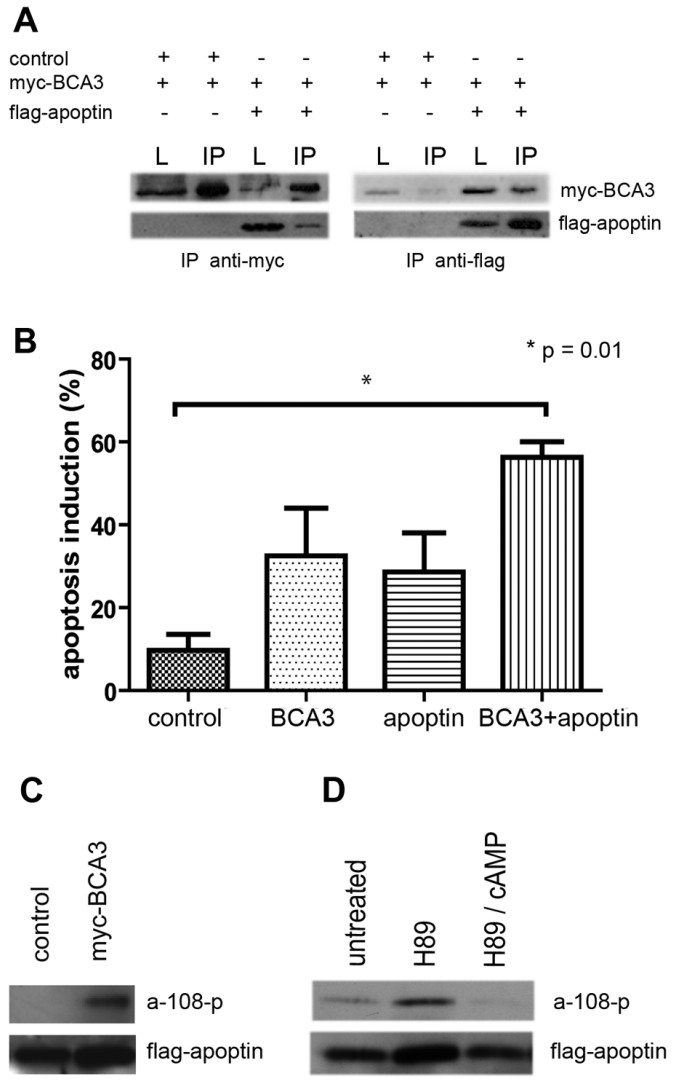

Figure 6.5 BCA3 interacts with apoptin and stimulates its activity. **A.** Apoptin interacts with BCA3 in a cellular background. Normal human foreskin fibroblasts were transfected with plasmids encoding myc-tagged BCA3 and Flag-tagged apoptin, or control plasmid in the indicated combinations. Total lysates (L) or protein complexes immunoprecipitated (IP) with antibody against the myc- (left panel) or Flag-tags (right panel) were separated by SDS-PAGE and analyzed by Western blot. **B.** Expression of myc-BCA3 together with Flag-apoptin results in increased induction of apoptosis. Human Saos-2 tumor cells were transfected with plasmids encoding Flag-tagged apoptin and myc-tagged BCA3, or vector control in the indicated combinations and grown on glass coverslips. Forty-eight hours post-transfection, slides were fixed and stained with appropriate antibodies for immunofluorescence analysis (right panel). pMaxGFP (Amaxa) was used as a negative control for apoptosis. Data are representative of 3 independent experiments, in which at least 100 cells were scored. **C.** Co-expression of myc-BCA3 enhances apoptin phosphorylation. Saos-2 cells transfected with BCA3 and apoptin, or apoptin alone, were lysed 6 hours post-transfection and analyzed for apoptin phoshphorylation by Western blot analysis. **D.** Inhibition of PKA results in increased apoptin phosphorylation. Saos-2 cells were transfected with Flag-tagged apoptin, and treated with the PKA inhibitor H89 (1h, 10μM) or activator cAMP (30 minutes, 10μM H89, followed by 30 minutes 1mM cAMP) 24hs post-transfection. Cells were then lysed and their contents analyzed by Western blot using indicated antibodies.

Down-regulation of PP2A B56δ subunit activates apoptin phosphorylation in normal cells

Ahn et al. reported that the PP2A B56δ subunit is phosphorylated by PKA, thereby increasing the overall activity of PP2A. The activity of a B56δ mutant that cannot be phosphorylated by PKA is significantly less than its wild-type counterpart (Ahn, et al., 2007). Therefore, one can assume that inhibition of PKA results in a diminished functional PP2A B56δ subunit, and hence aberrant PP2A activity.

We examined whether inhibition of the expression of the PP2A B56δ subunit in human F9 cells positively affected the phosphorylation of apoptin. Down-regulation of B56δ protein expression through RNAi was confirmed in normal F9 fibroblasts (Figure 6.4B). Co-expression of shRNA targeting B56δ mRNA and apoptin in normal F9 cells clearly resulted in the activation of apoptin phosphorylation, as compared to F9 cells transfected with apoptin and the RNAi control vector (Figure 6.4C).

Our results imply that PP2A complexes containing the regulatory subunits B56 γ and δ are essential for maintaining a normal cell environment, as the loss of either one of these subunits results in the activation of apoptin, which is typical for cancer cells.

Discussion

Activation of tumor-selective apoptosis-inducing proteins is an intriguing phenomenon, and revealing the molecular switch behind this process could allow important insights for developing anticancer therapies. Apoptin was the first protein known to harbor apoptosis activity selectively in transformed human cells (Danen-van Oorschot, et al., 1997). However, the molecular mechanisms underlying apoptin's tumor-selective activity are largely unknown (Grimm and Noteborn, 2010). Here, our studies reveal that apoptin becomes activated by inhibition of normal PP2A function. Two independent

lines of research pointed to a fundamental role of PP2A in the regulation of apoptin activity.

In one study, we examined the effect of specific domains within the transforming SV40 proteins LT136 and ST on the activation of apoptin. The C-terminal ST PP2A-binding transformation domain (Arroyo and Hahn, 2005; Pipas, 2009), when targeted to the nucleus of normal cells, turned out to be crucial for the tumor-characteristic activation of apoptin. We showed that a C103S mutation within the ST PP2A binding site (Chen, et al., 2004) abrogated the phosphorylation of apoptin induced by NLS-ST in normal human fibroblasts. RNA interference studies confirmed that inactivation of the B56γ protein promotes phosphorylation of apoptin in human fibroblasts.

A second line of research indicated that interference with the PP2A B56δ domain also led to the activation of apoptin. Over-expression of the apoptin- and PKA-interacting partner BCA3, or inhibition of PKA by treatment with H89 both resulted in enhanced phosphorylation and apoptosis activity of apoptin in human cancer cells. PKA is known to phosphorylate the B56δ subunit of PP2A leading to increased PP2A activity (Ahn, et al., 2007). RNA interference studies showed that inhibition of the synthesis of the PKA target PP2A-B56δ indeed activated apoptin phosphorylation in normal human cells.

Our studies, carried out from two different angles of research, reveal that apoptin senses PP2A inactivation during malignant cellular transformation. Interestingly, the delta and gamma subunits are the only nuclear PP2A B56 subunits (Chen, et al., 2004; McCright, et al., 1996), and nuclear localization is important for both LT/ST-induced cell transformation and apoptin-induced tumor-selective apoptosis. PP2A complexes containing B56δ domains prevent entry of cells into mitosis upon DNA damage (Virshup and Shenolikar, 2009), averting genetic instability that might contribute to cell transformation. B56γ mediates dephosphorylation and stabilization of the tumor suppressor protein p53 upon DNA damage, inhibiting cellular

proliferation and transformation (Li, et al., 2007). Studies have shown that derailment of B56γ results in aberrancies in functioning of e.g. cell cycle and tumor suppressor proteins, resulting in cell transformation (Chen, et al., 2004; Sablina and Hahn, 2008).

PP2A has also been shown to play a role in the activation of other tumor-selective apoptosis-inducing proteins. Besides apoptin, also the adenovirus E4orf4 protein has been shown to selectively induce apoptosis in human cancer cells (Branton, et al., 2001; Kleinberger, 2000). Direct interaction of E4orf4 with PP2A regulatory B domains is essential for the tumor-selective apoptosis activity of E4orf4 (Strichman, et al., 2000). Interaction of E4orf4 with the PP2A B55 subunit results in down-regulation of the expression of the Myc oncogene (Ben-Israel, et al., 2008). In addition to SV40 ST, other viral transforming proteins also interact with PP2A, showing its relevance in cellular transformation (Zhao and Elder, 2005). These findings corroborate earlier reports that aberrant PP2A functioning activates transforming processes, which then elicit the activation of tumor-selective apoptosis-inducing proteins such as apoptin or E4orf4.

Further steps within the development of tumorigenic cells seem at least not critical for apoptin's tumor-selective apoptosis characteristics. These conclusions are in accordance with the observations by others and ourselves that apoptin is able to induce apoptosis in a very broad panel of tumor types (Backendorf, et al., 2008; Grimm and Noteborn, 2010; Maddika, et al., 2006; Tavassoli, et al., 2005). If one assumes that tumor cells arise by a wide variety of mechanisms, all the while sharing a limited number of key characteristics, then apoptin simply needs to recognize one (or a subset) of these characteristics.

We have shown that inactivation of the nuclear PP2A B56 γ and/or δ subunits is sufficient to trigger apoptin's tumor-selective apoptosis activity. PP2A provides a central phosphatase activity affecting many cellular signaling pathways, and derailment of PP2A activity seems to be one of the

fundamental events occurring during oncogenic transformation. This implies that apoptin might be applied for the treatment of tumors arising from a wide range of origins.

Acknowledgements

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References

Ahn JH, McAvoy T, Rakhilin SV, Nishi A, Greengard P, Nairn AC (2007) Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56δ subunit. *Proc Natl Acad Sci U S A* 104, 2979-2984.

Alvisi G, Poon IK and Jans DA (2006) Tumor-specific nuclear targeting: promises for anti-cancer therapy. *Drug Resist Updat* 9, 40-50.

Arnold HK and Sears RC (2006) Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Cell Biol* 26, 2832-2844.

Arroyo JD and Hahn WC (2005) Involvement of PP2A in viral and cellular transformation. *Oncogene* 24, 7746-7755.

Backendorf C, Visser A, De Boer A, Zimmerman R, Visser M, Voskamp P et al. (2008) Apoptin: Therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Ben-Israel H, Sharf R, Rechavi G and Kleinberger T (2008) Adenovirus E4orf4 protein down-regulates Myc expression through interaction with PP2A-B55 subunit. *J Virol* 82, 9381-9388.

Boutros M and Ahringer J (2008) The art and design of genetic screens: RNA interference. *Nat Rev Genet* 9, 554-566.

Branton PE and Roopchand DE (2001) The role of adenovirus E4orf4 protein in viral replication and cell killing. *Oncogene* 20, 7855-7865.

Bruno P, Brinkmann C, Boulanger M, Flinterman M, Klanrit P, Landry M et al. (2009) Family at last: highlights of the first international meeting on proteins killing tumour cells. *Cell Death and Differentiation* 16, 184-186.

Bulun SE and Simpson ER (2008) Aromatase expression in women's cancers. *Adv Exp Med Biol* 630, 112-132.

Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC (2004) Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* 5, 127-136.

Chen Y, Xu Y, Bao Q, Xing Y, Li Z, Lin Z et al. (2007) Structural and biochemical insights into the regulation of protein phosphatase 2A by small t antigen of SV40. *Nat Struc Mol Biol* 6, 527-534.

Cho US, Morrone S, Sablina AA, Arroyo JD, Hahn WC, Xu W (2007) Structural basis of PP2A inhibition by small t antigen. *PLoS Biol* 5, e202.

Danen-Van Oorschot AAAM, Fischer DF, Grimbergen JM, Klein B, Zhuang Y-H, Falkenburg JHF et al. (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci U S A* 94, 5843-5847.

Danen-Van Oorschot AAAM, Voskamp P, Seelen MCMJ, Miltenburg MHAM, Bolk MW, Tait SW et al. (2004) Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11, 564-573.

Eichhorn PJ and Creyghton MP, Bernards R (2009) Protein phosphatase 2A regulating subunits and cancer. *Biochem Biophys Acta* 2795,1-15.

Fahrbach KM, Katzman RB and Rundell K (2008) Role of SV40 ST antigen in the persistent infection of mesothelial cells. *Virology* 379, 255-263.

Gjoerup O, Zaveri D, and Roberts TM (2001) Induction of p53-independent apoptosis by simian 40 small t antigen. *J Virol* 75, 9142-9155.

Grimm S and Noteborn M (2010) Anti-cancer genes: inducers of tumour-specific cell death signalling. *Trends in Molecular Medicine* 16, 88-96.

Kitching R, Li H, Wong MJ, Kanaganayakam S, Kahn H, and Seth A (2003) Characterization of a novel human breast cancer associated gene (BCA3) encoding an alternatively spliced proline-rich protein. *Biochim Biophys Acta* 1625, 116-121.

Kleinberger T (2000) Induction of apoptosis by adenovirus E4orf4 protein. *Apoptosis* 5, 211-215.

Li H, Cai X, Shouse GP, Piluso LG and Liu X (2007) A specific PP2A regulatory subunit, B56γ, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J* 26, 402–411.

Lochner A and Moolman JA (2006) The many faces of H89: a review. *Cardiovasc Drug Rev* 24, 261-274.

Maddika S, Mendosa FJ, Hauff K, Zamzow CR, Paranjothy T, Los M (2006) Cancer-selective therapy of the future: apoptin and its mechanism of action. *Cancer Biol Ther* 5, 10-19.

McCright B, Rivers AM, Audin S and Virshup DM (1996) The B56 family of protein phosphatise 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J Biol Chem* 271, 22081-22089.

Naviglio S, Caraglia M, Abbruzzese A, Chiosi E, Di Gesto D, Marra M et al. (2009) Protein kinase A as a biological target in cancer therapy. *Expert Opin Ther Targets* 13, 83-92.

Noteborn MHM, De Boer GF, Van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG et al. (1991) Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol* 65, 3131-3139.

Pipas J (2009) SV40: Cell transformation and tumorigenesis. *Virology* 384, 294-303.

Rohn JL and Noteborn MHM. (2004) The viral death effector apoptin reveals tumor-specific processes. *Apoptosis* 9, 315-322.

Rohn JL, Zhang Y-H, Aalbers RIJM, Otto N, Den Hertog J, Henriquez NV, et al. (2002) A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem* 277, 50820-50827.

Sablina AA and Hahn WC (2008) SV40 small T antigen and PP2A phosphatase in cell transformation. *Cancer Metastasis Rev* 27,137-146.

Sablina AA, Hector M, Colpaert N and Hahn WC (2010) Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res* 70, 10474-10484.

Sastri M, Barraclough DM, Carmichael PT and Taylor SS (2005) A-kinase-interacting protein localizes protein kinase A in the nucleus. *Proc Natl Acad Sci U S A* 102, 349-354.

Schmidt TGM and Skerra A (2007) The Strep-tag system for one-strep purification and high-affinity detection or capturing of proteins. *Nature Protocols* 2, 1528-1535.

Srinivasan A, McClellan AJ, Vartikar J, Marks I, Cantalupo P, Li Y et al. (1997) The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Mol Cell Biol* 17,4761-4773.

Strichman R, Sharf R and Kleinberger T (2000) Adenovirus E4orf4 protein interacts with both Balpha and B' subunits of protein phosphatase 2A but E4orf4-induced apoptosis is mediated only by its interaction with Balpha. *Oncogene* 19, 3757-3763.

Tavassoli M, Guelen L, Luxon BA and Gaken J (2005) Apoptin: specific killer of tumor cells? *Apoptosis* 10, 717-724.

Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H et al. (1998) Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B" (delta) regulatory subunit in vitro and identification of the phosphorylation sites. *FEBS Lett* 430, 312-316.

Van Kanegan MJ and Strack S (2009) The protein phosphatase 2A regulatory subunits B'beta and B'delta mediate sustained TrkA neurotrophin receptor autophosphorylation and neuronal differentiation. *Mol Cell Biol* 29, 662-674

Virshup DM and Shenolikar S (2009) From promiscuity to precision: protein phosphatases get a makeover. *Molecular Cell* 33, 537-545.

Wenner CE (2010) Cell signaling and cancer - possible targets for therapy. *J Cell Physiol* 223, 299-308.

Westermarck J and Hahn W (2008) Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* 14,152-60.

Yamashita K, Ikenaka Y, Kakutani T, Kawaharada H, and Watanabe K (1990) Comparison of human lymphotoxin gene expression in CHO cells directed by genomic DNA or cDNA sequences. *Agric Biol Chem* 54, 2801-2809.

Zhang YH, Kooistra K, Pietersen AM, Rohn JL, and Noteborn MH (2004) Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen. *J Virol* 78, 9965-9976.

Zhao RY and Elder RT (2005) Viral infections and cell cycle G2/M regulation. *Cell Res* 115, 143-149.