Apoptosis (2008) 13:483–494
DOI 10.1007/s10495-008-0193-x

ORIGINAL PAPER

Tumor-associated protein SPIK/TATI suppresses serine protease dependent cell apoptosis

Xuanyong Lu · Jason Lamontagne · Felix Lu · Timothy M. Block

Published online: 18 March 2008

Abstract Serine protease dependent cell apoptosis (SPDCA) is a recently described caspase independent innate apoptotic pathway. It differs from the traditional caspase dependent apoptotic pathway in that serine proteases, not caspases, are critical to the apoptotic process. The mechanism of SPDCA is still unclear and further investigation is needed to determine any role it may play in maintaining cellular homeostasis and development of disease. The current knowledge about this pathway is limited only to the inhibitory effects of some serine protease inhibitors. Synthetic agents such as pefabloc, AEBSF and TPCK can inhibit this apoptotic process in cultured cells. There is little known, however, about biologically active agents available in the cell which can inhibit SPDCA. Here, we show that over-expression of a cellular protein called serine protease inhibitor Kazal (SPIK/TATI/PSTI) results in a significant decrease in cell susceptibility to SPDCA, suggesting that SPIK is an apoptosis inhibitor suppressing this pathway of apoptosis. Previous work has associated SPIK and cancer development, indicating that this finding will help to open the doorway for further study on the mechanism of SPDCA and the role it may play in cancer development.

X. Lu (✉) · F. Lu · T. M. Block
Drexel Institute for Biotechnology and Virology Research, Department of Microbiology and Immunology, College of Medicine, Drexel University, 3805 Old Easton Road, Doylestown, PA 18902, USA
e-mail: xl34@drexel.edu

J. Lamontagne · T. M. Block
Institute for Hepatitis and Virus Research, Doylestown, PA 18902, USA

Keywords Serine protease inhibitor · Apoptosis · Serine protease dependent cell apoptosis · Caspase · Apoptosis inhibitor · Cancer

Abbreviations

BFA	Brefeldin A
CDCA	Caspase dependent cell apoptosis
CHX	cycloheximide
SPDCA	Serine protease dependent cell apoptosis
SPIK	Serine protease inhibitor Kazal

Introduction

Apoptosis, or programmed cell death, is essential for embryonic development, immune-system function, and the maintenance of tissue homeostasis in multicellular organisms. Ineffective regulation of apoptosis has been implicated in numerous pathological conditions, including neurodegenerative diseases, autoimmunity and cancer [1]. The execution of the apoptotic program has traditionally been considered the result of activation of a family of proteases known as caspases. Caspases are cysteinyl aspartate proteases cysteine proteases that cleave their substrates following an Asp residue. Caspase dependent cell apoptosis (CDCA) usually initiates by activating caspase-8 and 10 through proteolysis of their proenzymes, which further activates the executioner caspases, such as caspase-3 and caspase-7, resulting in the degradation of chromosomal DNA and cell death [2, 3]. CDCA can be blocked by cellular proteins called inhibitors of apoptosis (IAP), which directly suppress caspase activity to maintain cellular homeostasis [4, 5]. Recent evidence, however, has suggested that apoptotic cell death can also be promoted

and triggered by serine proteases in a caspase independent manner.

Serine protease dependent cell apoptosis (SPDCA) differs from CDCA in that serine proteases, not caspases, are critical to the apoptotic process [6–8]. SPDCA can be triggered by viral infection, and also by treating cells with certain synthetic agents. In 1997, Abate and Schroder found that lipopolysaccharide (LPS) caused a concentration-dependent toxicity in a macrophage cell line which was completely inhibited by the serine protease inhibitors tosylphenylalanylchloromethane (TPCK) and tosyl-lysyl-chloromethane (TLCK) [8]. In 2003, Egger et al. reported that treatment of brefeldin A (BFA) combined with cycloheximide (CHX) can induce apoptosis in R6 embryo fibroblasts and HeLa cells. They found that, unlike in CDCA, apoptosis induced by BFA/CHX was not blocked by the pan-caspase inhibitor Z-VAD, but was blocked by the serine protease inhibitor pefabloc [6]. SPDCA was also reported by Thorburn et al., when they infected prostate tumor cells with an adenovirus recombinant that expresses a Fas-associated death domain protein (FDD) to trigger CDCA [7]. Surprisingly, they found that the apoptosis induced by FDD was not completely blocked by Z-VAD, and FDD induced apoptosis could only be completely blocked by combining Z-VAD with the serine protease inhibitor AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride) [7]. Although the SPDCA pathway has been observed by different groups, the serine proteases and possible cellular inhibitors involved in the mechanism are still unknown.

Serine protease inhibitor Kazal (SPIK/SPINK1), which is also known as tumor-associated trypsin inhibitor (TATI) and Pancreatic secretory trypsin inhibitor (PSTI), is a small protein translated from a 240 nucleotide gene [9–11]. It was first discovered in the pancreas as an inhibitor of autoactivation of trypsinogen and has been identified as an important regulator of cellular proteases [12]. The SPIK gene is normally not expressed outside of the pancreas, however, its expression is elevated in numerous cancers such as pancreatic cancer, colorectal cancer, renal-cell carcinoma, intrahepatic cholangiocarcinoma (ICC) and hepatocellular carcinoma (HCC) [13–17]. Recent findings have also correlated increased SPIK expression with tumor growth and early recurrence of ICC and HCC in patients after surgical resection [16, 17]. The cause of SPIK up-regulation, as well as any role SPIK may play in cancer formation and development remains uncertain. It is possible, however, that SPIK works as an apoptosis inhibitor, preventing the apoptotic death of malignant cells, resulting in progression of cancer. With this in mind we explored the possibility that SPIK could inhibit cell apoptosis. Because SPIK is a serine protease inhibitor, we specifically explored the possibility that SPIK could inhibit SPDCA. Here, we report that over-expression of SPIK can greatly reduce the susceptibility of cultured cells to SPDCA, suggesting that SPIK is a likely SPDCA inhibitor.

Materials and methods

Plasmids and cell lines

Plasmid PWT was constructed from the pCMVscript vector (Stratagene, La Jolla, CA), and contains the entire SPIK gene under the control of a HCMV promoter. Plasmid PWT-neo was constructed from the same vector, but with the neomycin resistance gene as a selection marker. The plasmid L183 expressing SPIK siRNA was constructed with the pSilencer1.0 vector (Ambion, Austin, TX). The SPIK siRNA expression was controlled by a U6 RNA polymerase III promoter. To generate specific SPIK siRNA, sense SPIK oligonucleotides 19 bases long (^{183}GATATATGACCCTGTCTGT^{201}), and the reverse complementary antisense oligonucleotides, were linked by a 9 nucleotide hairpin forming spacer (TTCAAGAGA). This forced the antisense SPIK oligonucleotides to fold back and form a double SPIK RNA complex. The SPIK siRNA was generated after cleavage by ribonucleases in the RNA-induced silencing complex (RISC) (manufacturer's instructions, Ambion, Austin, TX).

Huh7T cells, a cell line derived from Huh7 cells which are susceptible to serine protease induced apoptosis, were kindly provided by Dr. Tian-lun Zhou (Nucleonics Inc, Horsham, PA). Cells were cultured and maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS).

Establishing a stable cell line over-expressing SPIK

To create an SPIK over-expressing cell line, Huh7T cells were transfected with plasmid PWT-neo, which contains the entire SPIK gene and the neomycin resistance gene as a selection marker. For the control cell line, cells were transfected with the PWT-neo vector, but lacking the SPIK gene. Three days post-transfection, the cells were treated with 1 mg/ml G418. Medium was changed every 3 days to remove dead cells, and after 2 weeks the surviving cells were reseeded in new plates at a very low density. Clones to be maintained were picked after 4 days and grown in 0.5 mg/ml G418 medium. Surviving cell lines were sustained in 0.2 mg/ml G418 medium.

Transfection and induction of SPDCA

For transfection, 10^5 cells were seeded in 6-well plates. After overnight incubation, 2 μg of plasmid were

transfected with 6 μl FuGENE 6 as described by the manufacturer (Roche Diagnostics, Indianapolis, IN). The transfection efficiency was determined by transfection and expression of green fluorescent protein (hGFP). After 3 days, cells were split into two daughter 6-well plates, and cultured for an additional one day. RNA was then isolated for northern blot analysis from half of the transfected cells and SPDCA was induced in the other half by treatment with BFA/CHX/Z-VAD (5 μg/ml/10 μg/ml/100 μM). Apoptosis was examined after 24 h of treatment. For the SPIK silencing study, HeLa cells were transfected with 2 μg PWT or co-transfected with PWT and 1 μg SPIK siRNA L183 plasmid following the transfection protocol above.

Northern blot analysis

For northern blot analysis, total RNA was isolated from cells 3 days post-transfection with TRIzol (Invitrogen, Carlsbad, CA). A 1% denaturing agarose gel was used to resolve 10 μg RNA, which was then transferred to a Nylon membrane. A SPIK specific probe was labeled with 32-P dCTP using SPIK DNA derived from PCR amplification as a template. After hybridization, the bands were visualized using a Phosphorimager. Equal sample loading was confirmed by ethidium bromide (EB) stained ribosomal RNA in each experiment.

Examination of apoptosis

The early stages of apoptosis were examined by observing both characteristic morphological changes and Annexin V-FITC staining. The middle stages of apoptosis were shown by Hoechst staining and the late stages were examined by DNA fragmentation. For the annexin staining, BFA/CHX/Z-VAD treated cells were washed with PBS and then incubated with Annexin V-FITC in 100 μl of binding buffer (Trevigen Inc., Gaithersburg, MD). After incubating for 30 min, the cells were washed with PBS and were then visualized with a fluorescent microscope. For flow cytometry (FACS) analysis, BFA/CHX/Z-VAD treated cells were released by limited trypsin digestion and washed with PBS. The cells were re-suspended in 100 μl total volume of Annexin V-FITC and binding buffer as per the manufacturer's instructions and incubated at room temperature in the dark for 25 min (Biosource International, Camarillo, CA). For cells double stained with Annexin V-FITC and propidium iodide (PI), 0.5 μg PI was added 3 min before the end of the Annexin V incubation. The cells were then washed with PBS, re-suspended in 400 μl binding buffer, and apoptosis was analyzed using a Becton Dickinson FACScan flow cytometer. For Hoechst staining, the cells were washed once with water after treatment of BFA/CHX/Z-VAD, followed by incubation with 100 μg/ml of dye at room temperature for 5 min. The cells were then washed two additional times, before visualization using a fluorescent microscope. Condensed nuclei, indicating apoptotic cells, and total cells were counted. The percentage of cells with condensed nuclei (condensed nuclei/total cell) was calculated based on the average of four fields of view. To analyze DNA fragmentation, treated cells were lysed with Tris–HCl, 0.05 M pH 8.0, 0.15 M NaCl, 0.1% NP-40, 1 mM EDTA, and 1 mg/ml proteinase K at 50°C for 1 h, then 37°C for 2 h. RNA was degraded by incubation with 0.2 mg/ml RNase A at 37°C for 1 h. Chromosomal DNA was isolated by phenol/chloroform extraction (repeated twice) and precipitated by adding 2.0 volumes of 100% ethanol at −20°C overnight. Pellets were dissolved in 0.01 M Tris–HCl, pH 8.0. 20 μg DNA was resolved on a 1.5% agarose gel and visualized by EB staining.

Caspase 3 activity analysis

Apoptosis was induced using BFA/CHX as previously described for SPDCA, or by treatment with 400 μM etoposide for 24–48 h for CDCA. Z-VAD was added to the BFA/CHX treatment to completely block caspase activity in SPDCA induction. The activity of caspase 3 was analyzed using BD ApoAlert caspase colorimetric assay kits (BioVision, Mountain View, CA). The results were read using a SLT Rainbow microplate reader at 405 nm.

Immunofluorescence and In-Cell Western

S2-3, SP23-5, and parental Huh7T cells were seeded on an 8-chamber slide for immunofluorescence or a 96-well plate for In-Cell Western analysis. After overnight growth, the cells were fixed by adding cold methanol and incubating at −20°C for 20 min. The cells were then blocked for 1 h at 37°C with 2% BSA/PBS. After washing with 0.05% Tween 20/PBS, cells were incubated for 2 h at 37°C with the anti-PSTI (SPIK) antibody (Boca Scientific, Boca Raton, FL), diluted 1:50 with 2% BSA/PBS. The cells were then washed three times with 0.05% Tween 20/PBS, and incubated for 1 h in the dark at 37°C with secondary antibody. For the immunofluorescence, the cells were incubated with FITC conjugated anti-mouse at a 1:2000 dilution. For the In-Cell Western, the cells were incubated with IRDye labeled anti-mouse antibody (LiCor Biosciences, Lincoln, Nebraska) at a 1:15,000 dilution. After incubation, the cells were washed four times with 0.05% Tween 20/PBS. For immunofluorescence analysis, the cells were visualized using a fluorescent microscope. For the In-Cell Western, the plate was read and color was determined using a LiCor Odyssey machine.

In vitro transcription and translation of SPIK

Plasmids PWT (wild-type SPIK), P32 (AUG mutant), and P21 (C39S mutant) were linearized by digestion with the endonuclease AatII. SPIK mRNA was produced using an in vitro transcription system (Promega, Madison, WI) following the manufacturer's instructions. RNA was resolved in a 1% agarose gel and either stained with EB or transferred to nylon membrane for northern blot analysis by hybridization with a SPIK specific probe. SPIK protein was translated from 1 μg PWT, P32, or P21 mRNA using the EasyXpress Random Biotin Labeled protein synthesis kit (Qiagen, Valencia, CA) as described by the manufacturer. The biotin labeled Lysine was added during incubation to label newly synthesized protein. Labeled proteins were resolved by SDS-PAGE, and transferred to a PVDF membrane. The membrane was blocked with 2% bovine serum albumin and incubated with a 1:2000 dilution of streptavidin-HPR (Abcam, Cambridge, UK). The bands were visualized with an ECL+ Chemiluminescence kit (Amersham, Piscataway, NJ).

Results

Over-expression of SPIK prevents SPDCA in HeLa cells

To determine whether SPIK could suppress SPDCA, HeLa cells were transfected with plasmid PWT, which contains the entire SPIK gene under the control of a HCMV promoter, and the impact of SPIK expression upon SPDCA was evaluated. Cells transfected with the vector of PWT or co-transfected with the plasmid L183 expressing SPIK siRNA targeting the SPIK sequence at location 183–201 (See Materials and methods) were used as controls.

Total RNA was isolated from transfected cells, transferred to a nylon membrane, and hybridized with a SPIK-specific probe. Northern blot shows that the endogenous and constitutive SPIK expression in normal HeLa cells is very low, and was below the level of northern blot detection (Fig. 1a, Vector). RT-PCR confirmed that very little endogenous SPIK was expressed in HeLa cells (data not

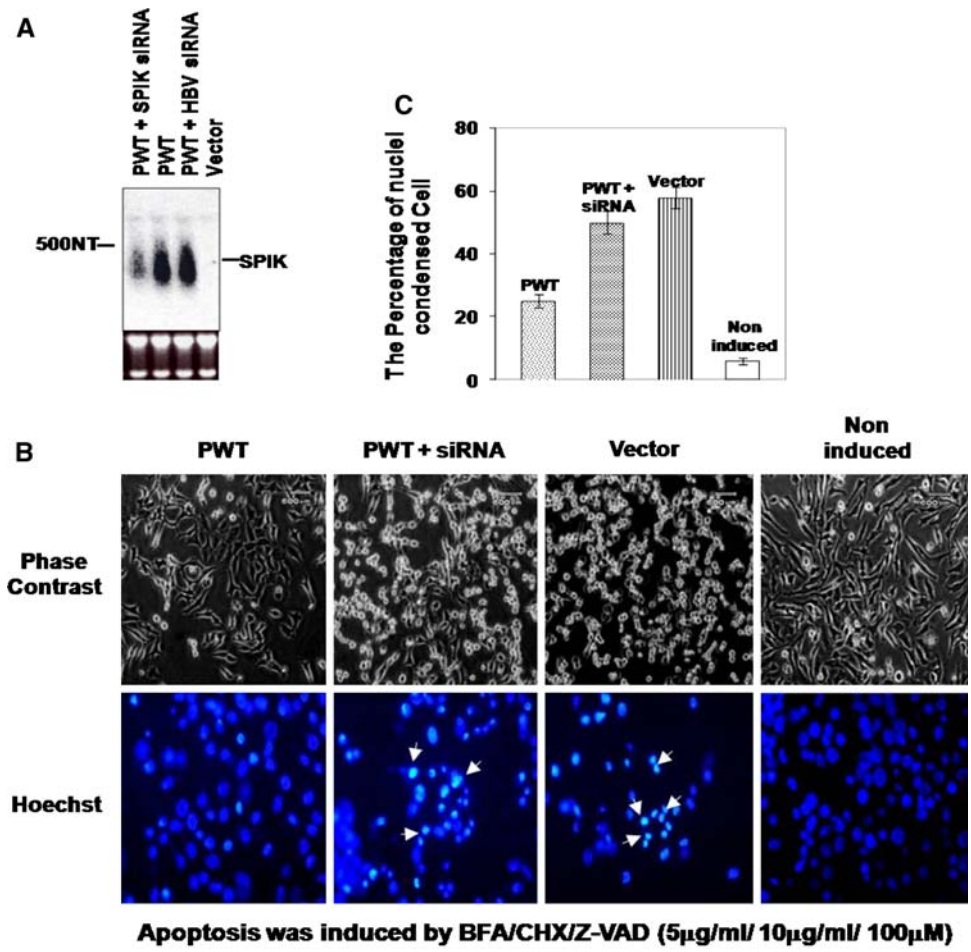

Fig. 1 SPIK expression suppresses apoptosis in HeLa cells. HeLa cells were transfected with plasmid PWT (SPIK) containing the entire SPIK gene. As a control, SPIK siRNA L183 was co-transfected with PWT. (**a**) Northern blot detects SPIK mRNA. 10 μg RNA from transfected cells or control cells was resolved on a 1% agarose gel, and hybridized with 32-dCTP labeled SPIK probe. The endogenous SPIK in HeLa cells is below the level of detection [17]. Ethidium bromide (EB) stained ribosome RNA is used as a loading control. (**b**) Cell apoptosis was examined after treatment with BFA/CHX/Z-VAD. Upper panels show morphological changes indicative of apoptotic cells under phase contrast. Bottom panels show DNA condensation in apoptotic cells stained with Hoechst dye. The arrows indicate typical apoptotic cells with DNA condensation. (**c**) The percentage of cells with condensed nuclei (condensed nuclei/total cells) in transfected cells

shown), which is in agreement with the observations of Lee et al. [17]. On the other hand, the expected ~400 nt transcripts associated with SPIK RNA were clearly detected in the cells transfected with PWT (Fig. 1a, PWT). Cells co-transfected with plasmid PWT and plasmid L183 expressing SPIK siRNA resulted in a greatly reduced level of SPIK RNA compared to cells transfected with PWT alone (Fig. 1a, PWT versus PWT+SPIK siRNA). The expression of SPIK in HeLa cells was not affected by co-transfection of a plasmid expressing hepatitis B virus (HBV) siRNA (Fig. 1a, PWT+HBV siRNA), confirming the specificity of the siRNA. Expression of GFP was unaffected by transfection of L183, which also confirms the specificity of the SPIK siRNA (data not shown).

Knowing that endogenous SPIK levels in HeLa cells are undetectable by northern blot, we reasoned that detectable SPIK gene products could be produced in a transfection dependent manner. With this in mind, we determined cell sensitivity to apoptosis as a function of transfected SPIK. SPDCA was induced in the transfected cells by incubation with BFA/CHX as described by Egger et al [6]. To ensure that SPDCA was selectively induced and CDCA was minimized, the pan-caspase inhibitor Z-VAD was also added. CDCA inhibition was supported by the absence of caspase 3 activity in the treated cells, which will be discussed later.

After a one-day treatment with BFA/CHX/Z-VAD, apoptosis was examined by observing morphological changes, such as cell shrinkage and membrane blebbing, as well as Hoechst staining of condensed nuclei. More than 58% of HeLa cells transfected with vector (lacking the SPIK gene) showed bright blue Hoechst staining, indicative of cells undergoing apoptosis. In contrast, around 25% of HeLa cells transfected with PWT were stained (Fig. 1b, bottom panel and 1c). When SPIK expression was suppressed via co-transfection with siRNA L183, the level of nuclear condensation in the cells clearly increased to nearly that observed for the control cells (Fig. 1b and c). These results were supported by clear changes in cell morphology in the cells transfected with vector or SPIK siRNA, but not in cells transfected with PWT (Fig. 1b, upper panel). Apoptotic resistance was not limited to transfection in HeLa cells, as the same results were also shown by transfection of SPIK into Huh7T cells (data not shown). Altogether, these results imply that SPIK expression plays a role in suppressing SPDCA.

Stable cell line over-expressing SPIK is more resistant to SPDCA

In order to minimize effects that may be due, in part, to variations in transfection efficiency, stable cell lines over-expressing SPIK were constructed using Huh7T cells transfected with plasmid PWT-Neo. As a control cell line, Huh7T cells were transfected with vector lacking the SPIK gene. Eight G418 resistant cell clones from the PWT-Neo transfection and seven clones from the vector transfection were selected and established as stable cell lines. No differences in morphology or growth were found between these clones and the parental Huh7T cell line (unpublished observation), suggesting that these cells are fundamentally the same as the parental cells.

SPIK expression in these newly created cell lines was analyzed by northern blot using a probe specific for SPIK. As expected, all seven clones (SP23-1 to SP23-7) acquired from transfection of vector produced low amounts of SPIK consistent with expression from the endogenous SPIK gene in the parental Huh7T cells. Of the eight PWT-Neo plasmid clones, two (S2-3, S1-2) produced very high amounts of SPIK. Compared to the parental Huh7T cells and the SP23 cells, both S2-3 and S1-2 accumulated threefold more SPIK mRNA based on northern blot quantification using a PhosphorImager (Fig. 2a). Apoptosis studies suggest that there is no detectable difference among the SP23 clones, or between the S2-3 and S1-2 cells (unpublished data), therefore, only the data from SP23-5 and S2-3 is presented here.

The SPIK protein level in S2-3, SP23-5 and parental Huh7T cells was examined by immunofluorescence and quantified by an In-Cell Western using a conformation dependent monoclonal anti-PSTI (SPIK) antibody (Boca Scientific, FL), since detection by western blot was not possible with these reagents. Fluorescent staining was visible only in the SPIK over-expressing S2-3 cells, while no visible fluorescence was seen in SP23-5 or Huh7T cells, or in S2-3 cells incubated with secondary antibody alone (Fig. 2b). Similar results were found using an In-Cell Western assay based on techniques developed by Li-Cor Biosciences (Lincoln, NE). This assay can directly quantify cellular protein by combining in situ immunofluorescent staining and visualization by the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Figure 2c shows that S2-3 cells expressed SPIK at amounts sixfold higher than either SP23-5 or Huh7T cells, confirming over-expression of SPIK protein in the stable cell line S2-3.

To analyze the resistance of the SPIK over-expressing S2-3 cells to apoptosis, SPDCA was induced in S2-3, SP23-5, and Huh7T cells by BFA/CHX/Z-VAD treatment and apoptosis was examined at various time points. The initiation and early stages of apoptosis were studied by fluorescent Annexin V staining and examination of changes in cell morphology. The later stages were examined by DNA fragmentation.

Fluorescence-labeled Annexin V, which identifies cell surface changes in the early stages of apoptosis, was seen in the SP23 and Huh7T cells, as were morphology changes

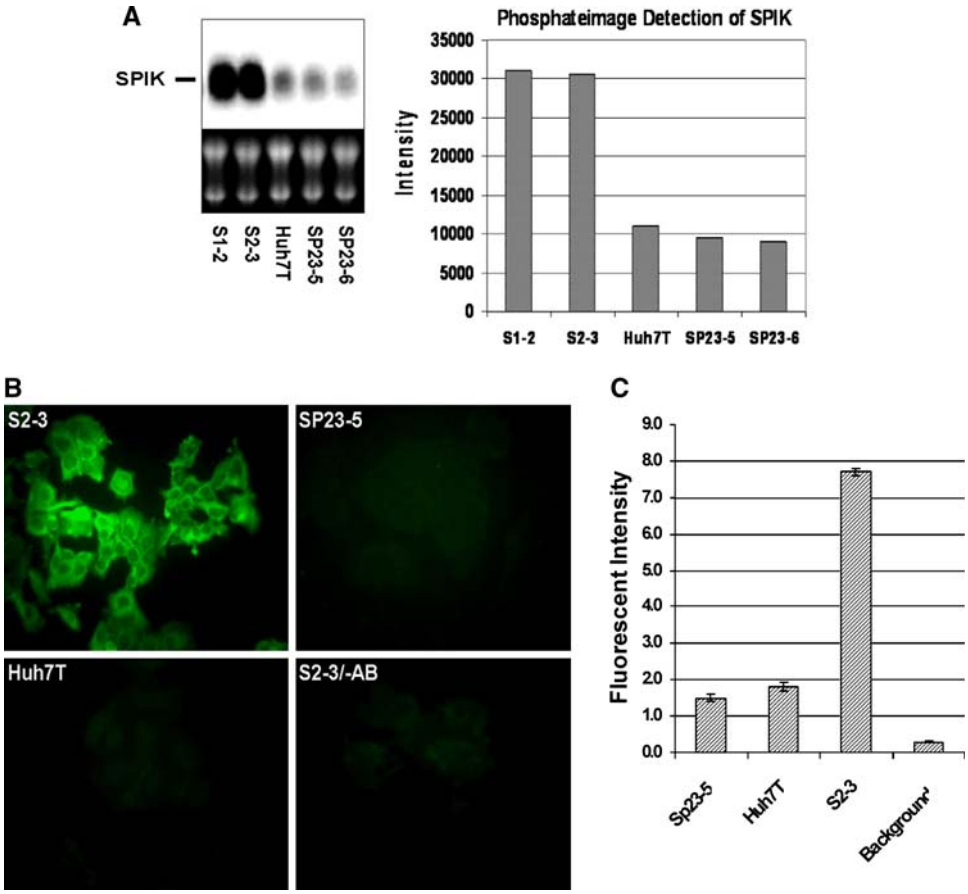

Fig. 2 SPIK expression in stable cell line S2-3. (**a**) Total SPIK mRNA was isolated from S2-3 and control cells SP23 and Huh7T, resolved on a 1% agarose gel, and hybridized with a SPIK specific probe for northern Blot analysis (*left panel*). The level of SPIK RNA was quantified by PhosphorImager (*right panel*). (**b**) SPIK protein in S2-3, SP23-5 and Huh7T cells was examined by immunofluorescent staining with a conformation dependent anti-SPIK antibody. (**c**) The SPIK protein was quantified by In-Cell Western

indicative of apoptosis. Very few S2-3 cells, however, showed positive Annexin V staining or morphological changes (Fig. 3a). Annexin V staining was also used in Flow cytometry (FACS) to quantify apoptotic cells. After initiation of SPDCA, the cells were double stained with Annexin V-FITC and propidium iodide (PI). The FACS data showed that more than 50% of the control cells underwent apoptosis, while only 26% of S2-3 cells appeared to be apoptotic (Fig. 3b). Additionally, there was no detectable difference between untreated controls and S2-3 cells (Fig. 3b).

Chromosomal DNA fragmentation is a characteristic of late stage apoptosis and therefore was used to detect apoptotic resistance. Total DNA was isolated from S2-3 and the control cells SP23 and Huh7T after treatment with BFA/CHX/Z-VAD. DNA was then resolved in a 1.5% agarose gel and stained with EB. Treatment with BFA/CHX/Z-VAD induced clear DNA fragmentation in the control cells, but not in the SPIK over-expressing S2-3 cells (Fig. 4a).

A clonogenic study of cell growth further supported the resistance of S2-3 cells to apoptotic death [18]. By reseeding BFA/CHX/Z-VAD treated cells, it showed that only the S2-3 cells retained the ability to proliferate in culture, while the Huh7T and SP23-5 cells formed no growth colonies (Fig. 4b, upper panel). The cell viability study confirmed the growth of S2-3 cells after re-seeding (Fig. 4b, bottom panel).

SPIK does not suppress CDCA

After demonstrating the correlation between SPIK expression and cell-resistance to SPDCA, we examined whether SPIK could also inhibit CDCA. To test for inhibition of CDCA, S2-3, SP23-5, and Huh7T cells were treated with etoposide, a frequently used CDCA inducer. After 24–48 h, strong fluorescence, indicating positive Annexin V staining, and changes in cell morphology indicative of apoptosis were seen in all cell lines (Fig. 5a). Approximately 62% of S2-3 cells, 58% of Huh7T, and 56% of SP23-5 cells showed positive Annexin V staining after 48 h of treatment (data not shown). This suggests that SPIK expression has no inhibitory effects upon CDCA, because apoptosis occurs similarly in these cell lines regardless of SPIK expression.

Caspase 3 activity was also used as a marker to determine CDCA induction. After a 48-h treatment with etoposide, caspase 3 activity was analyzed using a caspase 3 detection kit (Biovision, Mountain View, CA). Figure 5b shows that even though S2-3 produces sixfold more SPIK

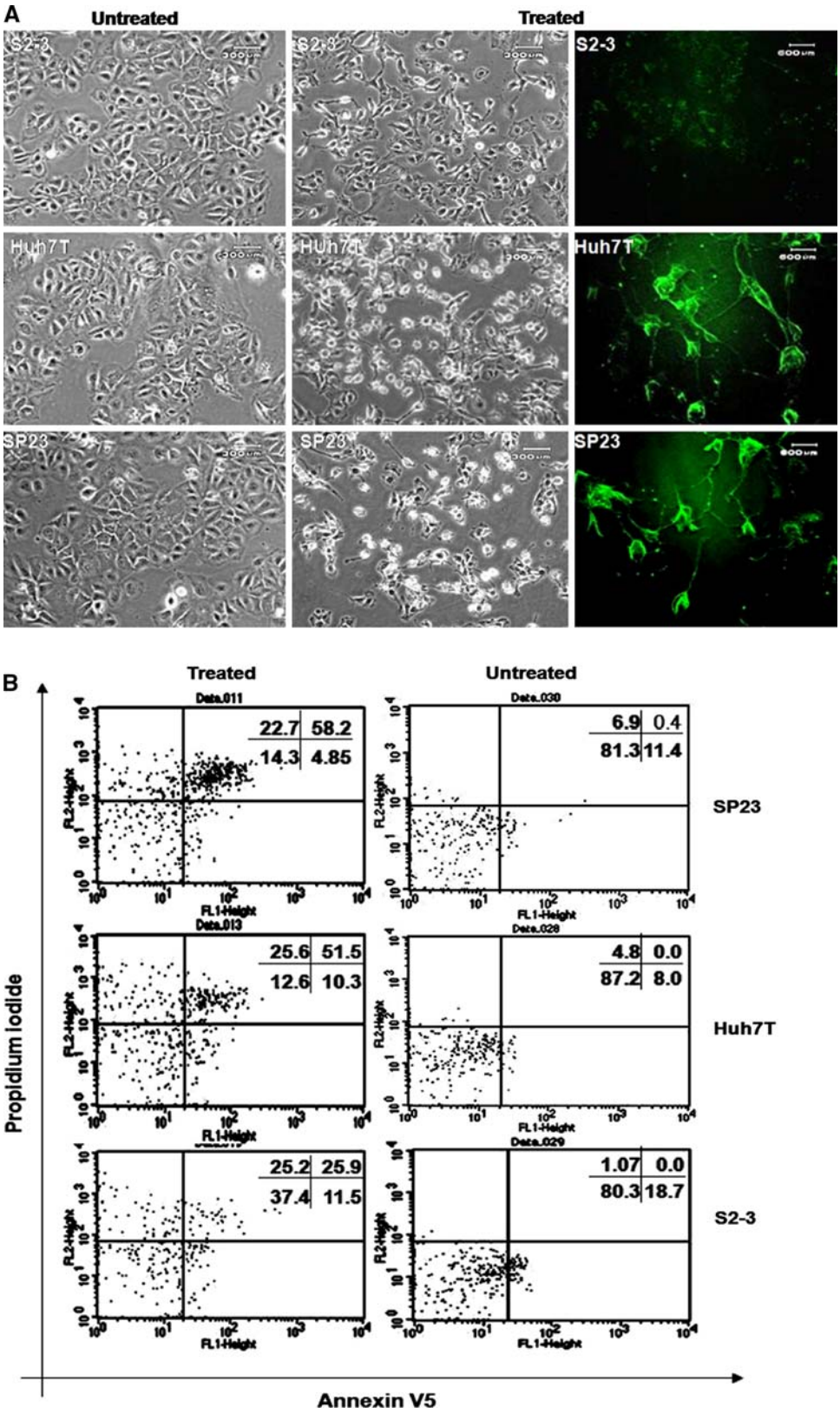

Fig. 3 Stable cell line S2-3 is more resistant to apoptosis. (**a**) S2-3, SP23-5 and Huh7T cells were treated with BFA/CHX/Z-VAD or left untreated. Apoptosis was examined by phase contrast (*left and middle column*) or fluorescence labeled Annexin V staining (*right column*). (**b**) S2-3, SP23-5, and Huh7T cells were treated with BFA/CHX/Z-VAD, and stained with PI and Annexin V-FITC for flow cytometry analysis. The cells in the top-right quadrant represent mid to late stage apoptotic cells

than SP23-5 and Huh7T cells (Fig. 2c), the induction of caspase 3 via etoposide treatment was similar in all three cell lines. In contrast, there was little or no caspase 3 activity in untreated cells or cells treated with the pan-caspase inhibitor Z-VAD (Fig. 5b). Additionally, the activity of caspase 3 did not increase in the cells treated

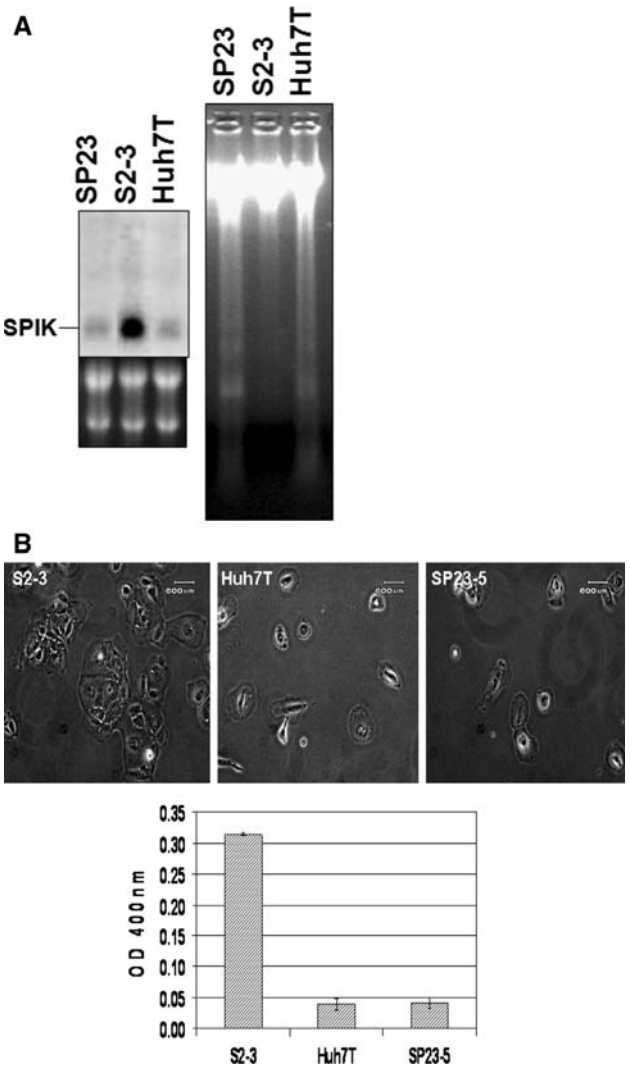

Fig. 4 DNA fragmentation and cell growth show S2-3 is more resistant to SPDCA. (**a**) DNA from S2-3, SP23-5, and Huh7T cells was isolated after treatment with BFA/CHX/Z-VAD. Ten micrograms DNA was resolved on a 1.5% agarose gel and the DNA ladder showing fragmentation was visualized by EB staining (*right panel*). Total RNA from the same cells was analyzed by northern blot (*left panel*). (**b**) Cell growth colonic studies. *Upper panel*: Cell growth colonies were analyzed in S2-3 and control cells. *Bottom panel*: The number of live cells was quantified by WST colorimetric assay (Roche Diagnostics, Indianapolis, IN). Cell viability was shown with high optical density (OD) at 400 nm

with the SPDCA inducer BFA/CHX, even with double the normal concentration (Fig. 5b, B/C/Z X2). These results confirm both that SPIK does not suppress CDCA, and that the apoptosis induced by BFA/CHX/Z-VAD is independent of caspase activity.

SPIK protein is responsible for the suppression of SPDCA

Although SPIK RNA expression and resistance to SPDCA have both been demonstrated, it was not clear if the SPIK polypeptide is a requirement for apoptotic resistance. Mutation studies were therefore performed to investigate the role of SPIK protein expression in suppression of apoptosis. For this study, three plasmids, each containing a HCMV promoter, T3 promoter and the SPIK gene, were constructed (Fig. 6a). The plasmid PWT was used as the wild-type SPIK and was used as the backbone for the mutant plasmids. For plasmid P32, the AUG start codon of SPIK was replaced with GCG, allowing production of SPIK mRNA but not SPIK protein (Fig. 6a). The mutant P21 retained the start codon for SPIK, but by replacing the second cysteine (C2) with a serine (C39S, Fig. 6a), any SPIK created would lose the C2–C4 disulfide bond. Breaking the C2–C4 disulfide bond of SPIK can disrupt the accessibility of the SPIK binding loop, leading to dysfunctional SPIK protein [19].

As shown in Fig. 6b, all three plasmids generated similar amounts of SPIK RNA in a bacterial in vitro transcription system. Neither mutation affected SPIK mRNA synthesis, which was confirmed by northern blot hybridization with a SPIK specific probe (Fig. 6b). The same result was obtained after transfection of these plasmids into Huh7T cells (data not shown). As expected, the AUG mutant P32 produced no recombinant SPIK protein in the in vitro protein synthesis system (Fig. 6c, lane "P32"). Biotin-labeled SPIK, with a molecular weight around 10 kD, can be observed by western blot only in the SPIK wild-type and C39S mutant samples (Fig. 6c, lane "PWT" and "P21"). The synthesis of other proteins, such as the internal system control, endogenous *E. coli* biotin carboxyl carrier protein (BCCP), was equivalent in all three samples, suggesting the translation was consistent (Fig. 6c).

Induction of apoptosis after transfection suggests that only the cells transfected with wild-type SPIK exhibited apoptotic resistance. Flow cytometry analysis showed that 43% of cells transfected with SPIK underwent apoptosis after BFA/CHX/Z-VAD treatment. In contrast, 65% of cells transfected with the AUG mutant and 64% of cells transfected with the C39S mutant underwent apoptosis (Fig. 7a), which was similar to the non-transfected control cells (72%). DNA fragmentation analysis was also done to confirm apoptotic resistance in wild-type SPIK transfected cells. DNA fragmentation was apparent in the cells transfected with the mutant plasmids, as well as the non-transfected control, but was not evident in the cells transfected with SPIK wildtype (Fig. 7b). Altogether, the mutant studies add significant evidence to support the role of SPIK protein in suppressing SPDCA.

Discussion

Our finding that SPIK is a SPDCA inhibitor makes possible further study into the mechanism and biological role of this

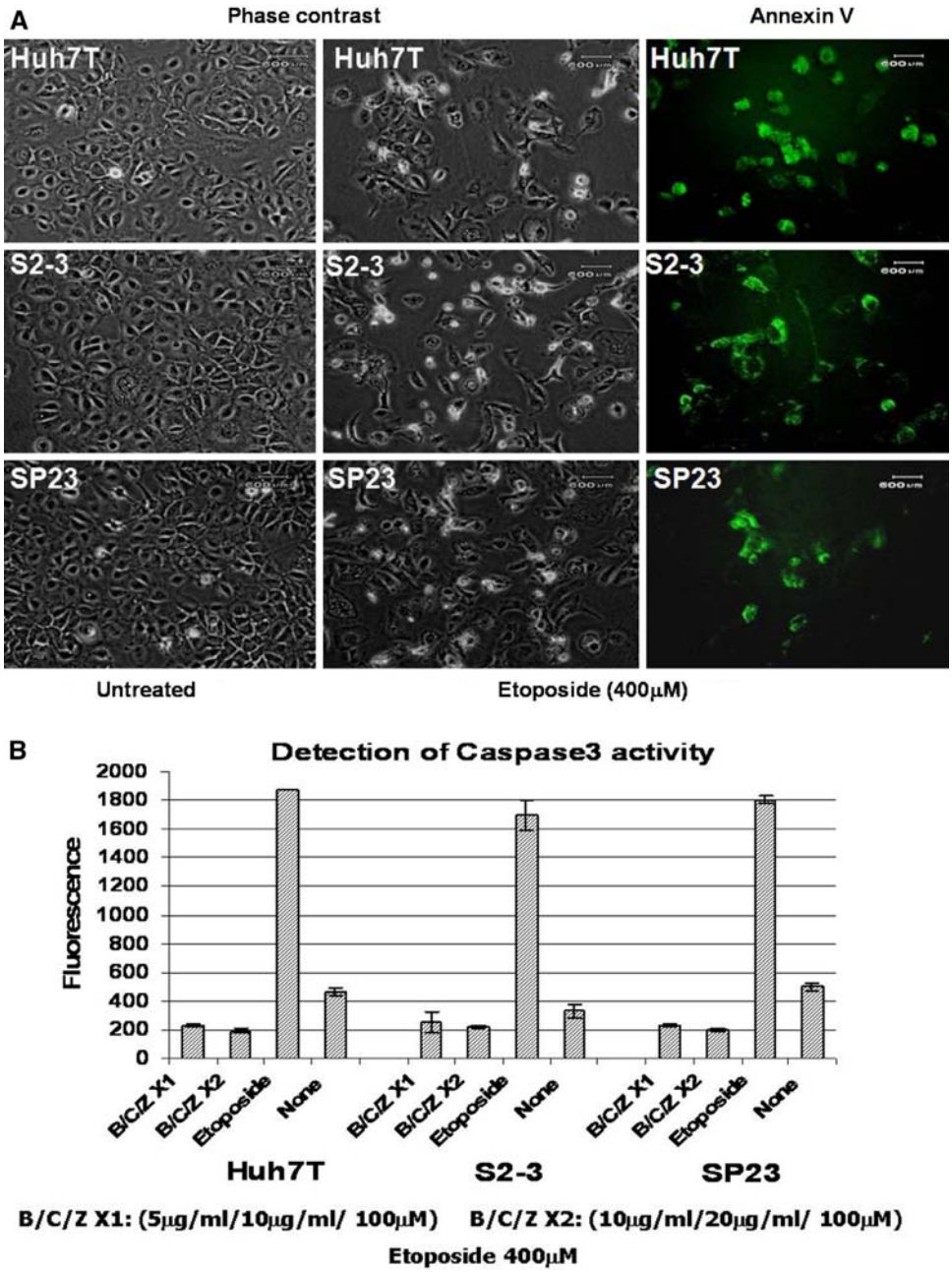

Fig. 5 SPIK has no effect on CDCA. S2-3, SP23-5 and Huh7T cells were treated with CDCA inducer etoposide or SPDCA inducer BFA/CHX with the caspase inhibitor Z-VAD. (**a**) After 48 h etoposide treatment, apoptosis was analyzed by observing changes in cell morphology (*middle column*) and Annexin V-FITC staining (*right column*). (**b**) The activity of caspase 3, a marker to determine CDCA activation, was assessed with a caspase 3 detection kit. CDCA was induced in the cells by treatment with etoposide, and SPDCA was induced in the cells with single or double dose of BFA/CHX along with treatment of Z-VAD. After 48 h the cells were lysed and caspase 3 activity was detected with a fluorescence reader

apoptotic pathway in the initiation and development of cancer. Though important for this reason, the discovery does not directly reveal the serine proteases involved in SPDCA. These unknowns raise difficulties in studying the mechanism of SPDCA and its biological roles. Some serine proteases, such as Omi/HtrA2, granzyme A/B, thrombin and AP24 (apoptotic protease of 24 KD) have shown apoptotic functionality, but it is noted that those serine proteases are either extracellular proteins or have been implicated as functioning along with the caspases [20]. Some of these may or may not be serine proteases involved in SPDCA, but those involved in the mechanism are likely cellular proteases which work in a caspase independent manner. Using co-immunoprecipitation, we are currently investigating possible cellular serine proteases which may interact with SPIK and be involved in SPDCA. We feel this will likely help to reveal the mechanism of SPDCA.

The biological role of SPDCA and SPIK as an apoptosis inhibitor, particularly in the initiation of cancer and other diseases, is also unclear. SPIK has been shown to be elevated in numerous cancers, suggesting that an increase in SPIK expression, and therefore prevention of SPDCA, may be associated with carcinogenesis. Since an IL-6-responsive element in an SPIK encoding gene was found [21], it was believed that up-regulation of SPIK might be related to cell inflammation. This is supported by the finding that

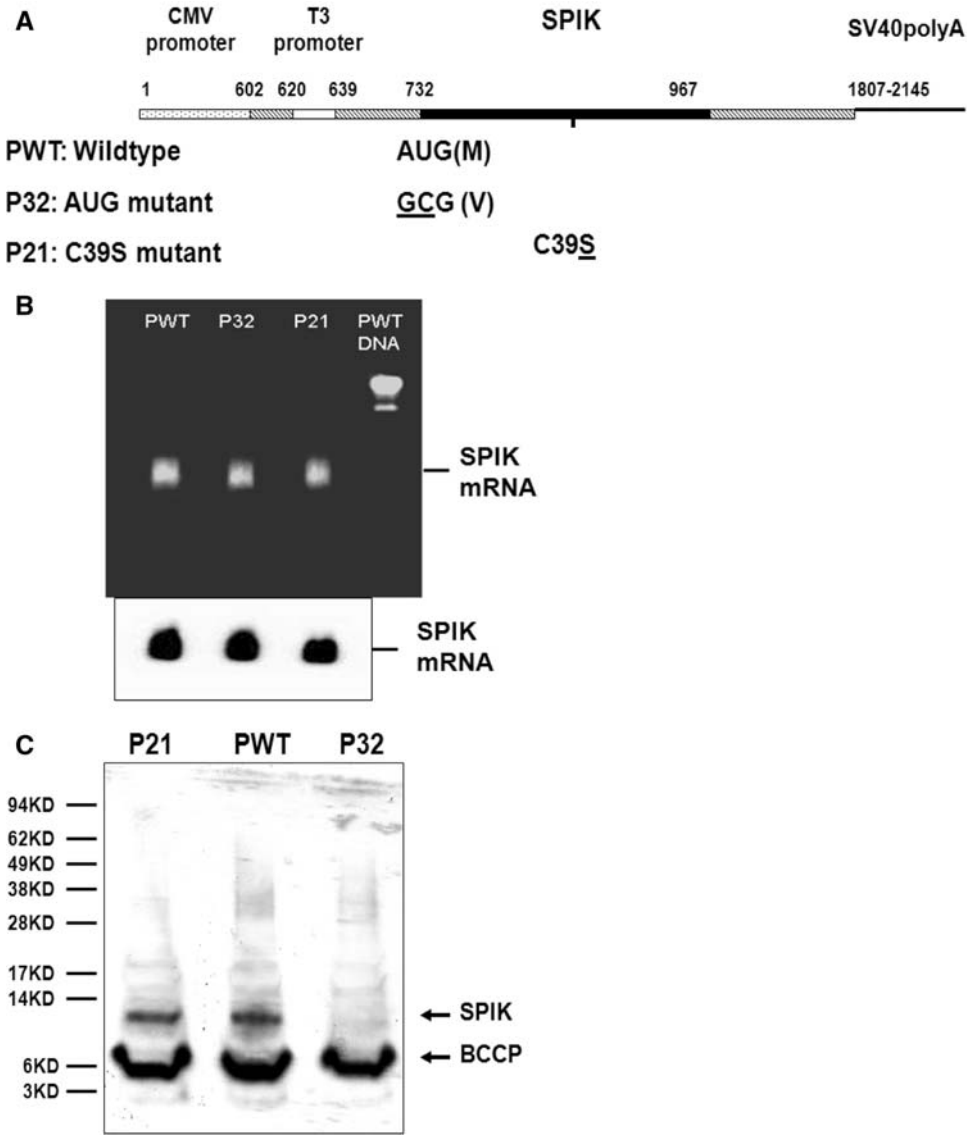

Fig. 6 Expression of SPIK. (**a**) A map of the wildtype and mutant plasmids used to study the SPIK protein. (**b**) SPIK mRNAs were made in vitro from mutants P32, P21 and wild-type PWT, and then resolved on 1% agarose gel. The upper panel shows ethidium bromide staining and the bottom panel shows a northern blot with a SPIK specific probe. (**c**) SPIK protein was expressed from P32, P21, and PWT plasmids using the EasyXpress in vitro translation kit. Protein was labeled with biotin-labeled lysine and visualized by western blot using Streptavidin-HRP. Synthesized SPIK protein is indicated. Endogenous *E. coli* biotin carboxyl carrier protein (BCCP) worked as an internal control for translation

SPIK can be activated as a reactant during hepatitis or liver inflammation. For example, SPIK was activated in rat liver cells to counter turpentine-induced liver inflammation/hepatitis [22]. SPIK was also activated during human chronic hepatitis B and hepatitis C in response to inflammatory cytokines [23, 24]. Up-regulation of SPIK can also be triggered by virus infection [7, 23]. Our unpublished data suggests that hepatitis B virus (HBV) and hepatitis C virus (HCV) replication can up-regulate SPIK expression. The infection of either of these viruses can ultimately cause chronic hepatitis and eventually induce liver cancer [25, 26]. Though viral infection can trigger SPDCA [7, 20], the up-regulation of SPIK due to virus replication and/or persistent inflammation, for example in chronic viral hepatitis, would result in inefficient clearance of virus-infected cells via the SPDCA pathway. This equates to malignant or viral infected cells escaping elimination, eventually leading to chronic infections and the development of cancer. This is consistent with the observation that in chronic viral hepatitis the clearance of viral infected cells is usually inefficient, which eventually leads to the development of HCC [27].

The finding that SPIK is an apoptosis inhibitor will be helpful in understanding the mechanism of SPDCA and its association with cancer development.

Conclusion

Serine protease dependent cell apoptosis is a recently described apoptotic pathway, which differs from the traditional caspase dependent apoptotic pathway. Although SPDCA is currently being investigated by more and more investigators, its mechanism and the roles it might play in

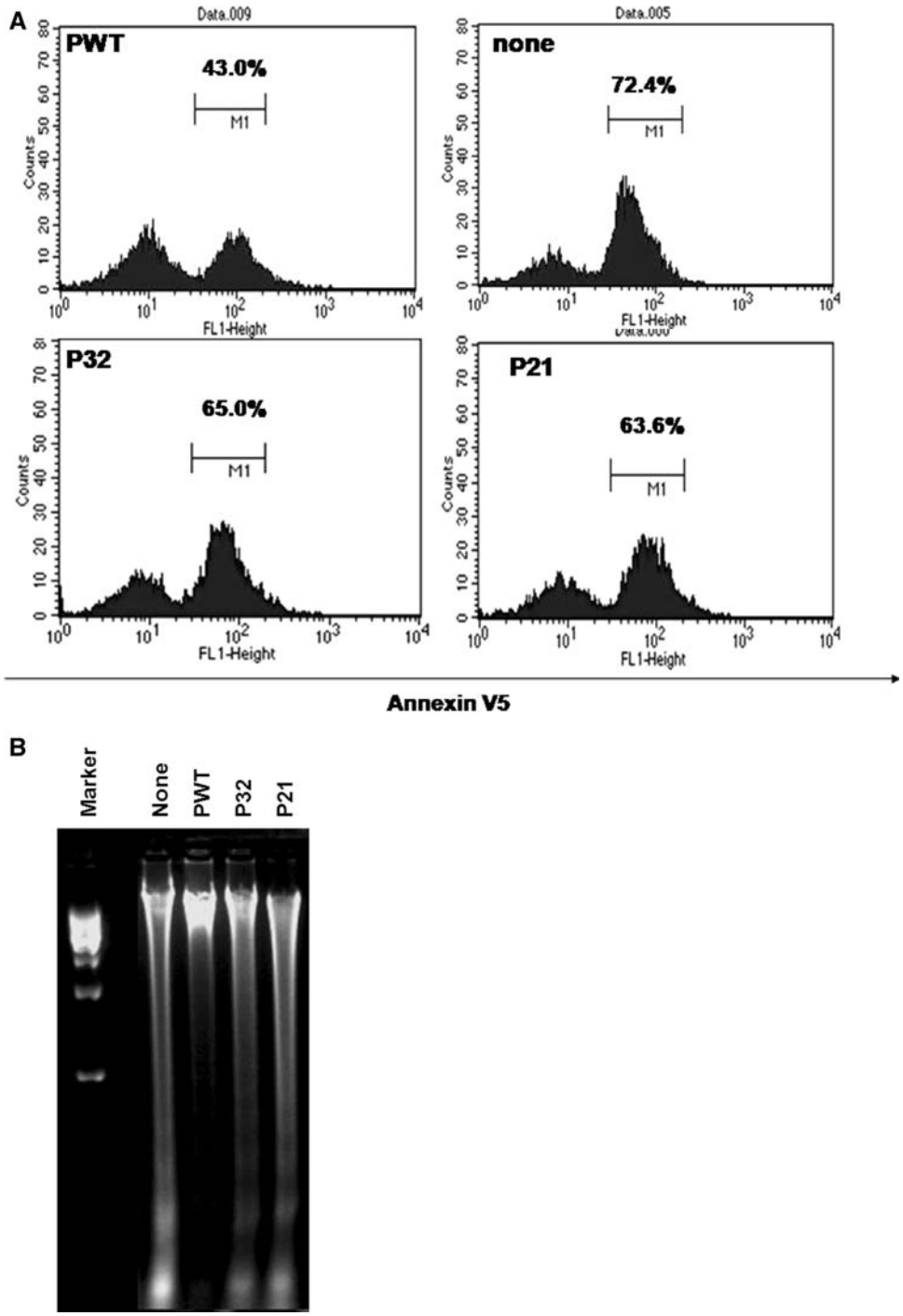

Fig. 7 SPIK protein is associated with suppression of apoptosis. Huh7T cells were transfected with SPIK mutants P32 and P21 or wild-type PWT. After 3 days, apoptosis was induced by BFA/CHX/Z-VAD treatment. (**a**) Apoptosis was quantified by flow cytometry using Annexin V-FITC staining. The percentages of apoptotic cells are indicated by M1 peak. (**b**) Apoptosis in transfected cells was examined by DNA fragmentation as previously described

cellular homeostasis and development of cancer and disease remain unknown. Our finding that SPIK is a SPDCA inhibitor is an important contribution towards the understanding of these unsolved questions. By establishing the inhibitory relationship between this cancer-associated protein and a relatively unknown pathway of apoptosis, we can conclude that the resistance of malignant cells and virus-infected cells to serine protease dependent apoptotic death could be an important factor in cancer development. This finding will be beneficial in further studies of the biological role of SPDCA, especially in cancer development.

Acknowledgments This work is supported by an Appropriation from the Commonwealth of Pennsylvania and National Cancer Institute, NIH. We thank Drs. C. Satishchandra and Tian-lun Zhou (Nucleonics Inc., Horsham, PA) for their helpful advice and for providing the Huh7T cell line. We also thank Drs. Andy Cuconati and Kunwar Shailubhai (Institute for Hepatitis and Virus Research, Doylestown, PA) for their critical reading.

References

1. Igney FH, Krammer PH (2002) Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2:277–288
2. Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. Trends Biochem Sci 22:299–306
3. Nunez G, Benedict MA, Hu Y, Inohara N (1998) Caspases: the proteases of the apoptotic pathway. Oncogene 17:3237–3245
4. Deveraux QL, Reed JC (1999) IAP family proteins–suppressors of apoptosis. Genes Dev 13:239–252
5. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM et al (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. Embo J 17:2215–2223
6. Egger L, Schneider J, Rheme C, Tapernoux M, Hacki J, Borner C (2003) Serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions. Cell Death Differ 10:1188–1203
7. Thorburn J, Bender LM, Morgan MJ, Thorburn A (2003) Caspase- and serine protease-dependent apoptosis by the death domain of FADD in normal epithelial cells. Mol Biol Cell 14: 67–77
8. Abate A, Schroder H (1998) Protease inhibitors protect macrophages from lipopolysaccharide-induced cytotoxicity: possible role for NF-[kappa]B. Life Sci 62:1081–1088
9. Graf R, Bimmler D (2006) Biochemistry and biology of SPINK-PSTI and monitor peptide. Endocr Metab Clin North Am 35: 333–43, ix
10. Stenman UH (2002) Tumor-associated trypsin inhibitor. Clin Chem 48:1206–1209
11. Paju A, Stenman UH (2006) Biochemistry and clinical role of trypsinogens and pancreatic secretory trypsin inhibitor. Crit Rev Clin Lab Sci 43:103–142
12. Greene LJ, Pubols MH, Bartelt DC (1976) Human pancreatic secretory trypsin inhibitor. Methods Enzymol 45:813–25
13. Ohmachi Y, Murata A, Matsuura N, Yasuda T, Uda K, Mori T (1994) Overexpression of pancreatic secretory trypsin inhibitor in pancreatic cancer. Evaluation of its biological function as a growth factor. Int J Pancreatol 15:65–73
14. Tomita N, Doi S, Higashiyama M, Morimoto H, Murotani M, Kawasaki Y et al (1990) Expression of pancreatic secretory trypsin inhibitor gene in human colorectal tumor. Cancer 66:2144–2149
15. Lukkonen A, Lintula S, von Boguslawski K, Carpen O, Ljungberg B, Landberg G et al (1999) Tumor-associated trypsin inhibitor in normal and malignant renal tissue and in serum of renal-cell carcinoma patients. Int J Cancer 83:486–490
16. Tonouchi A, Ohtsuka M, Ito H, Kimura F, Shimizu H, Kato M et al (2006) Relationship between pancreatic secretory trypsin inhibitor and early recurrence of intrahepatic cholangiocarcinoma following surgical resection. Am J Gastroenterol 101:1601–1610
17. Lee YC, Pan HW, Peng SY, Lai PL, Kuo WS, Ou YH et al (2007) Overexpression of tumour-associated trypsin inhibitor (TATI) enhances tumour growth and is associated with portal vein invasion, early recurrence and a stage-independent prognostic factor of hepatocellular carcinoma. Eur J Cancer 43:736–744
18. Anderson KM, Alrefai W, Bonomi P, Seed TM, Dudeja P, Hu Y et al (2003) Caspase-dependent and -independent Panc-1 cell death due to actinomycin D and MK 886 are additive but increase clonogenic survival. Exp Biol Med 228:915–925
19. Tian M, Kamoun S (2005) A two disulfide bridge Kazal domain from Phytophthora exhibits stable inhibitory activity against serine proteases of the subtilisin family. BMC Biochem 6:15
20. Moffitt KL, Martin SL, Walker B (2007) The emerging role of serine proteases in apoptosis. Biochem Soc Trans 35:559–560
21. Yasuda T, Ogawa M, Murata A, Ohmachi Y, Yasuda T, Mori T et al (1993) Identification of the IL-6-responsive element in an acute-phase-responsive human pancreatic secretory trypsin inhibitor-encoding gene. Gene 131:275–280
22. Uda K-I, Murata A, Nishijima J-I, Doi S, Tomita N, Ogawa M et al (1994) Elevation of circulating monitor peptide/pancreatic secretory trypsin inhibitor-I (PSTI-61) after turpentine-induced inflammation in rats: hepatocytes produce it as an acute phase reactant. J Surg Res 57:563–568
23. Ohmachi Y, Murata A, Yasuda T, Kitagawa K, Yamamoto S, Monden M et al (1994) Expression of the pancreatic secretory trypsin inhibitor gene in the liver infected with hepatitis B virus. J Hepatol 21:1012–1016
24. Lu X, Block T (2004) Study of the early steps of the hepatitis B virus life cycle. Int J Med Sci 1:21–33
25. Chisari FV, Ferrari C (1995) Hepatitis B virus immunopathogenesis. Annu Rev Immunol 13:29–60
26. Rocken C, Carl-McGrath S (2001) Pathology and pathogenesis of hepatocellular carcinoma. Dig Dis 19:269–278
27. Guidotti LG, Chisari FV (2006) Immunobiology and pathogenesis of viral hepatitis. Annu Rev Pathol 1:23–61