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Research Article

The effect of isoforms of the cell polarity protein, human ASIP, on the cell cycle and Fas/FasL-mediated apoptosis in human hepatoma cells

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Abstract. Human ASIP (hASIP) is expressed as numerous alternative splicing isoforms and there is an atypical protein kinase C (aPKC) phosphorylation site in exon 17b of the encoded sequence. We have identified an important role for exon 17b in cancer cells. Our results showed that hASIP-sa and sb had different effects on cell growth and Fas/FasL-mediated apoptosis in BEL-7404 human hepatoma cells. Human ASIP-sa modified the S phase of the cell cycle and might stimulate cell proliferation. Growth inhibition by hASIP-a antisense oligonucleotide confirmed the positive action of hASIP-sa. Compared with hASIP-sa, hASIP-sb accelerated Fas/FasL-induced apoptosis, examined by sub-G1 accumulation, chromatin condensation, nuclear fragmentation, PARP cleavage, caspase-8 degradation and mitochondria-regulated cell death. Treatment with aPKC inhibitor could enhance Fas/FasL-mediated apoptosis in hASIP-sa-overexpressing cells, suggesting that hASIP-sa and its interaction with aPKC might contribute to the malignant growth and the blocking of Fas/FasL-mediated apoptosis, while hASIP-sb might function as an antagonist of hASIP-sa.

Key words. hASIP; isoform; cell cycle; FasL; apoptosis; BEL-7404 human hepatoma cell.

Cell polarity is necessary for various cellular functions, development and tissue maintenance. Mammalian ASIP [mASIP, mammalian atypical protein kinase C (PKC)-isotype-specific-interacting-protein], a polarity protein, is a mammalian homologue of Caenorhabditis elegans PAR3 and Drosophila Bazooka. These proteins possess three PDZ domains and one aPKC-binding site and they cooperate with PAR6 and aPKC to play important roles in the cell polarity establishment of various cells [1]. Par3 is involved in establishing anterior-posterior polarity in C. elegans, while Bazooka is involved in the Drosophila germline cyst during oogenesis. In mammalian epithelial cells, mASIP is related to apical-basal polarity, regulates tight-junction formation through interaction with aPKC and is crucial for both cell-cell contacts and extracellular matrix (ECM) contacts [2]. Recent studies suggest that the mASIP/aPKC complex controls cell polarity in mammalian epithelial cells through tight junctions by its association with junctional adhesion molecule (JAM) [3]. Other experiments suggest an intriguing link between the contact-mediated inhibition of cell migration and the regulation of cell polarity controlled by ASIP [4]. The complex of mASIP/mPAR6/aPKC also contributes to axon formation in rat hippocampal neuronal polarity establishment, a process requiring the aPKC and phosphinositide 3-kinase (PI3-kinase) signaling pathway [5].

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Mammalian ASIP, PAR3 and Bazooka share three conserved regions (CRs): CR1, CR2 and CR3. CR2 contains three PDZ motifs implicated in protein-protein interactions [1]. Mammalian ASIP interacts with both the C-terminal PDZ-binding domain of JAM and the PDZ domain of mPAR6 through its first PDZ domain in the establishment of cell polarity [1, 6]. Mammalian ASIP interacts directly with aPKC through the consensus sequence from amino acids 712–936 in CR3 (aPKC-binding region) [7]. Mammalian ASIP regulates epithelial tight-junction formation and is phosphorylated at mASIP-Ser827 [3]. Human ASIP has numerous alternatively spliced variants, which have different aPKC-binding properties and effects on establishment of cell polarity [3, 8, 9]. The isoforms lack part of exon 17 (exon 17b), which encodes most of the CR3 region. Atypical PKC may regulate mammalian cellular polarity through different mASIP isoforms with or without exon 17b.

In the past decades, at least 200 genes that may promote or prevent cancer have been identified in the human genome [10]. We have reported five different isoforms of hASIP, including two long forms, hASIP-la (exon-17b-containing long isoform) and hASIP-lb (exon-17b-deleted long isoform), two short forms, hASIP-sa (exon-17b-containing short isoform) and hASIP-sb (exon-17b-deleted short isoform), and hPAR3 [9]. Our previous study found that the variants containing exon 17b (the supposed aPKC phosphorylation site [3]) were upregulated in human mammary carcinomas [unpublished data], while the expression of exon-17b-deleted variants were downregulated in human hepatocellular carcinoma (HCC) [9]. However, the functions of hASIP isoforms in tumors are still not well understood. Here, we report that hASIP-sa and hASIP-sb play different roles in the cell cycle and Fas/FasL-mediated cell death in human HCC cell line BEL-7404 cells. Human ASIP-sa could modify the cell cycle, while hASIP-sb could not. Overexpression of hASIP-sb in BEL-7404 cells accelerated Fas/FasL-mediated apoptosis independent of aPKC activity, but hASIP-sa-over expressing BEL-7404 cells were insensitive to Fas/FasL-mediated apoptosis which is dependent on aPKC activity, indicating that hASIP-sb may act as an antagonist of hASIP-sa. These results suggest that hASIP could regulate cell growth and cell death through the alternative hASIP splicing isoforms and their interaction with aPKC.

Materials and methods

Antibodies and reagents

Rabbit anti-PARP, anti-caspase-8, mouse anti-cytochrome c, anti-c-myc and anti-bcl-2 IgG were purchased from Santa Cruz Biotechnology. Rabbit anti-caspase-3 IgG and mouse anti-actin IgG were from Cell Signaling Technology and Neomarkers, respectively. PKC inhibitor, GF 109203X, Ro 31-8220 and aPKCζ-specific peptide inhibitor were from Biomol. Rabbit anti-hASIP PDZ1 domain serum was a gift from Prof. Z. Chen. Horseradish-peroxidase-coupled goat anti-mouse and anti-rabbit IgG (Bio-Rad) were used as second antibody. ECL reagent was produced from Amersham.

Expressing vectors and establishment of stable cell lines

The open reading frames of hASIP-sa and sb were inserted into the eukaryotic expressing vector pcDNA3.0. These expressing vectors were then transfected into BEL-7404 cells by DOTAP Liposomal Transfection Reagent (Roche) at a ratio of 1 : 6. After selection for 3–4 weeks in G418 selection medium (600 μg/ml; Geneticin, Sigma), hASIP stable cell lines were maintained in 200 μg/ml G418-containing medium. As a control, pcDNA3.0 was transfected into BEL-7404 cells. The enforced expression of hASIP-sa and sb in the transfected population cells and independent clones was detected by RT-PCR and Western blot analysis. More than 20 clones of hASIP-sa or sb transfectants were isolated and amplified. The transfected population cells and independent clones were used in subsequent experiments.

Cell culture, synchronization and transient transfection by electroporation

Human hepatoma BEL-7404 cells (Shanghai Cell Bank, Chinese Academy of Sciences) were grown at 37 ºC in a 5 % CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % newborn calf serum (Evergreen), 100 units/ml penicillin and 100 μg/ml streptomycin.

Different cell lines at the exponential stage were grown in 3-cm dishes for cell synchronization. Cells were arrested at the G1/S boundary by a double-thymidine (TdR) block. Briefly, BEL-7404 cells were treated for 14 h with medium containing 10 mM thymidine (Sigma), washed three times with PBS and released for 10 h in normal medium, and then treated again with 10 mM thymidine for 12 h. Thereafter, the cells were released in normal medium and harvested after incubation for the predetermined time. The phase of the cell cycle was confirmed by examining DNA contents.

For transient transfection, cells growing to exponential stage were collected, rinsed twice with D-Hanks saline solution and suspended in D-Hanks at 1.0×10^7 cells/ml. Recombinant pcDNA3.0 FasL expressing vectors and pcDNA3.0-GFP (a parallel control of transfection efficiency) were added to a final concentration of 100 μg/ml and incubated on ice for 10 min. Then, cells were electroporated once at 400 V, 25 μF and 100 Ω and incubated on ice for another 10 min. $1.0–1.5 \times 10^6$ cells were seeded in a 10 cm dish. In PKC inhibition experi-

ments, cells were treated with or without GF 109203X (10 μM), Ro 31-8220 (6 μM) or a cell-permeable aPKCζ-specific peptide inhibitor (20 μM). After 24 h, cells were collected for subsequent experiments.

RT-PCR and Western blot analysis

RNA was prepared with Trizol and RT-PCR was conducted according to the manufacturer's instruction (Promega). One microliter reverse transcription product was used for PCR. The primers for the hASIP sequence were: 5'-CTT GAT GAA TCG CCC AGC AGA AAT G-3'(sense) and 5'-CGG CCG TGG ACG ATG GAA AGG AAT A-3'(antisense), which amplified a 567-bp exon-17b-containing product and/or a 477-bp exon-17b-deleted product. The amplification conditions were an initial denaturation at 94 ºC for 2 min, followed by 25 cycles at 94 ºC for 45 s and 68 ºC for 1 min. The primers for the FasL sequence were: 5'-CGA GAG TCT ACC AGC CAG ATG C-3'(sense) and 5'-TCC CAA AGT GCT TCT CTT AGA GC-3'(antisense), which amplified a 520-bp product. The amplification conditions were an initial denaturation at 94 ºC for 5 min, followed by 25 cycles at 94 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 40 s. The PCR products were analyzed using 1.5 % agarose and ethidium bromide staining. For Western blot analysis, the cells were lysed in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 1 % 2-mercaptoethanol, 3 % SDS, 10 % glycerol and 0.1 % bromophenol blue) and heated in boiling water for 5 min. Protein concentration was determined using the Bradford reagent. An equal amount of cell samples were separated on SDS-PAGE, transferred onto nitrocellulose membrane and probed with an appropriate dilution of primary and secondary antibodies, then detected using the enhanced chemiluminescence system.

Antisense oligonucleotide treatment

With a 19-bp sense phosphorothioate ODN (5'-GTA TGT CAG AAA AAC GCA C-3') as control, the 19-bp antisense phosphorothioate ODN (5'-GTG CGT TTT TCT GAC ATA C-3') targeted against exon 17b of hASIP was synthesized and purified by Bioasia. For the cell proliferation assay, BEL-7404 cells were seeded onto 96-well plates at a density of 5×10^3 cells per well. ODNs were transfected into cells with FuGENE 6 Transfection Reagent (Roche) according to the instruction manual. BEL-7404 cells treated with FuGENE 6 alone were used as a control. Briefly, ODNs (1 μg/100μl final concentration) in serum-free medium were added to treat the cells for 4 h. Then, 100 μl H-DMEM growth medium containing 10 % newborn calf serum was replaced. A cell proliferation assay using MTT [11] was performed at 0, 2, 4 and 6 days after transfection. RT-PCR was used to detect the effect of ODNs on the mRNA level of hASIP-a isoforms in the cells that had been treated with 10 μg/ml ODNs or FuGENE 6 in 6-cm tissue culture dishes for 48 h.

Acridine orange/ethidium bromide staining and DNA fragmentation

Acridine orange/ethidium bromide (AO/EB) staining for morphologic examination was conducted as described elsewhere [12] with modifications. The transfected or control cells were seeded in 3-cm dishes. Twenty-four hours later, medium was collected and centrifuged. The suspended cells were resuspended in phosphate-buffered saline (PBS) and combined with adherent cells in dishes. The cells were stained and observed under a fluorescence microscope using wide-band blue excitation (wavelength 450–480 nm). Transfected or control cells were collected, rinsed twice in TBS (25 mM Tris-HCl, 2 mM KCl, 140 mM NaCl, pH 7.4) and lysed in 200 μl lysis buffer (50 mM Tris-HCl, 20 mM EDTA, and 1 % Triton X-100, pH 7.5) for 10 s, then centrifuged at 1600 g for 5 min. The supernatants were collected and incubated with 1 % SDS and RNase (100 μg/ml) at 37 ºC for 2 h and then further incubated with proteinase K (150 μg/ml) at 56 ºC for another 2 h. DNA was precipitated by adding 1/10 vol of 3 M NaAc and 2 vol of ethanol at –20 ºC overnight. DNA degradation was analyzed by 2 % agarose gel electrophoresis (100 V).

Cell fractionation and flow cytometric analysis

BEL-7404 cells (1×10^6) were collected and suspended in 100 μl transport buffer (20 mM Hepes-KOH, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM $MgAc_2$, 1 mM EGTA) containing 200 μg/ml digitonin, and then incubated on ice for 10 min. Cell lysates were centrifuged at 10,000 g, 4 ºC for 15 min. The supernatants were collected as cytosolic fractions and the pellets were mitochondrion-containing fractions. For cell cycle and apoptosis analysis, the cells were digested by trypsin, collected and fixed in sodium citrate at 4 ºC for 30 min. Then, the cells were incubated with 500 μl RNase (10 μg/ml) at 37 ºC for 30 min and stained with 20 μg/ml propidium iodide (PI; Sigma). The samples were analyzed by flow cytometry (FACScan; Becton Dickinson).

Results

Human ASIP-sa might stimulate the growth of BEL-7404 human hepatoma cells

Human ASIP has at least five alternative splicing variants [9]. They possess three typical conserved regions, CR1, CR2 and CR3, contain three PDZ motifs and one aPKC-binding site. Exon 17b of hASIP encodes the consensus sequence of the aPKC-binding domain which contains an aPKC phosphorylation site [1, 3] (fig. 1A). We have found that the exon-17b-containing isoforms are detected in most HCC tissues and surrounding non-tumorous liver tissues; exon-17b-deleted isoforms were present in surrounding non-tumorous liver tissues, but were undetect-

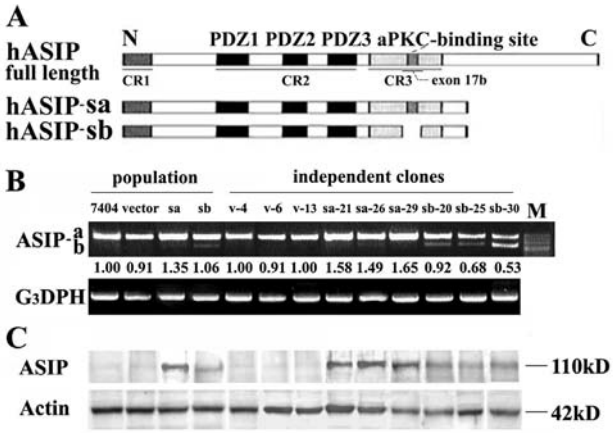

Figure 1. Establishment and detection of hASIP-sa- and sb-overexpressing stable cell lines. (A) Full-length hASIP posseses three typical conserved regions, three PDZ motifs and one aPKC-binding domain. We constructed hASIP-sa- and sb-expressing vectors to elucidate the function of exon 17b. (B) RT-PCR analysis of different stable cell lines overexpressing hASIP, to show the a and b isoforms of hASIP, G_3DPH was used as a control. Population cells and independent clones are shown. Population cells: 7404, BEL-7404 cells; vector, empty-vector-transfected BEL-7404 population cells; sa or sb, hASIP-sa- or sb-expressing-vector transfected cells. Independent clones: v, empty-vector-transfected cell line; sa, hASIP-sa-overexpressing cell line; sb, hASIP-sb-overexpressing cell line; Arabic numerals are the serial numbers of different clones; M, 100-bp DNA ladder. The 567 bp hASIP-a products have been aligned. Results were quantified by densitometry and the value from parent cells was taken as unity. (C) Western blot analysis of highly forced expression of hASIP-sa and sb in BEL-7404 cells with actin as a control.

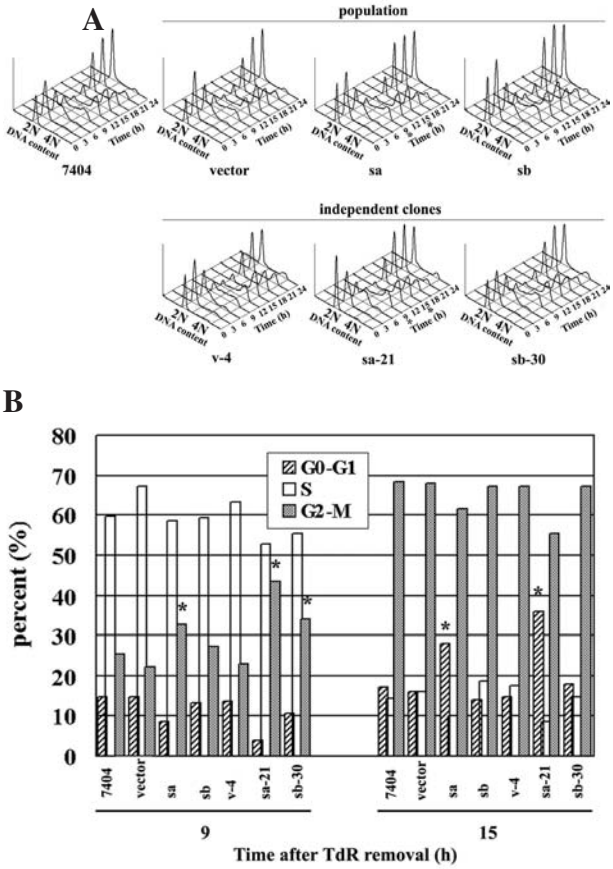

Figure 2. Cell cycle analysis indicated that hASIP-sa modified the S phase in BEL-7404 cells at the exponential stage. (A) Cells were synchronized at the G1/S boundary using thymidine and cell cycle distribution was analyzed at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h after release from the block. Analysis of cell cycle progression by DNA content is indicated in the figure. *, major difference. (B) Percentage of cells in each phase after TdR removal at 9 and 15 h is indicated in the figure. The S phase was shortened in hASIP-sa-overexpressing population cells and independent clone sa-21. These two cell lines entered into G2-M and re-entered the G0-G1 phase earlier than other cell lines. *, major difference. The data are representative results from three independent experiments. Population cells and independent clones are described in figure 1A.

able or downregulated in 52.6 % (10/19) of human HCCs [9]. To observe the function of exon 17b of hASIP (fig. 1A) in the cells, vectors expressing hASIP-sa and sb were constructed and transfected into BEL-7404 cells.

As shown in figure 1B, in population cells, the endogenous 567-bp hASIP-a isoforms were detectable in parent BEL-7404 cells and empty-vector-transfected BEL-7404 cells as in hASIP-sa and sb-overexpressing population cells. A remarkably increased amount of the hASIP-a isoforms was detected in hASIP-sa-transfected population cells. The 477-bp hASIP-b isoforms were produced in addition to the endogenous hASIP-a in the hASIP-sb-transfected population cells (fig. 1B). In the dependent clones, the results were consistent with the population cells. Western blot analysis showed a very weak signal in parent BEL-7404 cells and empty-vector-transfected BEL-7404 population cells and independent clones, but the clear 110-kDa band was detected in hASIP-sa- or sb-transfected population cells and independent clones (fig. 1C).

To analyze the effects of hASIP isoforms on the cell cycle of BEL-7404 human hepatoma cells, synchronized cells were released after double TdR block and collected at predetermined times. The cell cycle progression of different cell lines is shown in figure 2A. Human ASIP-sa-overexpressing population cells, sa, and independent clone, sa-21, first progressed from G1 to S phase and entered into the G2-M phase after release for 9 h. These two cell lines, sa and sa-21, also re-entered G0-G1 phase earlier than other cell lines, at 15 h. The FACS analysis of different cell phases at 9 and 15 h is shown in figure 2B. After release from TdR for 9 h, the proportion of G2-M phase for sa and sa-21 was 32.86 and 43.42 %, respectively, while the proportion of G2-M phase was lower in parent cells, empty-vector- or hASIP-sb-expressing-vector-transfected BEL-7404 population cells and independent clones. Similarly, the percentage of G0-G1 phase in sa and sa-21 was 28.03 % and 35.99 %, respectively, at 15 h; much higher than in control cell lines. The results suggested that hASIP-sa might be able to stimulate the growth of BEL-7404 cells via shortening the S phase. These data implied that hASIP-sa might possess a poten-

tial to cause proliferation and control cell growth through its modification of the cell cycle in BEL-7404 cells.

Antisense ODNs of hASIP-a inhibit the growth of BEL-7404 human hepatoma cells

To confirm the effect of the hASIP-sa isoform on the regulation of cell growth, the antisense ODNs specifically targeted against exon 17b of the hASIP gene were added to the medium of BEL-7404 human hepatoma cells which express hASIP-a isoforms abundantly, but do not express hASIP-b isoforms (fig. 3A). The 48-h treatment with antisense ODNs resulted in remarkable blocking of hASIP-a synthesis in BEL-7404 cells (fig. 3A) compared with the cells untreated or treated with FuGENE-6 and sense ODNs (fig. 3A). In a proliferation assay, 1 μg/100 μl antisense ODNs clearly inhibited the growth of BEL-7404 cells (fig. 3B) compared with parent cells, FuGENE-6-treated cells and sense-ODN-treated cells ($p < 0.01$). Although sense ODNs had some effects on BEL-7404 cells, sense-ODN-treated cells grew much quicker than antisense-ODN-treated cells. These data suggested that the hASIP-a isoforms including hASIP-sa might participate in the growth regulation of human hepatoma cells.

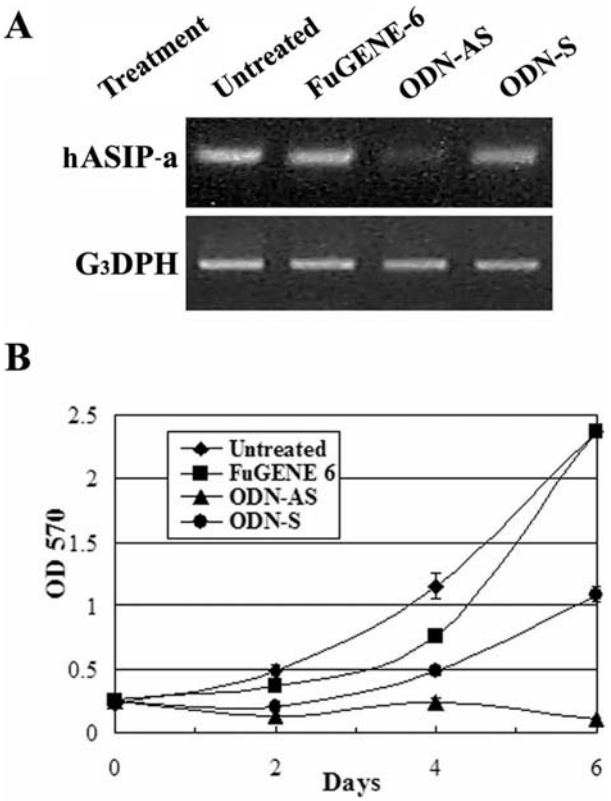

Figure 3. Effects of hASIP-a antisense ODNs on hASIP-a expression and growth of BEL-7404 cells. (A) RT-PCR analysis of hASIP-a in parent cells, cells treated with FuGENE-6, sense ODNs and antisense ODNs, with G_3DPH as a control. (B) Cells were seeded in 96-well plates and treated with antisense ODNs or sense ODNs as described before; cell proliferative ability was measured by the MTT method. Three independent experiments were averaged. The data are expressed as the means ± SE.

The human ASIP-sb isoform enhances Fas/FasL-mediated apoptosis in BEL-7404 cells

As described by Fang and Xu [9], the hASIP-a isoforms were ubiquitously expressed in normal liver and HCC cells. We showed that hASIP-sa might be a regulator of the cell cycle resulting in a stimulation of HCC cell growth. However, the hASIP-b isoforms were frequently downregulated in HCC cells [9] and hASIP-sb showed no stimulating activities on cell growth. Subsequently, we examined the effects of hASIP-sa and hASIP-sb isoforms on cell death. Fas/FasL-mediated cell death was explored in BEL-7404 cells and hASIP-isoform-transfected cells. BEL-7404 cells express endogenous Fas [13], but not FasL (fig. 4A). The FasL-expressing vector was transiently transfected into BEL-7404 cells, population cells, vector, sa, sb and independent clones, v-4, sa-21, sb-30. As shown in figure 4A, FasL was expressed in all cell lines transiently transfected with FasL. Flow cytometric analysis by PI staining showed there was 6.76 ± 2.26% in empty-vector-transfected parent BEL-7404 population cells (fig. 4C). FasL induced an increased apoptosis in parent BEL-7404 cells, population vector, sa and sb (14.43 ± 1.95%, 13.57 ± 1.46%, 20.46 ± 0.82% and 31.45 ± 3.59%, $p < 0.05$). Furthermore, hASIP-sb accelerated Fas/FasL-mediated cell death in comparison with hASIP-sa ($P < 0.05$). The sub-G1 population in FasL-induced apoptosis in parent BEL-7404 cells, independent clone v-4, sa-21 and sb-30 was 16.48 ± 2.14%, 16.18 ± 1.73%, 20.92 ± 3.12% and 33.75 ± 3.33%, respectively (fig. 4C). All the data showed there was an obvious increase in apoptosis in hASIP-sb-overexpressing cells, population sb and independent clone sb-30. A significant stimulating effect of the hASIP-sb isoform on Fas/FasL-mediated cell death in BEL-7404 cells, showing more chromatin condensation and nuclear DNA fragmentation than other cell lines, is revealed in figure 4B and 4D. The results confirmed that hASIP-sb significantly accelerated the Fas/FasL-induced apoptosis of BEL-7404 cells.

The aggregation of Fas, Fas-associating protein with a death domain (FADD) and pro-caspase-8 has been called the death-inducing signaling complex (DISC) and results in the proteolytic autoactivation of caspase-8 and the formation of the active enzyme complex, which triggers a downstream apoptotic cascade [14]. We also detected the degradation or proteolytic autoactivation of caspase-8 and PARP cleavage in Fas/FasL-mediated apoptosis in BEL-7404 cells, empty-vector- and hASIP-sa- or sb-expressing-vector-transfected population cells and independent clones (fig. 4E). The degradation of caspase-8 and PARP cleavage was greater in FasL transiently transfected hASIP-sb-overexpressing cells than in FasL transiently transfected hASIP-sa-overexpressing

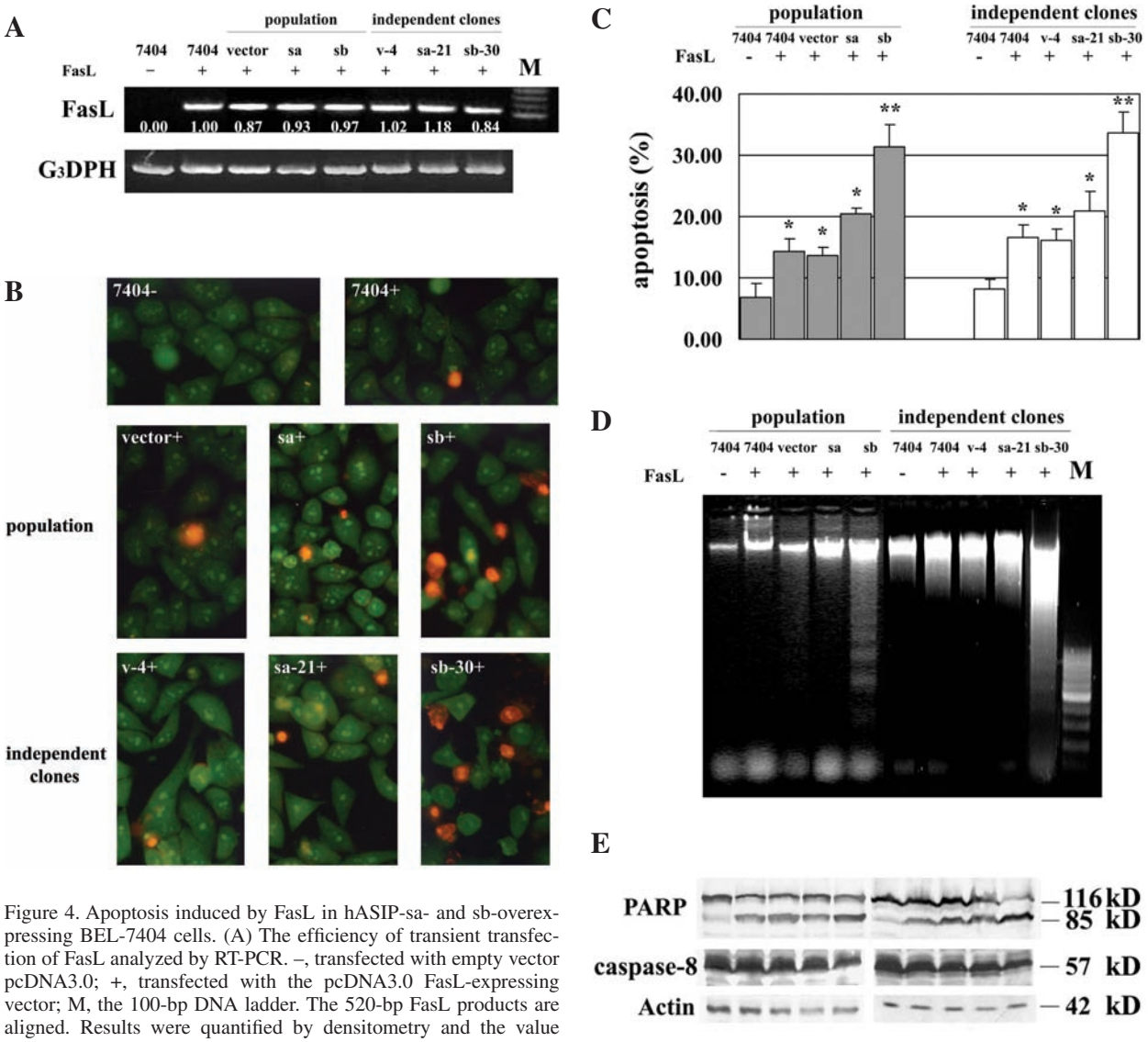

Figure 4. Apoptosis induced by FasL in hASIP-sa- and sb-overexpressing BEL-7404 cells. (A) The efficiency of transient transfection of FasL analyzed by RT-PCR. –, transfected with empty vector pcDNA3.0; +, transfected with the pcDNA3.0 FasL-expressing vector; M, the 100-bp DNA ladder. The 520-bp FasL products are aligned. Results were quantified by densitometry and the value from the second lane was taken as unity. (B) AO/EB staining for morphologic examination of FasL induced apoptosis in different cell lines. The red staining shows the condensed and fragmented nuclei. – and + as in A. Population cells and independent clones as in A. (C) Sub-G1 analysis by FACS with PI staining of FasL-induced apoptosis. Three independent experiments were averaged. The data are expressed as the means ± SE. *$p < 0.05$ in comparison with the parent cells; **$p < 0.05$ in comparison with pcDNA3.0 FasL transiently transfected BEL-7404 cells. (D) Effects of hASIP-sa and sb isoforms on FasL-induced DNA fragmentation in BEL-7404 cells. After transfection for 24 h, the cells were lysed and analyzed. M, the 100-bp DNA ladder. (E) PARP cleavage and degradation of caspase-8 were determined by Western blot analysis. Actin was used as a control. All experiments were repeated three times.

cells, vector-transfected cells and parent cells. All these data showed that hASIP-sa and sb might be involved in Fas/FasL-induced apoptosis in HCC. Human ASIP-sb might sensitize BEL-7404 cells to Fas/FasL-mediated apoptosis.

The mitochondria pathway may regulate Fas/FasL-mediated apoptosis in hASIP-sa- and sb-overexpressing BEL-7404 cells

We detected cytochrome c release, caspase-3 degradation and bcl-2 expression in FasL transiently transfected hASIP-sa- or hASIP-sb-overexpressing BEL-7404 cells by Western blot analysis. As shown in figure 5, after FasL transient transfection for 24 h, caspase-3 was only degraded in hASIP-sb-overexpressing BEL-7404 cells (population sb), but there was no significant change in other cells. Similar results were observed in independent clones as well. Simultaneously, cytochrome c release was significantly stimulated in hASIP-sb-overexpressing cells (population sb and independent clone sb-30) compared with parent cells or vector-transfected cells. The expression of Bcl-2 was doubled in hASIP-sa-overexpressing cells. Bcl-2 family members regulate apoptosis by controlling mitochondrial permeability

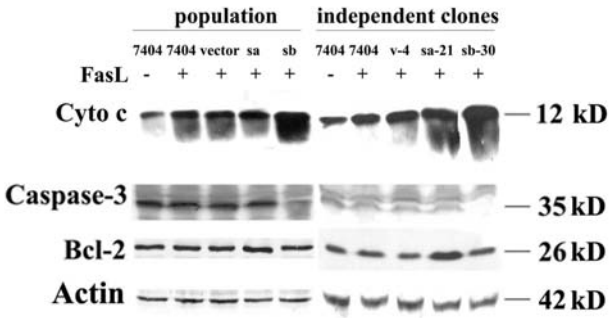

Figure 5. Mitochondrial-regulated Fas/FasL-mediated apoptosis in hASIP-sa- and sb-overexpressing BEL-7404 cells. Cytochrome c release, caspase-3 degradation and bcl-2 expression were detected by Western blot. All results showed that hASIP-sb accelerated the mitochondrial-regulated Fas/FasL-mediated apoptosis. Experiments were repeated at least three times.

and the release of cytochrome c [15, 16]. It is Bcl-2 that might inhibit the degradation of caspase-3 and decrease Fas/FasL-induced apoptosis in hASIP-sa-overexpressing cells. So we concluded that Fas/FasL-mediated apoptosis in hASIP-sa- and sb-overexpressing cells was regulated by the mitochondrial pathway and sensitive to Bcl-2.

Atypical PKC contributes to Fas/FasL-mediated apoptosis in hASIP-sa- and hASIP-sb-overexpressing BEL-7404 cells

The different effects of hASIP-sa and hASIP-sb on cell growth and cell death in BEL-7404 cells suggested the critical role of exon 17b in the hASIP gene. Mammalian ASIP interacts with aPKC through exon 17b that encodes the aPKC-binding site and mASIP-Ser827 phosphorylation site [3]. In addition, aPKC may catalyze mASIP as a substrate [17]. So we suspected that aPKC might play an important role in the participation of hASIP in cell death.

We examined the effect of aPKC activity on Fas/FasL-mediated apoptosis in hASIP-sa- and hASIP-sb-overexpressing BEL-7404 cells. The treatment of cells with Ro 31-8220, a broad-spectrum non specific PKC inhibitor (including aPKC and other PKCs), significantly increased Fas/FasL-induced cell death from 20.46 ± 0.82 % to 28.50 ± 3.58 % in the hASIP-sa-overexpressing BEL-7404 cell population sa and from 21.71 ± 3.59 % to 30.98 ± 1.11 % in independent clone sa-21 ($p < 0.05$) (fig. 6B). A slightly stimulating effect was also observed in parent BEL-7404 cells that contain the endogenous hASIP-sa isoform. The remarkable DNA fragmentation in Ro 31-8220-treated hASIP-sa-overexpressing cells (fig. 6A) also indicated the positive role of PKC in Fas/FasL-induced apoptosis. Ro 31-8220 had no effect on Fas/FasL-induced apoptosis in hASIP-sb-overexpressing cells. The aPKCζ specific inhibitor, a myristoylated peptide, was used to confirm the significance of aPKCζ and its interaction with exon 17b of the hASIP gene on Fas/FasL-induced apoptosis in hASIP-sa- and sb-overexpressing BEL-7404 cells. As shown in figure 6C and 6D, a definite effect of the aPKCζ specific inhibitor on hASIP-sa-overexpressing BEL-7404 cells was observed. Cell apoptosis was unchanged in GF 109203X- (an inhibitor of cPKC and nPKC) treated cells (data not shown). The results indicated that the interaction of aPKC with hASIP-sa substrate might correlate to the regulation of Fas/FasL-induced cell death. Further detection of PARP cleavage in Fas/FasL-induced apoptosis in the presence of the myristoylated peptide inhibitor in hASIP-sa- or sb-overexpressing cells also proved that its specific effect on Fas/FasL-induced apoptosis in hASIP-sa-overexpressing cells was very significant, but not in other cell lines (fig. 6E). All these results proved that the inhibition of aPKCζ activity might accelerate Fas/FasL-induced apoptosis in hASIP-sa-overexpressing cells, but not in hASIP-sb-overexpressing cells. So we consider that the aPKC activity and its interaction with the aPKC-binding site of hASIP might be important for Fas/FasL-mediated apoptosis in human hepatoma cells.

Discussion

An increasing number of studies are proving that alternative splicing is one of the most significant components of the functional complexity of the human genome [18–21]. Recent genome-wide analyses of alternative splicing indicate that 40–60 % of human genes have alternative forms [21]. Even if the difference is just several base pairs, the function of different isoforms of the same gene may be totally different, as is the case for BACH1 and BACH1t [18], CBFA1/Cbfa1 and Cbfa1/Osf2 [19] and Spi-B and delta Spi-B [20]. Correlative studies have found that various isoforms of the polarity protein, Par6 and Par3, have different functions [8, 22]. All of the Par3L/Par3 isoforms can associate with tight junctions in epithelial cells, but they show different binding properties with aPKCs [8]. Par6 family members localize differently when expressed in Madin-Darby canine kidney epithelial cells and have distinct effects on tight-junction assembly [22]. Our study found that the functions of hASIP-sa and sb isoforms in cell growth and cell death were entirely different. The results proved the important role of epigenetic regulation of hASIP gene expression and splicing in the control of cell life.

Par3 is required for the establishment of asymmetry in early C. elegans embryos [23, 24], maintenance of oocyte fate in Drosophila [25] and establishing epithelial cell polarity from C. elegans to mammals [26–28]. In recent years, studies have focused on the Par3/Par6/aPKC complex, their related molecules, such as Cdc42 [17, 29], JAM [6, 30, 31], ZO-1 [30, 32], E-cadherin and claudin-1 [32], and their regulation of cell polarity. Surprisingly,

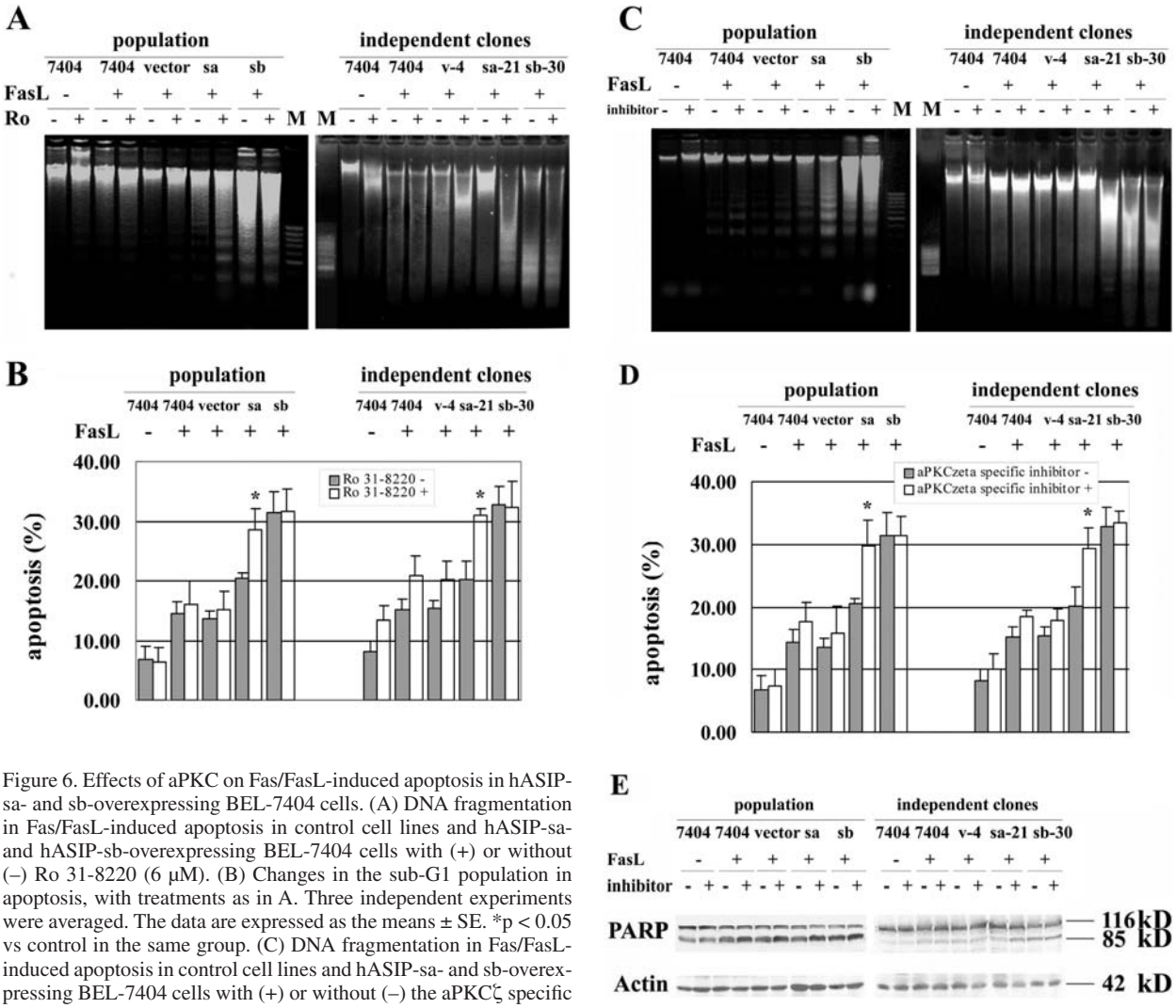

Figure 6. Effects of aPKC on Fas/FasL-induced apoptosis in hASIP-sa- and sb-overexpressing BEL-7404 cells. (A) DNA fragmentation in Fas/FasL-induced apoptosis in control cell lines and hASIP-sa- and hASIP-sb-overexpressing BEL-7404 cells with (+) or without (–) Ro 31-8220 (6 μM). (B) Changes in the sub-G1 population in apoptosis, with treatments as in A. Three independent experiments were averaged. The data are expressed as the means ± SE. *$p < 0.05$ vs control in the same group. (C) DNA fragmentation in Fas/FasL-induced apoptosis in control cell lines and hASIP-sa- and sb-overexpressing BEL-7404 cells with (+) or without (–) the aPKCζ specific peptide inhibitor (20 μM). (D) Changes in the sub-G1 population in apoptosis, with treatments as in C. Three independent experiments were averaged. The data are expressed as the means ± SE. *$p < 0.05$ vs control in the same group. (E) PARP cleavage in apoptosis; cells were treated as in C. All experiments were repeated at least three times.

our laboratory found that hASIP-b isoforms were down-regulated in HCC [9] and hASIP-a isoforms were upregulated in human mammary carcinomas (data not shown). So we concluded that hASIP-a and b might play opposite roles in cell growth and cell death, and the balance of the isoforms of hASIP might be involved in the progress of tumorigenesis. To gain insight into the function of hASIP isoforms in mammalian cells, we explored the effects of hASIP-sa and hASIP-sb in BEL-7404 cells. The different effects of hASIP short forms related to cell growth and Fas/FasL-mediated apoptosis in tumor cells confirmed our scenario. Researchers have also found that PAR3 overexpression suppressed contact-mediated inhibition of cell migration in MDCK cells [4]. The roles of hASIP isoforms in tumors and the relationship between cell polarity and tumorigenesis are definitely worthy of study.

However, what is the mechanism by which hASIP isoforms regulate the cell cycle and Fas/FasL-induced apoptosis in human hepatoma cells? The PKC family members play important roles in cell proliferation and apoptosis by transducing a variety of extracellular stimuli to the cellular signaling networks through lipid-derived second messengers [33]. PKCs can be subdivided into three classes: cPKC, nPKC and aPKC [34]. Unlike cPKC and nPKC isotypes, aPKC does not seem to be regulated directly by Ca^{2+}, phorbol esters or diacylglycerols. Although aPKC is crucial to cell growth and survival, the activation mechanism of aPKC is still unclear [35]. The difference between hASIP-sa and hASIP-sb isoforms is the presence or absence of exon 17b which contains an aPKC phosphorylation site at hASIP-Ser827 [3]. Researchers deemed that aPKC might regulate cell polarity and cell proliferation through interaction with PAR3 [17], p62 [36], lethal giant larvae (Lgl) [37–39], von Hippel-Lindau (VHL) tumor suppressor protein [40] and through the NF-κB signaling pathway [36] or PI-3K

pathway [41]. Atypical PKC is located at the center of the regulation of epithelial cell polarity by interacting with its substrate such as hASIP [17] and mLg1 [38]. Interestingly, we found the hASIP isoforms were closely related to the Fas/FasL-induced cell death via the interaction with aPKC. Our data suggested that the expression of the hASIP-sb isoform in BEL-7404 cells strongly antagonizes the growth-stimulating effect of hASIP-sa. It is the efficiency of aPKC interaction with hASIP isoforms that may decide cell fate. In other words, cell growth or cell death might be partially controlled or regulated by the binding activity of hASIP isoforms with aPKC. At the same time, the activation of PKC triggers cellular signals that inhibit Fas/FasL-mediated cell death [42]. Our study indicated that hASIP-sb, with the aPKC-binding site partly deleted, as an antagonist of hASIP-sa might reduce the activities of aPKC via competition with hASIP-sa to interact with aPKC molecules. The contribution of aPKC and its interaction with hASIP-sa in the protection from Fas/FasL-induced apoptosis in cancer cells was further proved by aPKC-specific inhibitor treatment. In this paper, we present evidence consistent with a hypothesis that an hASIP and aPKC complex might be involved in the control of cell growth and cell death in cancer.

Fas/FasL-mediated apoptosis is a kind of apoptosis mediated through the activation of the family of death receptors (Fas). Fas is expressed on several different cell types (mainly in the thymus, activated T and B lymphocytes, macrophages, liver, spleen, lung, testis and brain), and its expression can be augmented by cytokines, such as interferon-γ and tumor necrosis factor, and also by lymphocyte activation. In contrast, expression of FasL is more tightly regulated, often only inducible under specific conditions. FasL expression is restricted to immune cells, including T and B lymphocytes, macrophages, and natural killer cells, and to non-immune sites, such as the testis, kidney, lung, intestine and the eye [43]. Our experiment proved that hASIP-sb accelerated Fas/FasL-induced apopsis. In other words, hASIP-sb-overexpressing BEL-7404 cells are more sensitive to Fas/FasL-induced apoptosis than hASIP-sa-overexpressing BEL-7404 cells. As normal liver cells express hASIP-a and hASIP-b *in vivo*, and hASIP-b isoforms are antagonists of hASIP-a isoforms, we conclude that hASIP-b isoforms maintain natural growth and prevent malignant growth of normal liver cells. HCC cells, which only express hASIP-a, have proliferating potential and are insensitive to Fas/FasL-induced apoptosis, so they grow much quicker than normal liver cells. It is the balance of hASIP-a and b isoforms that participates in regulating the growth and apoptosis of human nomal liver cells and HCC cells. This may elucidate why human carcinoma cells in which hASIP-b isoforms are always downregulated can escape from Fas/FasL-induced apoptosis, survive and proliferate. Based on the sensitivity to bcl-2 protection, Fas-sensitive cells have been divided into two types. Type I cells utilize a mitochondria-independent pathway, bypassing a role for Bcl-2, and cannot prevent Fas-initiated apoptosis, while type II cell death involves the mitochondria, and is susceptible to Bcl-2 regulation [44]. Further research [42, 45] found that PKC activation blocked FADD recruitment and caspase-8 activation in both type I and II cells, and aPKCζ also plays an important role. We found that hASIP participation in Fas/FasL-induced apoptosis might depend on the efficiency of the interaction between hASIP isoforms and aPKC. This process might work through the degradation and activation of caspase-8. This is a typical mitochondria-regulated apoptosis, and belongs to type II cell death. However, what is the regulation mechanism of the interaction efficiency of aPKC and hASIP isoforms? How does the aPKC/hASIP complex act on Fas/FasL-mediated apoptosis in cancer cells? Several interesting questions remain to be elucidated.

In conclusion, our data suggested an intriguing link between the hASIP alternative splicing variants and the malignant growth potential or the regulation of cell death. The expression of exon-17b-containing hASIP-sa molecules and the interaction with aPKC contributed to the malignant growth and the blocking of Fas/FasL-mediated cell death in cancer cells. The exon-17b-deleted hASIP-sb molecules might function as an antagonist of exon-17b-containing hASIP-sa to regulate the growth of normal and cancer cells. The balance of hASIP molecules and their interaction with aPKC may regulate cell growth and cell death simultaneously; a loss in balance of the expression and function of hASIP isoforms might contribute to the development and progress of cancers.

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