Blackwell Publishing

Asian J Androl 2005; 7 (2):147–158
DOI: 10.1111/j.1745-7262.2005.00037.x

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·Original Article·

Saposin C stimulates growth and invasion, activates p42/44 and SAPK/JNK signaling pathways of MAPK and upregulates uPA/uPAR expression in prostate cancer and stromal cells

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Abstract

Aim: To determine the effect of saposin C (a known trophic domain of prosaposin) on proliferation, migration and invasion, as well as its effect on the expression of urokinase plasmonogen activator (uPA), its receptor (uPAR) and matrix metalloproteinases (MMP)-2 and -9 in normal and malignant prostate cells. In addition, we tested whether saposin C can activate p42/44 and stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) signal transduction pathways of the mitogen-activated protein kinase (MAPK) superfamily. **Methods:** We employed Western blot analysis, phospho-specific antibodies, cell proliferation assay, reverse transcriptase-polymerase chain reaction, *in vitro* kinase assays and migration and invasion to determine the effect of saposin C on various biological behaviors of prostate stromal and cancer cells. **Results:** Saposin C, in a cell type-specific manner, upregulates uPA/uPAR and immediate early gene c-Jun expression, stimulates cell proliferation, migration and invasion and activates p42/44 and SAPK/JNK MAPK pathways in prostate stromal and cancer cells. Normal prostate epithelial cells were not responsive to saposin C treatment in the above studies. **Conclusion:** Saposin C functions as a multipotential modulator of diverse biological activities in prostate cancer and stromal cells. These results strongly suggest that saposin C functions as a potent growth factor for prostatic cells and may contribute to prostate carcinogenesis and/or the development of hormone-refractory prostate cancer. *(Asian J Androl 2005 Jun; 7: 147–158)*

Keywords: saposin C; prostate cancer; uPA/uPAR; prosaposin; invasion; growth factor; SAPK/JNK; MAPK; MMP; c-Jun

1 Introduction

In addition to the fundamental role of androgens, polypeptide growth factors, neuropeptides and other trophic agents are also involved in normal and neoplastic growth of the prostate. Therefore, it is not surprising that in prostate cancer the expression of many of these

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Received 2004-09-13 Accepted 2005-01-31

non-androgenic effectors and their receptors is changed. Identification and characterization of pivotal growth factors that could affect diverse biological activities of prostate cancer cells will lead to a better understanding of the disease and potentially to the development of effective therapeutic approaches.

Prosaposin is a highly conserved glycoprotein (65–72 kDa, 527 amino acids) and the precursor of heat-stable small glycoproteins known as saposins A, B, C and D [1]. These mature saposins, through their interaction with glycosphinolipid hydrolases and their substrates, increase the lysosomal hydrolytic functions. In addition to its intracellular presence and function, prosaposin also exists as a secreted protein. Prosaposin and saposins are expressed by neuroglial-derived cells and tissues, various cell types and in body fluids (such as cerebrospinal fluid and milk) or present in seminiferous tubular fluid, in seminal plasma and in prostatic secretions [2].

As a secretory protein, prosaposin and its active mature domain (saposin C) are well-known neurotrophic molecules with involvement in neuro-embryological differentiation and development, ganglioside and sulfolipid synthesis, nerve regeneration and anti-apoptotic activity in neuro-glial cells [2, 3]. Based on several reports, the amino-terminal portion of saposin C contains a neurotrophic sequence that has been used as a source to generate a number of biologically active synthetic peptides (5–22 residues) called prosaptides (e.g., D5, TX14A) [2–4].

In spite of several important clues that signal the potential important cell-biological roles for prosaposin and saposin C, their function in cancer research in general and in prostate cancer specifically has not been addressed.

Using immunohistochemical analysis, we have recently reported the cell type-specific expression of prosaposin in human prostate cancer tissues and cells. In addition, we demonstrated that prosaptide TX14A prevents cell death induced by an apoptogenic molecule (sodium selenite), stimulates growth, migration and invasion and activates the p42/44 mitogen-activated protein kinase (MAPK) signaling pathway in prostate cancer cells [5].

Our previous report described only the effect of a synthetic peptide (TX14A) derived from saposin C and does not necessarily reflect or represent the potential biofunctional activities of saposin C itself [5]. Therefore, in the present report, we decided to test the effect of saposin C on normal prostate epithelial and stromal cells as well as on malignant prostate cells growth, migration and invasion. In addition, we also tested whether saposin C could affect the expression of matrix-degrading proteolytic enzymes (urokinase plasmonogen activator [uPA]/uPAR and matrix metalloproteinases [MMP]), p42/44 MAPK, and stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) signal transduction pathways in the above cells.

2 Materials and methods

2.1 Cell lines

Primary cultures of normal human prostate epithelial (PrEp) and stromal (PrSt) cells were purchased from Biowhittaker (Walkersville, MD, USA) and maintained in prostate epithelial growth medium (PrEGM) and stromal cell growth medium (SCGM), respectively. Androgen-independent (PC-3, DU-145) and -dependent (LNCaP) prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in defined media (PC-3 and DU-145 in DMEM-10 % FBS and LNCaP in RPMI-1640-10 % FBS supplemented with 1 mmol/L Na. Pyruvate, 10 mmol/L HEPES). Pheochromocytoma cell line, PC12, was purchased from ATCC and cultured as recommended by the supplier.

2.2 Cell proliferation assays

Cells were seeded in 96-well tissue culture plates in their respective complete culture media for 3 days. After changing the medium to serum-free, cells were treated with purified recombinant human saposin C (characterized before) [6]. After 2 days of incubation, the cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (based on a novel tetrazolium compound) using CellTiter 96 AQueous One Solution Cell Proliferation/Cytotoxicity Assay kit (Promega, Madison, WI). For each cell line, the total cell number was determined using a standard curve based on absorption (OD) versus cell number. We used twelve replicates for each treatment concentration. The assay was repeated at least three times.

2.3 *In vitro migration and invasion assays*

Cell migration and invasion assays were performed essentially as described previously with minor modifications [5]. The lower compartment of each transwell

unit contained 400 μL of 0.5 % Fetal Bovine Serum (FBS) supplemented medium (DMEM [PC-3 and DU-145], RPMI [LNCaP], SCBM [PrSt], or PrEBM [PrEp]) in the presence or absence of saposin C at 0.1, 1.0 or 10.0 nmol/L. After trypsinization, 10^4 cells in 100 μL of their respective serum-free media were placed in the upper compartment of the transwell unit. For cell invasion assays, transwell filters were coated with 20 μg of growth factor reduced Matrigel (BD Biosciences, Bedford, MA, USA). The rest of the protocol was followed exactly as described before [5]. Phase-contrast microscopy (× 200) equipped with an ocular grid (which divides each field equally) was used to determine migrated cells by counting the total number of the cells for each filter. Each sample was assayed in quadruplicate, and assays were repeated at least twice.

2.4 Western analysis and immunoprecipitation

Protein expression analysis was performed according to standard procedures [5]. Briefly, whole cell lysates were prepared by washing cell monolayer with cold-Phosphate Buffered Saline(PBS), lysing the cells on ice for 15 min with lysis buffer (20 mmol/L PIPES [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EGTA, 1 % Triton X-100, 1.5 mmol/L $MgCl_2$) supplemented with protease inhibitor cocktail (Roche Diagnostic, IN, USA) and 1 mmol/L sodium orthovandate, plus sodium dodecyl sulfate (SDS) at a final concentration of 0.1 %. The lysates were then centrifuged (15 min, 4 °C, 16 000 × g), and after collecting the supernatants, the protein concentration was determined by Bicinchoninic Acid (BCA) assay. Culture supernatants were concentrated 5- to 10-fold using a Centriprep-3 concentrator (with 3.0 kDa Molecular weight cut-off; Millipore, Billerica, MA, USA), and stored at –70 °C until use. Normalization of culture supernatants was based on the total cell number as well as on protein content. Each experiment was repeated at least three times. For Western analysis, membranes were blocked with 5 % BSA in the rinse buffer (150 mmol/L NaCl, 20 mmol/L Tris, 0.1 % Tween 20) for 1 h, washed in rinse buffer for 10 min and then incubated with the respective primary antibody at the indicated concentrations. The membranes were then washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature, washed for 10 min and four more cycles of 5 min and treated with enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ, USA). In some cases, when the signal was very weak or undetectable, we used ECL-plus (Amersham).

2.4.1 Effect of saposin C on the Raf-MEK-ERK-RSK-Elk-1 signaling cascade of MAPK

Cells were grown in their complete culture media up to 50–60 % confluency, washed twice with PBS and serum-deprived overnight in their respective basal media. After removing the culture medium, saposin C was added at indicated concentrations, incubated for 5 min, washed with cold PBS and lysed on ice in lysis buffer as indicated above. For each cell line, a representative plate was pre-treated with the specific MEK1/2 inhibitor (U0126; Cell Signaling Technology, Bedford, MA, USA) at 10 μmol/L for 1.5 h before treatment with saposin C (at 1 nmol/L). Clarified cell lysates (10 μg per lane for MEK1/2, Erk1/2 [p42/44], or p90RSK and 50 μg/lane for c-Raf and Elk-1 detection) were resolved by SDS-PAGE under reducing conditions. For immunoblotting, a phospho-Erk1/2 Pathway Sampler Kit (Cell Signaling Technologies) containing phospho-specific antibodies against c-Raf (Ser259), MEK1/2 (Ser217/221), p42/44 MAP Kinase (Thr202/Tyr204), p90RSK (Ser380), and Elk-1 (Ser383) were used. The remainder of the protocol was followed as indicated above. Primary antibodies against actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-Raf-1, p42/44 MAPK, MEK1/2, p90RSK, or Elk-1 were used for controls (Cell Signaling Technology).

2.4.2 In vitro SAPK/JNK kinase assays

Non-radioactive SAPK/JNK assay kits (Cell Signaling Technologies) were used to determine whether saposin C treatment of cells under serum starvation stress would lead to SAPK-activation. To evaluate SAPK/JNK activity, serum starvation of the cells was carried out for 18 h and after 5 min of treatment with saposin C (as described for p42/44 MAPK assay), SAPK/JNK was selectively pulled down using 250 μg protein of the cell lysates and an N-terminal c-Jun_{1-89} fusion protein bound to glutathione sepharose beads (GST-c-Jun_{1-89}). After washing the beads, the kinase reaction was carried out in the presence of cold ATP and phosphorylated c-Jun was detected by a phospho-c-Jun antibody. This antibody specifically detects SAPK-induced phosphorylation of c-Jun at serine 63. For control loading, 10 μg protein per sample from the same whole cell lysates were sub-

jected to Western analysis using monoclonal anti-actin antibody.

2.4.3 Analysis of c-Jun expression

Cells were cultured as indicated above and after serum starvation for 18 h, they were treated in the presence or absence of saposin C at 10 nmol/L (in basal media) for the indicated time periods. After each incubation period, cells were lysed in lysis buffer and clarified cell lysates (20 μg per lane for PC-3 and LNCaP, 35 μg/lane for DU-145 and 15 μg/lane for PrSt) were subjected to SDS-PAGE under reducing conditions. Immunoblotting was carried out using primary antibody against c-Jun or actin (1:100; Santa Cruz Biotechnology).

2.4.4 Expression of uPA and uPAR

Prostate cancer cells were grown up to 65–70 % confluency in their respective complete culture media and prostate stromal cells were maintained in SCGM (containing 5 % FBS). Several studies have demonstrated androgenic regulation of the expression of uPA/uPAR and cathepsins by prostatic cells [7]. Therefore, to reduce or eliminate such effects in androgen receptor (AR)-expressing cells (prostate stromal and LNCaP cells), after washing the prostate stromal cells and LNCaP with PBS, they were incubated for 24 h in phenol red (PR)-free RPMI-1640 (Invitrogen, Inc Carlsbad, CA, USA) supplemented with 5 % charcoal-stripped FBS (CS-FBS; Sigma, St Louis, MO, USA). Cells were then washed twice with PR-free RPMI (serum-free) and incubated overnight in this medium in the presence or absence of saposin C at 0.1, 1.0, or 10.0 nmol/L. PC-3 and DU-145 cells were washed twice with PBS and incubated for 24 h with or without saposin C in serum-free Dulbecco's modified eagle medium(DMEM). At the end of the incubation period, culture supernatants were collected and concentrated ten times using Centriprep-3 concentrator (Amicon) and 25 μg/lane was loaded for electrophoresis. Membranes were probed with uPA at 2 mg/mL (Clone #399; American Diagnostica, Greenwich, CT) or uPAR at 2 μg/mL (Clone #399R; American Diagnostica) primary antibodies. Normalization of culture supernatants was based on the total cell number as well as on protein content.

2.5 RNA extraction and RT-PCR

To study the mRNA expression, we used the same set of tissue culture plates that has been used for protein expression analysis of uPA and uPAR (see above). Total RNA was prepared from cell culture plates by using 1 mL of RNAzol B as specified by the manufacturer (TEL-TEST; Friendswood, TX, USA). The concentration and purity of the RNA were determined by spectrophotometry at 260 and 280 nm as well as by 1.2 % agarose gel electrophoresis. The first-strand synthesis of cDNA was conducted in a 50 μL reaction containing 7.5 μg of total RNA in the presence of 0.5 μg of random hexamer primers from the Pro-STAR First-Strand RT-PCR kit (Stratagene, Cedar Creek, TX). The 50 μL PCR reactions contained 33.85 μL of UltraPure water, 5 μL of cDNA, 5 μL of 10 × PCR buffer, 3 μL of 25 mmol/L $MgCl_2$, 0.4 μL of 100 mmol/L dNTP mix, 1.0 μL of both sense and antisense primers (at 10 μmol/L), and 0.75 μL of Taq polymerase (5 units/μL; Promega). All the primers were synthesized by Integrated DNA Technologies (Coralville, IA). The oligonucleotides used (according to the human uPA, uPAR, and beta 2 microglobulin [β_2-μGB] cDNA sequences deposited at the NCBI/genome data bank) were as follows: uPA sense, 5'-GTGGCCAAAAGACTCTGAGG-3' (positions 25–44) and uPA antisense, 5'-GCCGTACATGAAGCAGTGTG-3' (positions 209–190); uPAR sense, 5' GAGCTGGTG-GAGAAAAGCTG-3' (positions 248–267) and uPAR antisense, 5'-TGTTGCAGCATTTCAGGAAG-3' (positions 650–631); and β_2-mGB sense, 5'-ATGCCTGCCGTGT-GAACCATGT-3' (positions 327–347) and β_2-mGB antisense, 5'-AGAGCTACCTGTGGAGCAACCT-3' (positions 632–613). PCR was carried out using the T-gradient model (Biometra, Horsham, PA, USA) under the following conditions: 19 to 35 cycles (depending on the gene of interest) at 95°C for 90 s, Tm as indicated below, 72°C for 1.5 min with a final 10 min extension cycle at 72°C.

The sizes of the amplified cDNA fragments, the optimized Tm/time period for PCR were 230 bp, 59.6 ºC/ 90 s for uPA, 403 bp, 59 ºC/ 90 s for uPAR, 590 bp, and 286 bp for β_2-μGB (was amplified either alone or in a duplex PCR). The number of PCR cycles was optimized for each cell line to provide the best resolution (30 cycles for PC-3 and DU-145, 35 cycles for prostate stromal, and 40 cycles for LNCaP). The PCR products were confirmed as a single band using 1.2 % agarose gel electrophoresis and loading was also normalized with the β_2-μGB. The experiment was repeated three times independently and each PCR experiment included non-template control wells.

3 Results

3.1 Saposin C stimulates growth, migration and invasion in prostate stromal and cancer cells

PrEp cells did not show any proliferative response to saposin C. However, saposin C in a dose-dependent manner, was able to stimulate proliferation of PrSt cells by 43 %–135 %, PC-3 cells by 25 %–45 %, DU-145 cells by 33 %–66 % and LNCaP cells by 44 %–102 %, as compared to control values (Figure 1A). It is note-

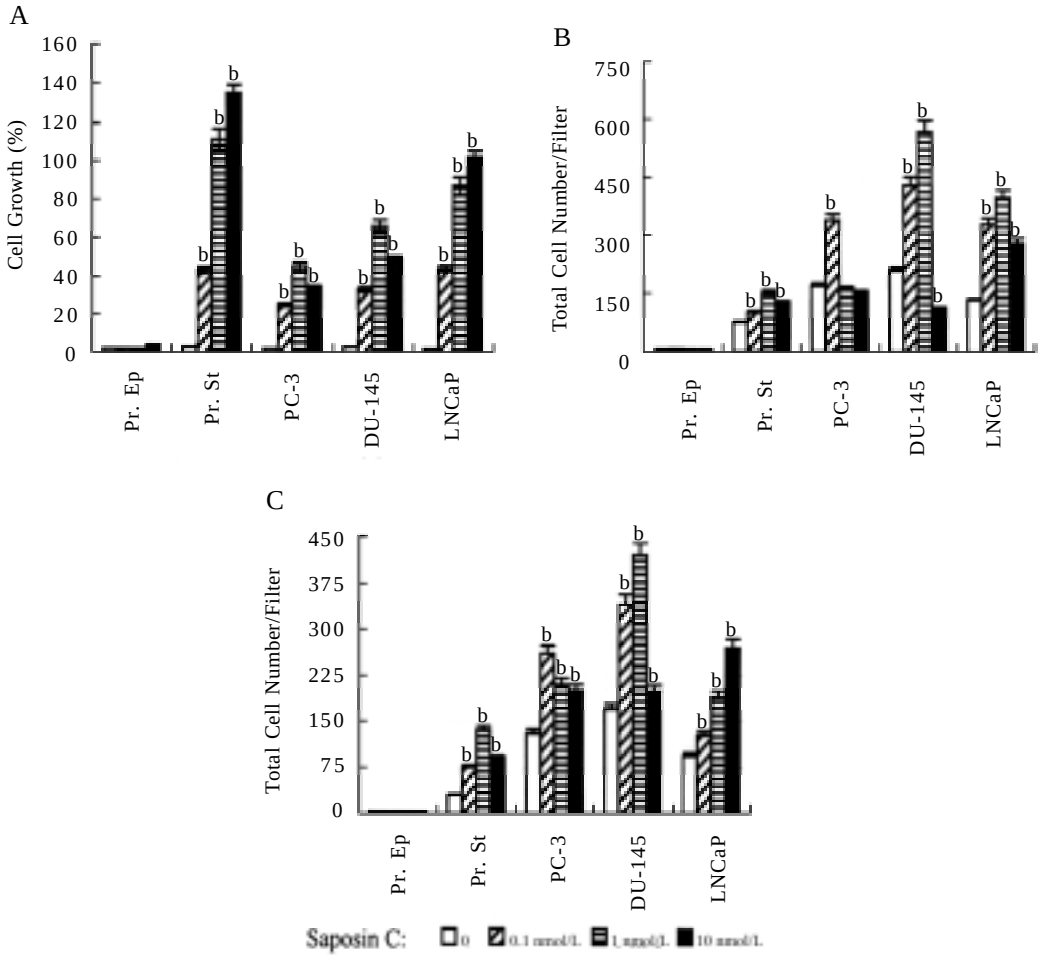

Figure 1. Effect of saposin C on prostate normal and cancer cell proliferation, migration, and invasion. (A) Proliferation assay. Cells were seeded at equal numbers per well in 96-well plates in complete culture media for 3 days. Saposin C-supplemented serum-free medium was added to the cells at indicated concentrations and after 2 days, the cell number was determined by MTS assay. For each cell line, the total cell number was determined using OD/cell number standard curve. Twelve replicates were used for each treatment condition and the assay was repeated at least three times. Cell migration (B) and invasion (C) assays were performed as described in the materials and methods sections of the present paper. The lower transwell unit contained 400 μL of 0.5 % FBS supplemented culture medium in the presence or absence of saposin C at the indicated concentrations. After trypsinization, 10^4 cells in 100 μL of the respective serum-free culture media were placed on top of non-coated (for migration) or coated with GFR-Matrigel (for invasion) transwell filters. After a 20 h incubation period, the non-migratory cells on the upper surface of the filter were removed and the cells attached to the lower surface of the filter were fixed/stained with Diff-Quick. The total number of cells was counted using phase-contrast microscopy (× 200 magnification) equipped with an ocular grid that shows each filter in divided sections. Each sample was assayed in triplicate, and the assays were repeated three times. PrSt; normal human prostate stromal cells, PrEp; normal human prostate epithelial cells. PC-3 and DU-145 were used as androgen-independent and LNCaP cells were used as androgen-dependent prostate cancer cell lines. Data represent the average of three independent experiments in triplicate samples; *bars*, ± SEM. Statistical significance was determined by one-way ANOVA with Bonferroni's corrections. $^bP < 0.05$, compared with controls.

worthy that the pattern of proliferative response to saposin C in LNCaP and prostate stromal cells was different from the androgen-independent PC-3 and DU-145 cells. Unlike a peak response at 1.0 nmol/L for these AI cells, a dose-dependent growth response to saposin C in AD LNCaP cells and prostate stromal cells was noticed.

Various steps in the complex process of invasion are mediated by soluble migration and invasion stimulatory factors (such as cytokines, growth factors and trophic peptides) through autocrine or paracrine loop(s) created among different cellular compartments within the tumor-host microenvironment. We examined the growth factor potential of saposin C on normal and malignant prostate cells for effects on cell migration and invasion. Normal prostate epithelial cells were proved to be non-responsive in these assays (Figure 1B and 1C). However, saposin C in a dose-dependent manner stimulated migration of prostate stromal cells by 100 % and invasion of them by 350 %. Saposin C stimulated migration of PC-3 cells by 100 %, DU-145 cells by 171 %, and LNCaP cells by 196 %. It stimulated invasion of PC-3 by 100 %, DU-145 by 170 % and LNCaP by 187 %. This result demonstrates that in addition to growth-promoting effect, saposin C acts a potent cell motility and invasion stimulatory factor for both prostate stromal and cancer cells.

3.2 Saposin C activates p42/44 and SAPK/JNK MAPK signal transduction pathways and upregulates the expression of immediate early gene protein, c-Jun in prostate stromal and cancer cells

Activation of p42/44 MAPK by prosaposin, saposin C, or TX14A has been demonstrated in neuronal- or glial-derived cells [4, 5, 8, 9]. Interestingly, inactivation of p42/44 MAPK in prostate epithelium was also observed in mutant prosaposin homozygous mice [10, 11]. The importance of MAPK signaling as a core signaling pathway influencing diverse cell biological activities (that are often dysregulated in malignant cells), encouraged us to examine the effect of saposin C on the Raf-MEK-ERK1/2 (p42/44)-p90RSK-Elk-1 signaling cascade that links extracellular ligands to cell transcription machinery. PC-12, pheochromocytoma cells, served as an external positive control cell line. PrEp cells showed a constitutively high level of activation that remained steady in the presence of saposin C. However, in PrSt and cancer cells, the basal level of activity of various downstream effectors in the above signaling cascade was either very low or undetectable (Figure 2). Saposin C increased the phosphorylative activity of all members of the Raf-MEK-ERK-p90RSK-Elk-1 signaling cascade in a biphasic pattern with an initial increase at 0.1mol/L, a decrease at 1.0 nmol/L and a final increase at 10.0 nmol/L as compared to the control basal levels. Induction of the linear MAPK signaling cascade (MEK-ERK-RSK) was substantially inhibited by pretreatment of the cells with a specific MEK1/2 inhibitor (U0126). The activation of the MAPK pathway by saposin C was also dependent on cell density or the state of confluency of tissue cultured cells. Saposin C did not affect basal MAPK activity when cells are confluent or sub-confluent. Likewise, in agreement with a previous study, saposin C-treated PC-12 cells also showed that the activation of p42/44 MAPK and MEK1/2 inhibitor prevented this effect (Figure 2) [8]. The p42/44 MAPK pathway is primarily a mitogenic pathway regulating cell growth and differentiation, while the other member of the MAP kinase superfamily, JNK, also known as SAPK, is primarily involved in the regulation of apoptosis and stress responses [12]. However, recent evidence suggests that there are exceptions to this rule. In certain cell types, activation of p42/44 MAPK has been shown to protect cells from apoptosis induced by various extracellular stimuli or agents, whereas activation of SAPK/JNK promotes apoptosis in other cell lines [13]. Therefore, we investigated whether there is a cell type-specific and/or divergent regulation of SAPK/JNK in response to saposin C. Using a non-radioactive immunoprecipitation/*in vitro* kinase assay, we found that saposin C upregulated SAPK/JNK activity in both androgen-dependent (AD) and -independent (AI) prostate cancer cells that led to the induction of phosphorylation of c-Jun at serine 63 (Figure 3A). This site is an important position for c-Jun-dependent transcriptional activity. Our results demonstrate a cell type-specific response to saposin C for SAPK/JNK activation and might suggest that saposin C utilizes multiple pathways in the MAPK superfamily to induce proliferative response in the cells.

Since transcriptional activity of c-Jun could at least partially be responsible for diverse biological activities of saposin C as with many other neurotrophic molecules and neuropeptides, we carried out time-course studies to evaluate the effect of saposin C on immediate early gene c-Jun expression. We observed a moderate to strong induction of c-Jun expression in both AD- and AI-prostate cancer cells after 5–15 min treatment with 10 nmol/L saposin C (Figure 3B), followed by a modest down regulation (returning) to baseline level in LNCaP and PC-

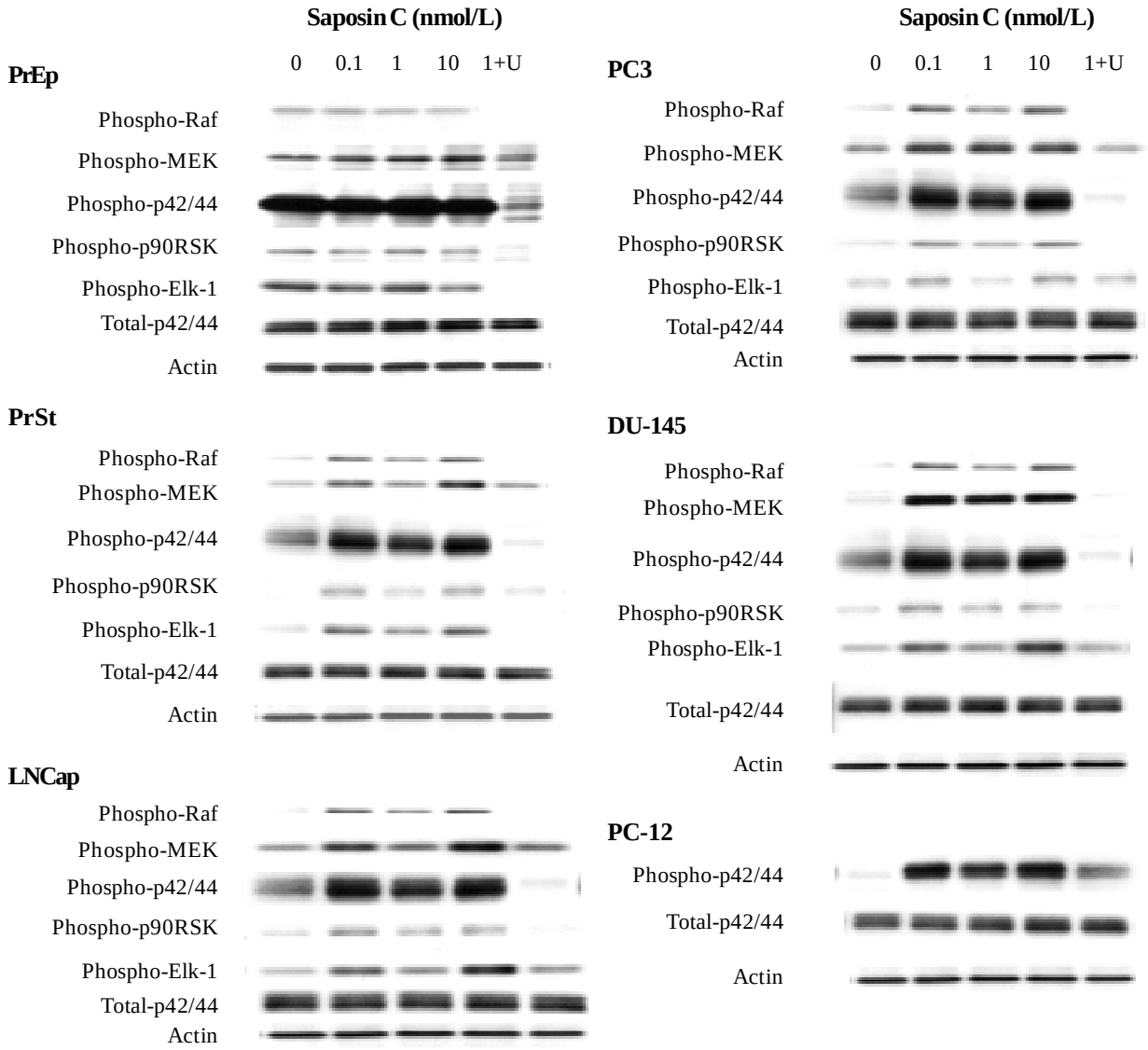

Figure 2. Effect of saposin C on linear Raf-MEK-ERK-RSK-ELK-1 signaling pathway of MAPK in prostate epithelial, stromal, and cancer cells. Cells were grown up to 50 %–60 % confluency, serum-starved overnight, and then stimulated with saposin C for 5 min at the indicated concentrations. After preparation of the cell lysates, 10 μg (for MEK, ERK, or p90RSK) or 50 μg (for c-raf and Elk-1) per lane was analyzed for phosphorylation using phospho-specific antibodies and immunoblotting. For each cell line a representative culture plate was pre-treated for 1.5 h with U0126, specific MEK1/2 inhibitor (10 μmol/L). The loading normalization was determined by re-probing the membranes with control antibodies against actin or p42/44 MAPK. Human pheochromocytoma cell line, PC-12, was used as an external positive control cell line. PrSt; normal human prostate stromal cells, PrEp; normal human prostate epithelial cells. PC-3 and DU-145 were used as androgen-independent and LNCaP cells were used as androgen-dependent prostate cancer cell lines.

3 at 30 or 60 min and then an increase again for the remaining period of the study (up to 240 min). A similar observation was made in DU-145 cells, where saposin C increased c-Jun expression significantly at 5 min and remained at the same level at 15 min, down modulated at 30 min and then increased over time from 60 to 240 min reaching to maximum level at 4 h. Prostate stromal cell response was different from the cancer cell responses. Unlike prostate cancer cells, saposin C upregulated c-Jun expression after 15 min and lasted for 3 h at the

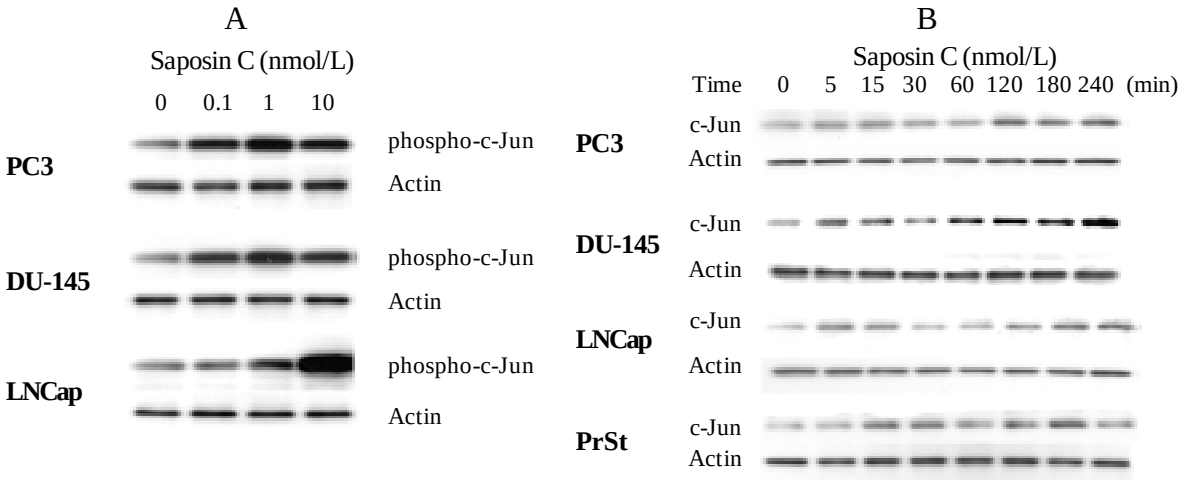

Figure 3. Effect of saposin C on SAPK/JNK signaling pathway and immediate early gene (c-Jun) expression. (A) Cells were grown up to 70 % confluency in their maintenance media, serum-deprived for 18 h, and treated with saposin C. SAPK/JNK was selectively pulled down using 250 µg protein and an N-terminal c-Jun$_{1\text{-}89}$ fusion protein bound to glutathione sepharose beads (GST-c-Jun$_{1\text{-}89}$). *In vitro* kinase reaction was carried out in the presence of cold adenoisine triphosphate(ATP) and SAPK-induced phosphorylation of c-Jun at serine 63 was detected by a phospho-specific-c-Jun antibody. For control, 10 µg protein per sample from the same whole cell lysate was subjected to SDS-PAGE and immunoblotting using monoclonal anti-actin antibody. (B) To analyze c-Jun expression, cells were serum-deprived as above and treated in their basal media with or without 10 nmol/L saposin C for the indicated time periods. Cells were then lysed and clarified cell lysates (20 µg per lane for PC-3 and LNCaP, 35 µg/lane for DU-145, and 15 µg/lane for PrSt cells) were subjected to SDS-PAGE under reducing conditions. Immunoblotting was carried out using primary antibodies against c-Jun or actin (both at 1:100). PrSt; normal human prostate stromal cells. PC-3 and DU-145 were used as androgen-independent and LNCaP cells were used as androgen-dependent prostate cancer cell lines.

same level and then was down-regulated at 240 min. Overall the above data provide a collective body of evidence for the effect of saposin C on p42/44 MAPK and SAPK/JNK of MAPK superfamily and transcriptional regulation of c-Jun that could subsequently lead to diverse but profound biological activities (such as proliferation) in both prostate stromal and cancer cells.

3.3 Saposin C upregulates uPA/uPAR mRNA and protein expression in prostate stromal and cancer cells

To obtain a mechanistic understanding of what underlies the migration and invasion stimulatory effect of saposin C, we investigated the effect of saposin C on expression of two major families of proteases: uPA and its receptor uPAR and MMPs, all of which have been shown to be either under androgenic regulation or have been implicated in prostate cancer invasion and metastasis [14]. We employed a standard androgen-deprived culture condition in androgen-responsive (and AR-expressing) cells (PrSt and LNCaP) using phenol red-free RPMI and charcoal stripped-FBS.

Saposin C treatment of prostate stromal and cancer cells did not affect the expression of MMP-2 and -9 using gelatin zymography and Western analysis (data not shown). We examined the expression of uPA/uPAR protein and mRNA by Western blotting of concentrated conditioned media and RT-PCR analysis, respectively. Urokinase plasminogen activator and its receptor were detected at approximately 54 and 55–60 kDa, respectively. In prostate stromal cells, saposin C increased uPA/uPAR protein and mRNA expression at concentration as low as 0.1 nmol/L and remained at the same level at higher concentrations. In the AI PC-3 cell line, saposin C elicited a biphasic response for uPA/uPAR protein expression with an initial increase at 0.1, a decrease at 1.0 nmol/L and a final increase at 10 nmol/L. A similar biphasic response was also observed for uPA mRNA (Figure 4). The uPAR mRNA expression was also increased and reached to a maximum level at 10 nmol/L. In DU-145 cells, saposin C was able to increase the expression of secreted uPA/uPAR protein and their mRNA. To detect secreted uPA and uPAR protein in PrSt cells, we concentrated the supernatant 10 times and loaded 30 µg protein per lane. To intensify the signal, we used the

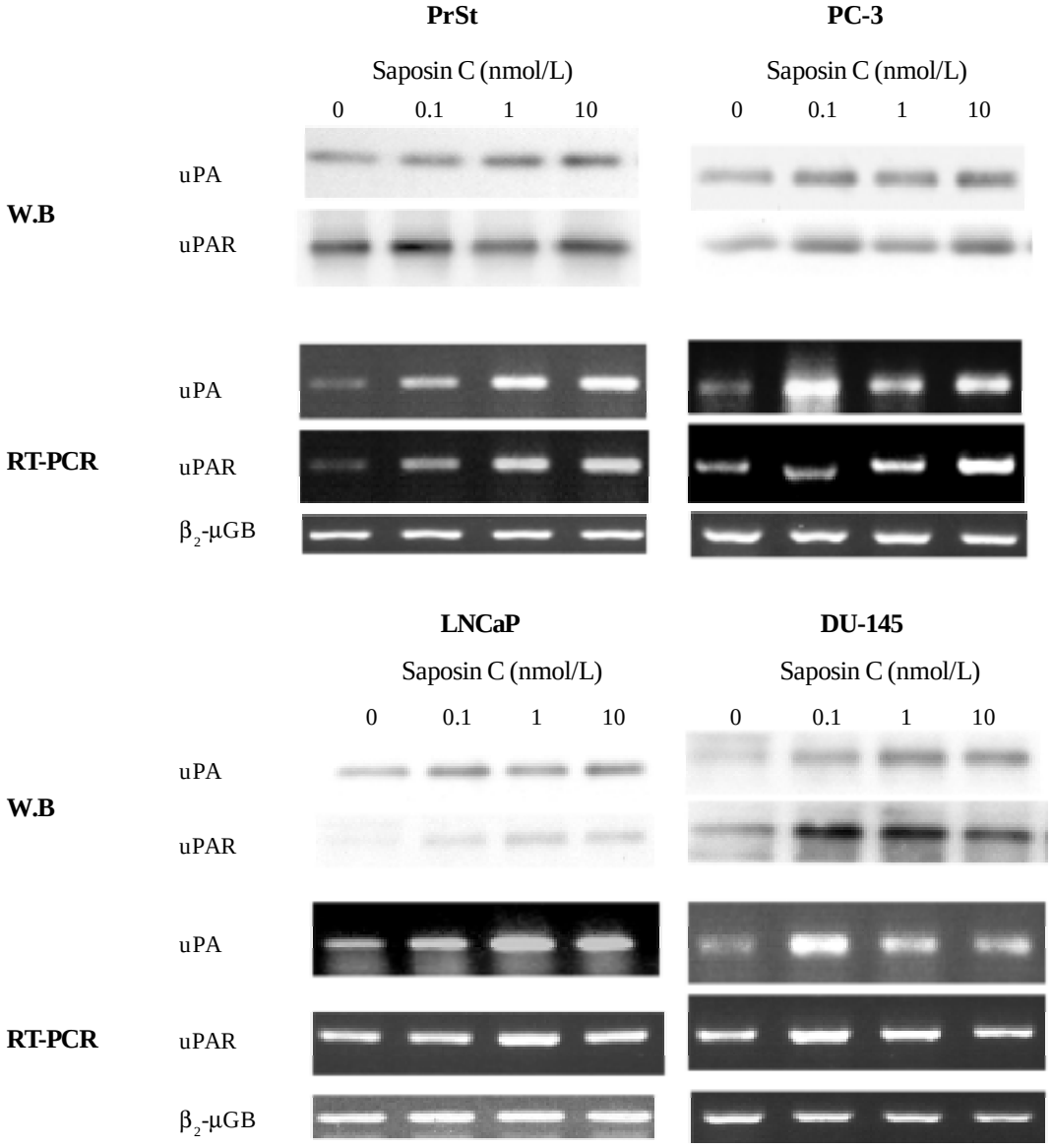

Figure 4. Induction of uPA/uPAR expression by saposin C in prostate stromal and cancer cells. Prostate stromal and cancer cells were grown up to 65–70% confluency. AR-expressing (PrSt and LNCaP) cells were washed with PBS, incubated for 24 h in phenol red-free RPMI supplemented with 5 % CS-FBS, washed with phenol red and serum-free RPMI twice, and incubated for 24 h in this medium in the presence or absence of saposin C. Androgen-independent PC-3 and DU-145 cells were also treated in their basal medium with saposin C as above. Cell-free culture supernatants were concentrated and 25 or 30 μg per sample was subjected to SDS-PAGE under reducing conditions. Membranes were probed with uPA (2 μg/mL) or uPAR (2 μg/mL) primary antibodies. Normalization of culture supernatants was based on the total cell number as well as on protein content. PrSt; normal human prostate stromal cells. PC-3 and DU-145 were used as androgen-independent and LNCaP cells were used as androgen-dependent prostate cancer cell lines. For mRNA expression analysis, total RNA was extracted from the above tissue culture plates. Briefly, total RNA was extracted using 1 mL of RNAzol B. After determination of the concentration and purity of the RNA by spectrophotometry as well as by agarose gel electrophoresis, first strand cDNA was synthesized and used for PCR as described in details in the materials and methods section of the present paper. Optimal PCR condition (Tm, cycle number, etc) was determined empirically for each of the genes under investigation using a thermal gradient-PCR machine. β_2-μGB was used to monitor loading control. The PCR product was confirmed as a single band using 1.2 % agarose gel electrophoresis. The experiment was repeated three times independently and a non-template control was used for each PCR experiment. PrSt; normal human prostate stromal cells. PC-3 and DU-145 were used as androgen-independent and LNCaP cells were used as androgen-dependent prostate cancer cell lines.

ECL-plus detection reagent (Amersham) and exposed the film for more than 15 min. To detect the uPA/uPAR mRNA by RT-PCR, we had to increase the cycle number to 35 or more. Using these modifications, we were able to detect uPA and uPAR proteins in conditioned media as well as their mRNAs. Saposin C increased uPA protein level at 0.1 nmol/L and remained steady at higher concentrations. There was also a slight increase in the level of secreted uPAR. RT-PCR analysis also confirmed the upregulation of both proteins. Upregulation of uPA/uPAR by saposin C might at least partially account for the cell migration and invasion stimulatory response of prostate stromal and cancer cells under investigation.

4 Discussion

Although androgens remain the most important native stimulus to androgen receptor in normal and malignant prostatic cells, polypeptide growth factors, cytokines, neuropeptides, protein kinase activators and some other trophic agents may also play a substantial role in the initiation of prostate cancer at its AD stage, its progression toward the AI regrowth, or its clinical regression in response to various anti-androgen treatment strategies [15, 16].

Homozygous inactivation of *prosaposin* gene in mice led to early death at the age of 35–40 days mainly due to neurological deficits or disorders [17]. Additional studies in the knock-out mice model revealed a number of interesting findings specifically in the male reproductive organs. Among these are atrophy of testes, prostate gland, epididymis, and seminal vesicles. Microscopic analyses also showed a reduced spermatozoa and reduction in the overall size of the tubuloalveolar glands and their epithelial cell lining [10]. In spite of these abnormalities, radio-immunoassays of blood samples in mice with homozygous-inactivation of prosaposin revealed a normal or higher testosterone level compared to prosaposin-heterozygous (+/–) or control-mice (+/+).

We have demonstrated that saposin C stimulates proliferation, migration and invasion of prostate stromal and cancer cells in a dose-dependent manner. While normal prostate epithelial cells, by contrast, proved to be non-responsive in those assays (Figure 1A). The effect of saposin C on cell growth was higher in prostate stromal and AD LNCaP cells than the AI PC-3 and DU-145 cells. The pattern of cell type-specific growth response was similar to TX14A. However, proliferative-response to saposin C was more than TX14A [5]. The presence of prosaposin and saposin C in complete culture medium in our previous study might mask the proliferative effect of TX14A in the cells under investigation [5]. Prostate stromal cells also showed strong migratory and invasive response (Figure 2B and C) to saposin C. Likewise, cell migration and stimulatory response to saposin C was similar to TX14A [5]. With the exception to normal prostate epithelial cells, saposin C in a dose-dependent manner increased migration and invasion of all cells investigated. However, at the highest treatment concentration (10 nmol/L), it inhibited DU-145 cell migration. Although the exact mechanism for this observation is not known, this effect or the differences in AI and AD prostate cancer cells response to saposin C could be related to cell type-specific characteristic phenotypes such as receptor density and turnover, post-receptor occupancy events, the presence or absence of certain positive and negative feedback mechanisms and/or signaling pathways among others.

Like many other malignancies, growth or trophic factors produced by prostate cancer and/or stromal cells can influence the multistep process of migration/invasion by regulating the expression and/or activity of matrix-degrading proteolytic enzymes such as uPA and MMPs. Most of the activity of uPA is mediated while the protease is bound to uPAR allowing focal digestion of the surrounding matrix in tumor microenvironment [18]. In cancer patients, elevated levels of uPA or uPAR also exist as soluble forms in extracellular matrix and spaces (i.e., blood) and serve as a pool that is accessible by tumor or stromal cells [19]. In addition to its proteolytic function, the uPA/uPAR system can also affect cell motility and proliferation. These phenotypes may be mediated by activated signal transduction pathways rather than by a proteolytic mechanism [17].

Using immunoblotting and gelatin zymography, we discovered that saposin C had no effect on expression or activity of MMP-2 and -9 (data not shown). Although conflicting reports for the presence or absence of uPA or uPAR expression in LNCaP cells exist, using the experimental conditions described before, our LNCaP cell line expresses both uPAR and uPA protein (soluble) and mRNA and saposin C treatment increased their expression (Figure 4). In addition, our data demonstrate that uPA and uPAR are not only expressed by prostate stromal and AI cancer cells, but that their expression is also upregulated by saposin C. Upregulation of uPA and uPAR

expression by saposin C could at least partially account for the increased cellular migration and invasion.

We also found that saposin C activates the Raf-MEK-ERK-Elk-1 signaling cascade of p42/44 MAP kinase and SAPK/JNK pathway and upregulates the expression of immediate early gene c-Jun expression in prostate stromal and in prostate cancer cells under investigation (Figure 2). It is worth noting that basal levels of active p42/44 MAPK in normal prostate epithelial cells were the highest among all other cells investigated and were not affected by saposin C treatment. These data might suggest high basal level of MAPK activity, as a potential characteristic phenotype for well-differentiated normal prostate epithelial cells. The functional significance of this phenotype requires further investigation. The bimodal phosphorylative response of various effectors in p42/44 MAPK pathway could be due to a number of factors such as down-modulation of the responding receptor and/or enhanced phosphatase activity (Figure 2). A similar biphasic response to TX14A or prosaposin has been also reported in PC12 and in nerve regeneration experiments with guinea pig sciatic nerve [8].

Prostatic stroma may well contribute to prostate carcinogenesis by production of soluble growth or trophic factors either under androgenic influence or paracrine stimulation from cancer or other mesenchymal cells. Special consideration has been given to soluble pluripotent effectors secreted by tumor or stromal cells and present in the tumor microenvironment. These molecules mediate aberrant interactions among tumor and stromal cells that lead to the formation of the "vicious cycle" as initially introduced and formulated by Chung *et al.* [20]. With respect to saposin C as a soluble multipotential growth factor affecting various biological activities in prostate stromal and cancer cells and the significant importance of prostate cancer cells-stromal interactions in the pathophysiology of prostate cancer, we hypothesize that saposin C or its precursor (prosaposin), might also mediate or contribute to the formation of a vicious cycle. In addition to the in vitro finding presented here, it is noteworthy to mention that our immunohistochemical analysis showed positive prosaposin staining in reactive stroma (as defined histopathologically) and very strong anti-prosaposin immunoreactivity in the endothelial cell lining of blood vessels and specifically in venules adjacent to the inflammatory regions [5]. Overall, prosaposin immunostaining in mesenchymal cells showed to be more prevalent and in close proximity with positively stained tumor foci. This might increase not only the chance of cell-cell contact, but also might lead to the creation of paracrine loop(s) between stromal cells, endothelial cells, or inflammatory cells and tumor cells through the release of prosaposin among them.

In summary, the present study for the first time provides evidence that indicates saposin C, a previously known neurotrophic factor, functions as a potent growth factor in human prostate stromal and cancer cells. Using various *in vitro* bio-functional and expression assays, we found that saposin C stimulates cell growth, migration and invasion and upregulates the expression of the matrix-degrading proteolytic enzyme uPA and its receptor in prostate cancer cells. While normal prostate epithelial cells were non-responsive to saposin C, prostate stromal cell responsiveness in the assays investigated suggests that saposin C may be a potential mediator of tumor-stromal interactions in prostate cancer. Like many other growth factors, saposin C upregulates the expression of immediate early gene, c-Jun and activates p42/44 and SAPK/JNK signaling pathways of MAPK superfamily. Overall, by modulating diverse biological activities, saposin C or its precursor (prosaposin) may contribute to prostate carcinogenesis at its early AD-stage or late hormone-refractory state.

Acknowledgment

We are grateful to Ms Nicole Barron for her editorial assistance. This research was supported by the Stanley S. Scott Cancer Center at Louisiana State University Health Sciences Center, New Orleans, LA.

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