Regulation of survivin by PI3K/Akt/p70S6K1 pathway

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PI3K activation is commonly observed in many human cancer cells. Survivin expression is elevated in cancer cells, and induced by some growth factors through PI3K activation. However, it is not clear whether PI3K activation is sufficient to induce survivin expression. To investigate the role of PI3K pathway in the regulation of survivin, we expressed an active form of PI3K, v-PI3K in chicken embryonic fibroblast cells (CEF), and found that overexpression of PI3K-induced survivin mRNA expression. Forced expression of wild-type but not mutant tumor suppressor PTEN in CEF decreased survivin mRNA levels. PI3K regulates survivin expression through Akt activation. To further investigate downstream target of PI3K and Akt in regulating the expression of survivin mRNA, we found that PI3K and Akt-induced p70S6K1 activation and that overexpression of p70S6K1 alone was sufficient to induce survivin expression. The treatment of CEF cells by rapamycin decreased the survivin mRNA expression. This result demonstrated that p70S6K1 is an important target downstream of PI3K and Akt in regulating survivin mRNA expression. The knockdown of survivin mRNA expression by its specific siRNA induced apoptosis of cancer cells when the cells were treated with LY294002 or taxol. Taken together, these results demonstrated that PI3K/Akt/p70S6K1 pathway is essential for regulating survivin mRNA expression.

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1. Introduction

Apoptosis is an important process for cell and tissue homeostasis. Apoptotic effector molecules and disordered apoptosis are involved in various diseases. The inhibitor of apoptosis protein (IAP) inhibits apoptosis by inactivating several caspases [1,2]. Survivin is a newly described member of the IAP family [3]. Survivin is constitutively expressed in most cancers, including carcinomas of the lung, colon, pancreas, prostate, breast, stomach, ovarian and in most hematopoietic malignancies, and is an unfavorable prognostic marker [4]. Survivin can inhibit apoptosis by blocking a common step downstream of mitochondrial cytochrome c release by inhibiting terminal effectors caspase-3 and caspase-7, and by interfering with caspase-9 activity and processing [5,6]. Overexpression of survivin can lead to resistance to apoptotic stimuli including chemotherapy.

Survivin is expressed primarily in fetal, but not adult tissues. The expression of survivin is cell cycle dependent. It is selectively expressed at the G2/M phase of the cell cycle in a cell cycle-regulated manner, and localized with caspase-3 to mitotic spindle microtubules [7]. Hematopoietic and vascular remodeling cytokines, STAT3-dependent signaling and phosphotyidinositol-3-kinase (PI3K) activity affect survivin expression through non-cell cycle dependent mechanisms [8]. PI3K/Akt signaling pathway has been implicated to play an important role in the upregulation of survivin in both vascular endothelial cells and tumor cells. For example, in endothelial cells, angiopoietin-1 inhibits cell apoptosis via the Akt/survivin pathway [9]. VEGF binding to VEGF-R2 activates the PI3K pathway and induces the expression of survivin in endothelial cells [10]. In cancer cells, survivin protein level is upregulated by coexpression of human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) through PI3K/Akt signaling pathway in breast cancer cells [11]. Hematopoietic cytokine GM-CSF exerts the anti-apoptotic and mitogenic effects partially by increasing survivin levels through the activation of PI3K, while PI3K inhibitor LY294002 inhibits the effect of GM-CSF in acute myeloid leukemia cells by decreasing survivin expression at mRNA and protein levels [12]. Exposure of human neuroblastoma cells to exogenous VEGF results in an increased expression of survivin protein and phosphorylated Akt, and inhibition of PI3K abrogates those effects [13]. Inhibition of PI3K pathway also downregulates survivin expression, and enhances TRAIL-mediated apoptosis in neuroblastomas [14]. Geranylgeranyltransferase I inhibitors (GGTIs) induce apoptosis in both cisplatin-sensitive and -resistant human ovarian epithelial cells by inhibition of PI3K/Akt and survivin pathways [15]. These results...
suggest that PI3K/Akt signaling pathway is involved in regulating survivin expression in response to cytokines, growth factors, and chemotherapeutic drugs. However, it is unknown whether PI3K is sufficient to induce survivin expression.

Epithelial ovarian cancer is the fourth biggest cause of cancer-related death in women. Taxol is one of the first-line chemotherapeutic drugs for ovarian cancer, especially for advanced ovarian cancer [16]. Mitotic deregulation by survivin in ErbB2-overexpressing breast cancer cells and ovarian cancer cells is proposed to contribute to taxol resistance [17,18]. A recent study has shown that survivin counteracts the therapeutic effect of microtubule destabilizers by stabilizing tubulin polymers, which may contribute to taxol resistance through microtubule-targeting [19]. Our previous studies have demonstrated that PI3K/Akt/p70S6K1 is an important signaling pathway in regulating ovarian tumorigenesis and angiogenesis [20–22]. Since high levels of survivin protein are detected in advanced ovarian carcinomas [23], we hypothesize that PI3K is sufficient to induce survivin expression. In this study, we will use chicken embryo fibroblast (CEF) cells as a model to test: (1) whether PI3K activation alone induced endogenous survivin expression, (2) whether inhibition of PI3K signaling by PTEN and LY294002 decreased survivin expression; and (3) what are the downstream molecules of PI3K that regulate survivin expression. These results will allow us to identify the role of PI3K in mediating survivin expression, and to understand the mechanism of PI3K in regulating survivin expression.

2. Materials and methods

2.1. Reagents and cell culture

Total RNAs were isolated using the Trizol reagent from Invitrogen (Carlsbad, CA, USA). The primers for the survivin and GAPDH were from Gene Scrip Inc. (Piscataway, NJ). The antibodies against survivin were from Santa Cruz Biotechnology (Santa Cruz, CA) and the antibodies against phospho-Akt (Ser473), total Akt, p70S6K1, and phospho-p70S6K1 (Thr421/Ser424) were from Cell Signaling Technology (Beverly, MA). The antibody against β-actin was from Sigma (St. Louis, MO). The horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were from Perkin Elmer Life Sciences (Boston, MA). The human ovarian cancer cell line OVAR-3 (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% FBS, 0.2% insulin and antibiotics at 37 °C with 95% air and 5% CO2.

2.2. Plasmid constructs

SiRNA was designed using siRNA converter software (Ambion, Austin, TX), and the sequence was searched for homology with Basic Local Alignment Search Tool (BLAST) software. The 21-mer oligonucleotides specific to survivin were sense strand siRNA: UGGCGUAAACCCAGGAGGCTt, and antisense strand: GCCUCCUG GUUCAACCCGATt. Two annealed sense and antisense DNA oligonucleotides containing the 21-mer sequences directed against survivin were ligated into a pSilencer 2.1-U6 vector (Ambion, Austin, TX). DNA oligonucleotides containing the scramble sequence having no homology to any human genomes were also ligated into the vector to be used as a control. The plasmid that expresses siRNA against human survivin was named as si-survivin. The negative control plasmid was named as si-SCR. The plasmid constructs used were v-P3k, Myr-Akt, PTEN and PTEN mutant (C124S) expressed by the avian retrovirus vector RCAS as we previously described [24–26]. P70S6K1 (E389D3E) was first subcloned into an adaptor vector pBSFl, then inserted into a modified avian retrovirus vector RCAS.Sfi.

Chicken embryo fibroblasts (CEF) were plated at 2 × 10^5 cells/well in six-well plates, cultured overnight, and transfected with 2 μg of plasmid DNA using Lipofectamine reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instruction. After transfection, the cells were passaged for 2 weeks to ensure that the actively replicating retroviral vector spreads through the culture.

2.3. The cells that stably express survivin siRNA or si-SCR

OVCAR-3 cells were plated into 6 well plates. After cells were grown to 50–60% confluence, 2 μg of si-survivin or si-SCR was transfected into OVCAR-3 cells using Lipofectamine, and cultured for 24 h in complete medium. Then the cells were switched to medium containing 500 μg/ml G418 (Sigma), and cultured for 2 weeks. The resistant cells were pooled and passaged in medium containing 250 μg/ml G418 as needed.

2.4. RNA isolation and RT-PCR

Total RNAs were extracted with Trizol reagent (Invitrogen). RNAs were quantitated by measuring absorbance at 260 nm. CDNAs were prepared by incubating 1 μg of total RNAs in 25 μl reaction buffer that has AMV Reverse Transcriptase, an oligo(dT) primer, RNase inhibitors and dNTP (Promega) for 60 min at 37 °C. The CDNA fragment was amplified by PCR using following specific primers: survivin forward, 5′−GAG CTG CAG GTT CCTTAT C-3′; survivin reverse, 5′−ACA GCA TCG ACC CAA GTC AT-3′; GAPDH forward, 5′−CACCCATGCAAATTCATGCGA-3′; GAPDH reverse 5′−TCTAC AGGCGAGTCGTCGACC-3′. PCR was carried out in a thermal cycle programmed at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and amplified for 30 cycles. The amplified PCR products were visualized on 2% agarose gels.

2.5. Northern blot analysis

Total RNAs were extracted using Trizol reagent according to manufacturer’s instruction. Aliquots of total RNAs (10 μg) per lane were separated by formaldehyde gel, and transferred to nylon membranes by capillary transfer with a downward transfer system (Schleicher and Schuell). After UV crosslinking, the membranes were prehybridized for 1 h at 42 °C in 10 ml of UltraHyb buffer (Ambion). Then the membrane was hybridized to [32P] labeled chicken survivin cDNA fragment, which was labeled with [α-32P] dCTP by random priming using the RadPrime DNA labeling system (Invitrogen), and purified with the ProbeQuant G-50 Micro Columns (Amersham Biosciences, Piscataway, NJ).

2.6. Western blotting

Cells were washed with cold PBS, and collected by scraping. They were lysed in ice-cold lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholicacid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF] supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM pepstatin A. After centrifugation at 12,000 rpm for 15 min, the supernatant was used for protein determination using Bio-Rad protein assay reagent (Richmond, CA). The proteins were resolved on 8% SDS–PAGE, transferred onto nitrocellulose membrane, and incubated with antibodies against total Akt, phospho-Akt (Ser473), total p70S6K, phospho-p70S6K (Thr421/Ser424) and β-actin. The membranes were washed with PBS buffer containing 0.05% Tween 20, followed by incubation with the appropriate HRP-linked secondary antibodies. The levels of specific proteins were detected with the enhanced chemiluminescence reagent (NEN, Boston, MA).
2.7. Tunnel assay

Cells were washed three times in PBS buffer, and seeded into a V-bottomed 96-well microplate at 100 l/well in 2 x 107 cells/ml PBS. Cells were transferred to a freshly prepared fixation solution (100 l/well) and added to cell suspension. Cells were resuspended and incubated 60 min at 15–25°C. Fixative solution was removed by flicking off the microplate. Cells were washed once with PBS. The microplate was centrifuged at 300g for 10 min. Cells were resuspended in 100 l/well permeabilisation solution for 2 min on ice. Cells were washed twice with PBS, and resuspended in Tunnel reaction mixture. The microplate was incubated for 30 min at 37°C in a humidified atmosphere in the dark. Samples were washed twice in PBS. Cells were transferred in a tube to a final volume of 250–500 l in PBS. Samples were analyzed by flow cytometry using an excitation wavelength at 540 nm and detection wavelength at 620 nm.

2.8. Statistical analysis

The data were analyzed using SPSS statistics software package (SPSS, Chicago, IL). All of the results are expressed as means ± SD from three independent experiments. The values were considered significant difference with P < 0.05.

3. Results

3.1. PI3K/PTEN mediates survivin mRNA expression

PI3K has been implicated in the regulation of survivin induced by growth factors. To investigate the direct role of PI3K in the regulation of survivin, we expressed v-P3k by avian retroviral RCAS vector. The oncogene v-P3k codes for a constitutively active form of PI3K catalytic subunit [27]. Northern blot analysis using total RNAs from CEF expressing RCAS alone or RCAS carrying v-P3k demonstrated that overexpression of v-P3k increased the levels of survivin mRNA expression (Fig. 1A), indicating that activation of PI3K is sufficient to induce survivin expression. The tumor suppressor PTEN is a phosphoinositide 3-specific phosphatase that dephosphorylates PtdIns(3,4,5)P3 and PtdIns(3,4)P2 to PtdIns(4,5)P2 and PtdIns(4)P, respectively, antagonizing the effect of PI3K signaling pathway. The wild-type PTEN but not PTEN kinase dead mutant (C124S) inhibited survivin mRNA expression (Fig. 1B). These results indicate that PI3K/PTEN regulates survivin mRNA expression.

3.2. Akt is downstream target of PI3K/PTEN for regulating survivin mRNA expression

Akt is an important downstream target of PI3K. Next, we asked whether PI3K-inducing survivin expression was mediated by Akt activation. Overexpression of v-P3k increased the expression of p-Akt (Ser473), and PI3K inhibitor LY294002 treatment decreased the level of p-Akt (Ser473) (Fig. 2A). Similarly, overexpression of wild-type PTEN, but not mutant PTEN, inhibited Akt activation (Fig. 2B). To assess whether overexpression of a constitutively active form of Akt (Myr-Akt) is sufficient to induce survivin mRNA expression, CEF cells were infected with RCAS alone or RCAS carrying Myr-Akt. As we expected, overexpression of Myr-Akt increased survivin mRNA levels (Fig. 2C), suggesting that forced expression of Akt is sufficient to induce survivin expression.

![Fig. 1. PI3K/PTEN signaling pathway mediates survivin mRNA expression. (A) Overexpression of active form of PI3K is sufficient to induce survivin at mRNA level. RCAS carrying Pten or Pten mutant (C124S) was transfected into CEF cells and cultured for 2 weeks. Cells were cultured to 80% confluence and then changed to serum-free medium for 8 h. Total RNAs (10 µg) were analyzed by Northern blotting. (B) Overexpression of wild-type Pten, but not mutant Pten, inhibits survivin mRNA expression. CEF cells were transduced as Fig. 1B. Cells were cultured to 80% confluence and total RNAs (10 µg) were analyzed by Northern blotting.](image)

![Fig. 2. Akt is downstream target of PI3K/PTEN for regulating survivin mRNA expression. (A) Overexpression of active form of PI3K is sufficient to induce Akt activation. RCAS alone or RCAS carrying v-P3k (active form of PI3K) was transfected into CEF cells and cultured for 2 weeks. CEF cells expressing RCAS vector control or RCAS-Pi3k were cultured in complete medium until 70–80% confluence, changed to serum-free medium for 12 h and then treated with complete medium without or with 10 µM LY294002 for 1 h. Total cellular extracts were prepared and subjected to immunoblotting analysis using antibody specific to phosphorylation of Akt (Ser473) and total Akt. (B) Overexpression of wild-type PI3K, but not mutant PI3K, inhibits Akt activation. CEF cells were transduced by RCAS carrying PI3k or PI3k mutant (C124S) as above and the level of Akt phosphorylation at Ser473 or total Akt was detected by immunoblotting using phospho-Akt (Ser473) or total Akt antibodies. (C) Overexpression of Akt is sufficient to induce survivin expression at mRNA level. Cells were cultured to 80% confluence and then changed to serum-free medium for 8 h. The mRNA level of survivin was detected by Northern blotting.](image)
3.3. PI3K/Akt pathway upregulates survivin mRNA expression through mTOR/p70S6K1

mTOR/p70S6K1 signaling is involved in protein synthesis, glucose metabolism, cell cycle progression, and apoptosis [28]. To test whether p70S6K1 activation is required for PI3K- and Akt-mediating survivin mRNA expression, we showed that PI3K and Myr-Akt-induced p70S6K1 activation in CEF cells and that mTOR inhibitor rapamycin abolished PI3K- and Myr-Akt-inducing p70S6K1 activation (Figs. 3A and C), suggesting that p70S6K1 is the downstream molecule of PI3K and Akt in the cells. Similarly, rapamycin treatment inhibited the mRNA expression levels of survivin induced by PI3K (Fig. 3B) and by Myr-Akt (Fig. 3D), indicating that PI3K/Akt induces survivin mRNA expression via mTOR/p70S6K1 activation. When compared to that of RCAS control, forced expression of p70S6K1 induced survivin expression (Fig. 3E). The treatment of cells by rapamycin inhibited p70S6K1-inducing survivin mRNA expression (Fig. 3E). These results suggest that p70S6K1 is an essential downstream of PI3K/Akt for regulating survivin mRNA expression.

3.4. Expression of siRNA against survivin (si-survivin) in ovarian cancer cells induced apoptosis when the cells were treated with LY294002 or taxol

Increased survivin expression is a negative prognostic marker in many tumors, including ovarian cancer. To investigate the role of survivin in ovarian cancer, we generated the ovarian cancer cells that stably expressed si-survivin. Cells expressing si-survivin had a decreased expression levels of survivin mRNA when compared to that of cells expressing siSCR control (Fig. 4A). Expression of si-survivin also decreased the expression levels of survivin protein (Fig. 4B). PI3K plays an important role in cell survival and cell growth. It was reported that taxol-mediated mitotic arrest of cancer cells is associated with a survival pathway and taxol resistance to [29,30]. To test the effect of PI3K inhibitor or taxol on the cell survival of ovarian cancer cells expressing si-survivin or si-SCR, we found that PI3K inhibitor LY294002 treatment induced much higher apoptosis in the cells expressing si-survivin than those expressing siSCR control (Fig. 4C). Similarly taxol treatment also induced much higher apoptosis in OVCAR-3 cells expressing si-survivin when compared to those expressing siSCR (Fig. 4D). These results suggest that increased levels of survivin expression were associated with increased resistance to apoptosis induced by PI3K inhibitor and taxol in cancer cells.

4. Discussion

Recent studies demonstrated that PI3K/Akt activation has been involved in the upregulation of survivin induced by GM-CSF [12], Ang-1 [9,31], and VEGF [32–34]. However, there is no information on the direct effect of PI3K alone on survivin expression. In this study, we demonstrated that forced expression of PI3K alone increased levels of survivin mRNA when compared to the control cells. The chemical inhibitor of PI3K, LY294002 inhibited PI3K-inducing survivin expression, and overexpression of PTEN also decreased survivin mRNA level. These survivin mRNA expression levels are correlated with Akt activation, suggesting that Akt may be a downstream target. Forced expression of Akt also increased survivin mRNA levels, demonstrating that PI3K/PTEN regulates survivin expression through Akt activation. Activation of PI3K and Akt-induced p70S6K1 and increased survivin mRNA expres-

![Fig. 3. PI3K/Akt pathway upregulates survivin mRNA expression through p70S6K1.](image-url)
tion levels; while rapamycin abolished PI3K- and Akt-inducing survivin mRNA expression. These results may help to understand some effects of mTOR inhibitors that have been developed as a class of anticancer agents and used in clinical trials [28]. In addition, the expression of survivin was induced by overexpression of constitutively activated p70S6K1 (E389D3E), and inhibited by rapamycin treatment. This suggests that p70S6K1 regulates survivin mRNA expression through mTOR-dependent pathway.

Anti-apoptosis is another hallmark of cancer cells. In recent years, researchers have made considerable efforts to develop strategies for inducing apoptosis in cancer. Considering that apoptosis is the primary mode of cell death induced by several classes of anticancer agents and ionizing radiation, a possible role of survivin in determining the chemo- and radio-sensitivity profiles of tumor cells has been hypothesized [29,30,35,36]. We showed that expression of survivin siRNA in ovarian cancer cells induced much higher apoptosis when the cells were treated with LY294002 or taxol. These results are consistent with previous reports. Although PI3K regulates survivin expression, siRNA survivin still shows some synergistic effects with LY294002 for inducing apoptosis, suggesting that there may be other signaling molecules downstream of PI3K that are involved in survival.

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