The Proto-oncogene PKCi Regulates the Alternative Splicing of Bcl-x Pre-mRNA

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Abstract

Two splice variants derived from the Bcl-x gene via alternative 5' splice site selection (5'SS) are proapoptotic Bcl-x(s) and antiapoptotic Bcl-x(L). Previously, our laboratory showed that apoptotic signaling pathways regulated the alternative 5' SS selection via protein phosphatasease-1 and de novo ceramide. In this study, we examined the elusive prosurvival signaling pathways that regulate the 5'SS selection of Bcl-x pre-mRNA in cancer cells. Taking a broad-based approach by using a number of small-molecule inhibitors of various mitogenic/survival pathways, we found that only treatment of non–small cell lung cancer (NSCLC) cell lines with the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (50 μmol/L) or the pan-protein kinase C (PKC) inhibitor G66983 (25 μmol/L) decreased the Bcl-x(L)/(s) mRNA ratio. Pan-PKC inhibitors that did not target the atypical PKCs, PKCi and PKCζ, had no effect on the Bcl-x(L)/(s) mRNA ratio. Additional studies showed that downregulation of the proto-oncogene, PKCi, in contrast to PKCζ, also resulted in a decrease in the Bcl-x(L)/(s) mRNA ratio. Furthermore, downregulation of PKCi correlated with a dramatic decrease in the expression of SAP155, an RNA trans-acting factor that regulates the 5'SS selection of Bcl-x pre-mRNA. Inhibition of the PI3K or atypical PKC pathway induced a dramatic loss of SAP155 complex formation at ceramide-responsive RNA cis-element 1. Finally, forced expression of Bcl-x(L) "rescued" the loss of cell survival induced by PKCi siRNA. In summary, the PI3K/PKCζ regulates the alternative splicing of Bcl-x pre-mRNA with implications in the cell survival of NSCLC cells. Mol Cancer Res; 10(5); 660–9.

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Introduction

Numerous studies have shown that overexpression of Bcl-x(L) in cells confers resistance to many apoptotic stimuli and cooperates with oncogenic factors (e.g., c-Myc) in tumorigenesis (1–10). Furthermore, many cell types spontaneously resistant to chemotherapeutic agents also show increased levels of Bcl-x(L) (11–16). The regulation of Bcl-x(L) expression is a complex mechanism consisting of both transcriptional and posttranscriptional processes. In regard to posttranscriptional processing, the Bcl-x gene, via alternative 5' splice site (SS) selection within exon 2, produces either the proapoptotic Bcl-x(s) (upstream 5' SS selection) or the antiapoptotic Bcl-x(L) (downstream 5' SS selection).

Several studies have shown that the Bcl-x splice variant, Bcl-x(s), in contrast to Bcl-x(L), promotes apoptosis (9, 17–20), and the mechanism of alternative 5'SS selection of Bcl-x pre-mRNA has emerged as a potential target for anticancer therapeutics in non–small cell lung cancer (NSCLC). For example, Taylor and colleagues showed that Bcl-x alternative splicing was specifically modulated using an antisense oligonucleotide targeting an RNA sequence surrounding the Bcl-x(L) 5'SS (21). Hybridization of this oligonucleotide to Bcl-x pre-mRNA induced an increase in the expression of Bcl-x(s) mRNA with a concomitant decrease in the expression of Bcl-x(L) mRNA resulting in sensitization of the NSCLC cells to cisplatin and eventually inducing apoptosis after long-term exposure (>48 hours; ref. 21). These findings were also shown by Mercatante and colleagues who extended these findings to several different cancer types (22). Thus, regulation of the 5'SS selection within the Bcl-x exon 2 is a critical factor in determining whether an NSCLC cell is susceptible or resistant to apoptosis in response to chemotherapy (21–25).

To this end, previous studies by our laboratory defined the generation of de novo ceramide and the activation of protein phosphatasease-1 (PP1) as major components of an apoptotic signaling pathway regulating the 5'SS selection of Bcl-x exon 2 in response to chemotherapeutic agents (26, 27). Recent studies by Boon-Unger and colleagues (28) and Chang and colleagues (29) verified these early findings and extended the
list of chemotherapeutic agents to emetine, a potent protein synthesis inhibitor, and amiloride, a potassium-conserving diuretic. Mechanistic studies from our laboratory identified the ceramide-responsive RNA cis-element 1 (CRCE-1) located in exon 2 of Bcl-x pre-mRNA 277 to 295 bp upstream from intron 2 (30). In further mechanistic studies, the involvement of the splicing factor, SAP155, as an RNA trans-factor that interacted with CRCE-1 and regulated the 5'SS selection of Bcl-x pre-mRNA was elucidated (31). Importantly, this RNA trans-factor was required for the effect of ceramide on the alternative 5'SS selection of Bcl-x, and downregulation of SAP155 by siRNA technology was as effective as ceramide in reducing the Bcl-x(L)/(s) mRNA ratio as well as the viability of NSCLC cells (31).

This study sought to examine the hypothesis that Bcl-x alternative splicing was dysregulated in certain cancer cells and, thus linked to a specific oncogenic signaling pathway. In this regard, we show that the ratio of Bcl-x(L)/(s) mRNA is significantly increased in a large number of NSCLC tumors. On the basis of these findings, we examined the survival/mitogenic pathways responsible for regulating the alternative 5'SS selection of Bcl-x pre-mRNA in malignant NSCLC cells. Whereas a classical protein kinase C (PKC) pathway has been implicated in nontransformed cells by Revil and colleagues, the signaling pathways in transformed cells has remained elusive. Indeed, classical PKC inhibitors had no effect on Bcl-x RNA splicing in cancer cells in the Chabot study (32). In this study, we identify the phosphoinositide 3-kinase (PI3K) pathway as a key survival pathway regulating the alternative 5'SS selection of Bcl-x pre-mRNA to produce the antiapoptotic Bcl-x(L) isoform. Furthermore, we show that the atypical PKC (aPKC), PKCζ, is downstream of PI3K and regulates this alternative splicing mechanism and the expression of SAP155. Finally, the presented study showed that Bcl-x(L) plays an important role in the survival function of PKCζ in NSCLC cells. Overall, this report defines a survival/oncogenic pathway regulating the alternative splicing of Bcl-x pre-mRNA in NSCLC cells.

Materials and Methods

Cell culture

The NSCLC cell lines, A549, H292, H226, and H520 cells were grown in 50% RPMI-1640 (Invitrogen Life Technologies) and 50% Dulbecco’s Modified Eagle’s Medium (Invitrogen Life Technologies) supplemented with l-glutamine, 10% (v/v) FBS (Sigma), 100 units/mL penicillin G sodium (Invitrogen Life Technologies), and 100 μg/mL streptomycin sulfate (Invitrogen Life Technologies). A549 cells were purchased from American Type Culture Collection. Cells were maintained at less than 80% confluence under standard incubator conditions (humidified atmosphere, 95% air, 5% CO₂, 37°C).

Quantitative/competitive real-time PCR

Total RNA from cell lines was isolated using the RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer’s protocol. Total RNA (1 μg) was reverse-transcribed using Superscript III reverse transcriptase (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen) and PCR was carried out for Bcl-x splice variants as previously described (31, 33). The final PCR products were resolved on a 5% Tris-borate EDTA (TBE) acrylamide gel electrophoresis, stained with SYBR Gold (Invitrogen), and visualized using a Molecular Imager FX (Bio-Rad) with a 488 nm excision (530 nm BYPASS) laser.

Quantitative real-time PCR

Total RNA was reverse-transcribed using Superscript III reverse transcriptase (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen) and quantitative real-time PCR (qRT-PCR) was carried out with SAP155 and 18S-specific primers (Applied Biosystems) using the TaqMan PCR master mix in an Applied Biosystems 7500 Real-Time PCR System as previously described (34).

Western immunoblotting

Cells were lysed using CelLytic Lysis Buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein samples (5 μg) were subjected to 10% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad), and blocked in 5% milk/1% PBS-0.1% Tween (M-PBS-T) for 2 hours. Primary antibodies were anti-PKCζ (1:1,000, Santa Cruz), anti-PKCζ (1:1,000, Santa Cruz), anti-SAP155 (1:1,000), anti-hnRNP K (1:2,000, Santa Cruz), anti-hnRNP L (1:2,000, Santa Cruz), anti-hnRNP F/H (1:2,000, Santa Cruz), anti-Sam68 (1:2,000, BD Biosciences), anti-Bcl-x(L) (1:2,000, Cell Signaling), and anti-β-actin (1:5,000, Sigma-Aldrich). Secondary antibodies were horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit (1:5,000, Santa Cruz). Immunoblots were developed using Pierce enhanced chemiluminescence (ECL) reagents and Bio-Max film.

siRNA transfection

For inhibition of PKCζ, PKCζ, or hnRNP K expression, cell lines were transfected with PKCζ SMARTpool multiplex, PKCζ SMARTpool multiplex, SAP155 SMARTpool multiplex, hnRNP K SMARTpool multiplex, or scrambled control siRNA (Dharmacon) using DharmaFECT 1 transfection reagent (Dharmacon) as previously described (33, 35, 36). Briefly, cell lines were plated in 6-well tissue culture dishes and allowed to rest overnight. At 50% confluency, cells were transfected with siRNA (100 nmol/L) using DharmaFECT 1 in Opti-Mem I–reduced serum medium. Forty-eight hours posttransfection, RNA and/or protein were isolated.

Inhibitor studies

For Inhibitor studies, A549 cells (1.5 × 10⁵) were plated into 6-well tissue culture plates. The following day media were removed and replaced with the appropriate complete growth media. Cells were subsequently treated with sham control (1:1,000) or the appropriate concentration of active inhibitor (1:1,000, Calbiochem). Twenty-four hours post-treatment, total RNA and/or protein was isolated.
Clonogenic assays
For clonogenic assays, A549 cells were transfected with control siRNA (100 nmol/L) or PKCα siRNA (100 nmol/L). After 24 hours, cells were then infected with either control adenovirus or Bcl-x(L) adenovirus (10 multiplicity of infection; Applied Biological Materials Inc.). After an additional 24 hours, 125 viable cells were seeded into 6-well tissue culture dishes with complete growth media. After 12 days, colonies were counted following fixation with methanol and staining (0.1% crystal violet; colonies ≥ 40 cells).

Electromobility shift assays
The following sequence was used for the fluorescein isothiocyanate (FITC)-tagged CRCE-1 RNA oligonucleotide (5'FL-GAG GGA GGC AGG CGA CGA C-3'). RNA-binding reactions were conducted in a final volume of 25 μL containing: 600 ng CRCE-1 fluorescein-oligonucleotide, 40 μg nuclear protein extract, 40 U R Nasin, and 11.3 μg tRNAs in buffer composed of 10 mmol/L HEPES, 1 mmol/L dithiothreitol (DTT), 120 mmol/L KCl, 3 mmol/L MgCl2, and 5% glycerol as previously described by us (31). The reaction mixtures were incubated at 4°C for 25 minutes. Samples were loaded on a 5% TBE-PAGE (37.5:1 acrylamide/bis-acrylamide) for electrophoretic separation of RNA-protein(s) complexes. The gel was then scanned using Molecular Imager FX (Bio-Rad) with a 488 nm excision (530 nm BP) laser.

Pathologist-verified tumor samples
TissueScan Cancer and Normal Tissue cDNA Arrays used in this study were obtained from OriGene (Lung cancer and normal tissue samples, catalog no. HLRT102; Breast, Cervical and esophageal cancer and normal tissue samples, catalog no. CSRT103; see Supplementary Tables SI and SII).

Statistical analysis
When appropriate, the data are presented as the mean ± SE or SD as indicated in the specific figure. Data points were compared using a 2-tailed, independent samples t test, and P values calculated. P values less than 0.05 were considered significant.

Results
The alternative splicing of Bcl-x is dysregulated in NSCLC tumors
Previously, our laboratory showed that the alternative splicing of Bcl-x pre-mRNA was regulated by apoptotic stimuli (27, 37). In this study, we expanded our investigations to test the hypothesis that the alternative splicing of Bcl-x was dysregulated (in a prosurvival manner or increased Bcl-x(L)/(s) mRNA ratio) in cancer phenotypes, specifically NSCLC tumors. Using total RNA samples from pathologist-verified human NSCLC tumor samples (Supplementary Table SI), quantitative/competitive RT-PCR analysis was conducted to determine the degree of dysregulation in the Bcl-x(L)/(s) ratio as compared with normal lung tissue controls. Tumor samples were categorized into 3 groups respectively: normal, a Bcl-x(L)/(s) mRNA ratio of < 5.0; moderately dysregulated, a Bcl-x(L)/(s) mRNA ratio between 5.0 and 7.5; and highly dysregulated, a Bcl-x(L)/(s) mRNA ratio of > 7.5 (Fig. 1). These classifications about the degree of dysregulation were due to the observation that the normal lung tissue samples had a Bcl-x(L)/(s) ratio of 4.75 ± 0.15 (N = 4), which closely correlated with the report from Minn and colleagues showing that Bcl-x(s) overcame Bcl-x(L) expression at a Bcl-x(L)/(s) ratio of approximately 4.0 (9). Furthermore, Bauman and colleagues showed using RNA oligonucleotides to modulate the endogenous ratio of Bcl-x(L)/(s) mRNA that a ratio less than 5.0 for Bcl-x(L)/(s) mRNA >20% Bcl-x(s) led to a loss of cell viability for cancer cells (38). Furthermore, a ratio of about 5.0 was induced in the lung tumors of mice in the same study, which corresponded to a 75% loss in tumor mass (38). Hence, a Bcl-x(L)/(s) mRNA ratio > 5.0 indicates a prosurvival/dysregulated ratio compared with normal lung tissue. The classification for moderately dysregulated versus highly dysregulated was based on the findings of both Taylor and colleagues and Mercatante and colleagues. These studies showed that cancer cells with a ratio of Bcl-x(L)/(s) mRNA of >7.5 correlated with strong resistance to various chemotherapies whereas cancer cells with a ratios between 5.0 and 7.5 were partially resistant (e.g., PC-3 cells; refs. 21, 22, 24).

Using the aforementioned parameters, 78% (N = 41) of NSCLC tumors presented with a more than 25% increase in the Bcl-x(L)/(s) mRNA ratio as compared with normal lung tissue controls. Even more dramatic, 32% of NSCLC tumors examined presented with a more than 50% increase in the Bcl-x(L)/(s) mRNA ratio (Fig. 1). Thus, the ratio of Bcl-x(L)/(s) mRNA is dramatically increased in a high percentage of NSCLC tumors. Interestingly, a dysregulated Bcl-x(L)/(s) mRNA ratio correlated with tumor stage as 10 of 18 tumors classified as stage II or higher presented with a Bcl-x(L)/(s) mRNA ratio more than 7.5 (Fig. 1C). Therefore, these data indicate that a significant portion of NSCLC tumors present with a dysregulated ratio of Bcl-x(L)/(s) mRNA favoring a prosurvival/pro-oncogenic phenotype.

Next, our laboratory determined whether our findings in NSCLC tumors on the Bcl-x(L)/(s) mRNA ratio translated to other tumor types. Using total RNA extracted from pathologist-verified human breast, cervical, and esophageal tumors samples, quantitative/competitive RT-PCR analysis was again conducted to determine the degree of dysregulation in the Bcl-x(L)/(s) ratio as compared with normal tissue controls. Of the samples examined, 43% of tumors of the esophagus (N = 14) presented with a more than 25% increase in the Bcl-x(L)/(s) mRNA ratio as compared with normal esophageal tissue controls (Supplementary Table SII). However, only 17% of breast tumor samples (N = 23) and 20% of cervical tumor samples (N = 10) presented with a more than 25% increase in the Bcl-x(L)/(s) mRNA ratio as compared with normal tissue controls (data not shown). Therefore, dysregulation in the alternative splicing of Bcl-x pre-mRNA translates to multiple cancer types, albeit to a lesser extent in both breast and cervical tumors.
The PI3K pathway regulates the 5′SS selection of Bcl-x pre-mRNA

In this study, we hypothesized that a major survival/mitogenic pathway regulates the alternative processing of Bcl-x pre-mRNA to favor the production of anti-apoptotic Bcl-x(L), thereby increasing the Bcl-x(L)/(s) mRNA ratio. To investigate this hypothesis, these pathways were examined for regulation of the alternative splicing of Bcl-x pre-mRNA using small-molecule inhibitors well characterized in the scientific literature. Treatment of the NSCLC cell line, A549 cells, as previously described by us (39), with the following inhibitors; the mitogen-activated protein (MAP) kinase inhibitor, PD98059 (10 μmol/L); the MAP/ERK (MEK)1/2 inhibitor, U0126 (10 μmol/L); the Rho-kinase inhibitor, Y-27632 (10 μmol/L); and an Src kinase inhibitor (25 μmol/L), had no significant effect on the alternative splicing of Bcl-x pre-mRNA (Supplementary Table SIII). In contrast, treatment of A549 cells with the PI3K inhibitor, LY294002 (50 μmol/L), resulted in a significant reduction in the ratio of Bcl-x(L)/(s) splice variants compared with the inactive, structurally related compound, LY303511 (50 μmol/L; Fig. 2A). Specifically, the Bcl-x(L)/(s) mRNA ratio decreased from 6.00 ± 0.16 for LY303511 control-treated samples to 3.40 ± 0.19 for LY294002-treated samples (P < 0.01; N = 6). Therefore, these data show that PI3K regulates the alternative splicing of Bcl-x pre-mRNA in an anti-apoptotic/prosurvival manner.

To determine the translatability of the mechanism, H226 and H292 were also treated with LY294002 (Fig. 2B and C). As with the A549 cells, the Bcl-x(L)/(s) ratio was dramatically decreased (H226 cells from 5.87 ± 0.13 to 3.15 ± 0.12; H292 cells from 6.51 ± 0.12 to 3.50 ± 0.19). Importantly, the effect of PI3K inhibition of Bcl-x 5′SS selection translated to the protein level as a decrease in the protein levels of Bcl-x(L) was observed in all 3 cell lines after LY294002 treatment (Fig. 2D). To further confirm the involvement of PI3K in regulating the alternative splicing of Bcl-x, we used a cell-permeable Wortmannin 1β-hydroxy analog (HWT) that acts as an irreversible PI3K inhibitor. Treatment of A549 cells with HWT (10 μmol/L) also resulted in a significant reduction in the ratio of Bcl-x(L)/(s) splice variants as compared with dimethyl sulfoxide (DMSO) controls (Fig. 2E). Specifically, the Bcl-x(L)/(s) mRNA ratio decreased from 6.05 ± 0.18 for DMSO control–treated samples to 3.62 ± 0.05 for HWT-treated samples (P < 0.01; N = 6). Therefore, the PI3K pathway regulates the alternative splicing of Bcl-x, and this signaling cascade translates to multiple NSCLC cell lines.

Figure 1. The Bcl-x(L)/(s) mRNA ratio is dysregulated in NSCLC tumors. A, a representation of the RT-PCR analysis of matched normal and tumor samples used to determine the degree of Bcl-x(L)/(s) dysregulation. N, normal lung tissue; T, tumor tissue. B, quantitative/competitive PCR analysis of Bcl-x splice variants show that 46% of NSCLC tumors present a moderately dysregulated Bcl-x(L)/(s) mRNA ratio (Bcl-x(L)/(s) ratio of 5.0–7.5) and 32% of NSCLC tumors present a highly dysregulated Bcl-x(L)/(s) mRNA ratio (Bcl-x(L)/(s) ratio > 7.5; N = 41), as determined by densitometric analysis of PCR products. C, the lung NSCLC samples (N = 41) used above and SCLC samples (N = 3) were grouped according to tumor stage to depict the correlation between tumor stage and degree of Bcl-x(L)/(s) mRNA dysregulation.
PKCι regulates the activation of the Bcl-x(s) 5'SS in A549 cells

Akt/PKB, αPKCs (ζ and τ), mTOR/S6 kinase, and PKCd are downstream of PI3K. To investigate the downstream effect of PI3K responsible for regulating the alternative splicing of Bcl-x pre-mRNA, well-characterized small-molecule inhibitors were again used. Treatment of A549 cells with the small-molecule Akt1/2 inhibitor, AKT VIII (25 μmol/L); the classical PKC (cPKC) inhibitor, GF109203X (10 μmol/L); the cPKC/novel PKC (nPKC) inhibitor, Go6976 (10 μmol/L); or the mTOR/S6 kinase inhibitor, rapamycin (10 μmol/L), had no effect on the Bcl-x(L)/(s) mRNA ratio (Supplementary Table SIII). On the other hand, treatment of A549 cells with the pan-PKC (cPKCs, nPKCs, and aPKCs) inhibitor, Go6983 (10 μmol/L; Fig. 3A), significantly decreased the Bcl-x(L)/(s) mRNA ratio from 6.04 ± 0.18 for control samples to 3.16 ± 0.29 (P < 0.05; N = 6). The effects were specific to the 5'SS selection of Bcl-x pre-mRNA as the alternative splicing of caspase 9, another de novo ceramide target, was previously reported by us to be unaffected by Go6983 in A549 cells (39). Therefore, these data, via the process of elimination, suggest that the PI3K survival pathway regulates the alternative splicing of Bcl-x via an αPKC.

To confirm a role for an αPKC in this mechanism, siRNA to both human αPKC isoforms, PKCι and PKCζ, was used. In contrast to PKCζ, downregulation of PKCι, a known downstream target of the PI3K, induced the activation of the Bcl-x(s) 5'SS, decreasing the Bcl-x(L)/(s) ratio from 6.12 ± 0.12 for siControl-treated cells to 4.01 ± 0.11 for siPKCι-treated cells (Fig. 3B and C), congruent with the inhibition of αPKCs and PI3K by small-molecule inhibitors. Furthermore, co-treatment of A549 cells with both siPKCι and LY294002 could not further decrease the Bcl-x(L)/(s) ratio, showing a linear pathway with PKCι as the downstream effector of PI3K (Fig. 3D). Thus, the αPKC, PKCι, regulates the alternative splicing of Bcl-x pre-mRNA in a prosurvival fashion.

The PI3K/PKCι pathway regulates SAP155 expression

Our laboratory has previously reported that the RNA trans-factor, SAP155, regulates the 5'SS selection of Bcl-x pre-mRNA. Here, we again show that downregulation of SAP155 via siRNA decreased the Bcl-x(L)/(s) mRNA ratio from 6.10 ± 0.08 for siControl-treated cells to 3.21 ± 0.12 for siSAP155-treated cells (Fig. 4A). To determine whether the expression of SAP155 was a distal mechanism regulated by PI3K/PKCι, the LY294002 and Go6983 inhibitors, and siRNA to PKCι and PKCζ were again used to examine the expression of SAP155. Inhibition of PI3K, αPKCs and knockdown of PKCι by siRNA, but not knockdown of PKCζ by siRNA, induced a significant decrease in the levels of SAP155 (Fig. 4B), analogous to a level of expression shown to significantly lower the Bcl-x(L)/(s) ratio (31). The observed decrease in SAP155 protein levels induced by LY294002 treatment and PKCι downregulation was mimicked at the RNA level (Fig. 4C and D). Specifically, treatment of A549 cells with LY294002 reduced the levels...
of SAP155 mRNA by 47% in comparison with LY303511 control samples (Fig. 4C). Similarly, treatment of siPKCi reduced the levels of SAP155 mRNA by 25% in comparison with siControl samples (Fig. 4D). Interestingly, no changes in the expression of additional RNA trans-factors implicated in regulating the 5′SS selection of Bcl-x pre-mRNA (e.g., hnRNP K, Sam68, hnRNP L, or hnRNP F/H) were observed following downregulation of PKCi (Fig. 4E; refs. 40–42). These data provide evidence for PKCi regulation of the 5′SS selection of Bcl-x pre-mRNA via modulation of SAP155 levels.

We next investigated that the role of hnRNP K on the alternative splicing of Bcl-x in NSCLC cell lines as this RNA trans-factor has been implicated in regulating the 5′SS selection of Bcl-x pre-mRNA (e.g., hnRNP K, Sam68, hnRNP L, or hnRNP F/H) were observed following downregulation of PKCi (Fig. 4E; refs. 40–42). These data provide evidence for PKCi regulation of the 5′SS selection of Bcl-x pre-mRNA via modulation of SAP155 levels.

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The PI3K/aPKC pathway regulates association of SAP155 with CRCE-1

Our laboratory has previously identified CRCE-1 located 277 to 295 bp upstream of intron 2, within exon 2 of Bcl-x pre-mRNA (30). Furthermore, we have previously shown that SAP155-deficient nuclear extracts resulted in the loss of a specific protein complex to CRCE-1, showing that SAP155 specifically interacts with CRCE-1 (31). Indeed, Supplementary Fig. S2 shows that addition of a SAP155 antibody blocks the formation of the SAP155:CRCE-1 complex in A549 nuclear extracts revalidating our previous report (31). Importantly, A549 nuclear extracts from LY294002-treated cells also resulted in the loss of the SAP155:CRCE-1 complex (Fig. 5A) in contrast to the classical/novel PKC inhibitor, G66976 (data not shown). These data show that the PI3K/aPKC pathway regulates the formation of the SAP155:CRCE-1 complex analogous to the observed loss of SAP155 expression.

**PKCi requires Bcl-x(L) expression to sustain the survival of NSCLC cells**

Previous studies have shown that the activation of the Bcl-x(s) 5′SS at the expense of Bcl-x(L) sensitized cells to chemotherapy and ultimately led to apoptosis (21, 26). Furthermore, the expression of PKCi was previously shown to have an important function in the survival of NSCLC cells (43), and our results presented within this study link PKCi to regulating both Bcl-x 5′SS selection and Bcl-x(L) levels. Therefore, we hypothesized that Bcl-x(L) expression due to
the activation of the Bcl-x(L) 5′SS by PKCι activation/expression was a key mechanism for the survival of NSCLC cells. Indeed, we examined the ability of forced expression of Bcl-x(L) to "rescue" the effects of downregulation of PKCι on the survival of NSCLC cells. As shown by colony formation assays, knockdown of PKCι resulted in decreased clonogenic survival of A549 cells in agreement with previous reports (Fig. 6A and B; ref. 43). Importantly, forced expression of Bcl-x(L) was able to completely "rescue" this effect dramatically inhibiting the ability of siPKCι to suppress clonogenic survival (Fig. 6A and B). These data support the hypothesis that the activation of PKCι enhances cell survival of NSCLC cells via activation of the Bcl-x(L) 5′SS at the expense of the Bcl-x(s) 5′SS use.

Discussion

The presented study began from our first observation that the alternative splicing of Bcl-x pre-mRNA was dysregulated in a large percentage of NSCLC cells. This led to the hypothesis that a survival pathway of signal transduction regulated the alternative splicing of Bcl-x pre-mRNA. Indeed, we have previously shown that a contrasting pathway in apoptotic signaling existed for the activation of the Bcl-x(s) 5′SS. Specifically, our laboratory showed that the production of Bcl-x(s) via alternative splicing was dependent on the generation of de novo ceramide and the activation of PP1 (26, 27). Therefore, a survival pathway regulating this key distal mechanism and balancing the cell between apoptosis and survival was likely to exist as well. The data presented in this study show that the aPKC, PKCι, is a major regulator of the alternative splicing of Bcl-x pre-mRNA in A549 cells, acting downstream of the major survival/oncogenic enzyme, PI3K. Therefore, in contrast to published reports showing a classical PKC responsible for regulating the 5′SS selection of Bcl-x pre-mRNA in nonmalignant/nontransformed cells basally and in response to DNA-damaging agents (32), an oncogenic aPKC regulates the 5′SS selection in a prosurvival fashion in transformed phenotypes of lung cancer. Interestingly, the aPKC, PKCζ,
has been implicated in regulating the alternative splicing of caspase-2 (44). Although PKCζ has been implicated in regulating the alternative splicing of Bcl-x, factors, hnRNP K and the hnRNPs F/H, have also been implicated in regulating the alternative splicing of Bcl-xL in NSCLC cells. However, this downregulation, suggesting an alternative to the sensitivity of cancer cells to apoptotic stimuli (44).

Once the upstream components in the survival signaling pathway were determined, our laboratory focused on linking these upstream signals to downstream RNA trans-factors known to modulate the 5′SS selection of Bcl-x pre-mRNA. For this purpose, we turned to the literature as well as our own work for these RNA trans-factors. In a collaborative study with Paronetto and colleagues, SAM68, a well-established RNA trans-factor with roles in cell signaling and transformation, was shown to regulate the 5′SS selection in HEK293 cells. However, this finding did not completely translate to NSCLC cells, as siRNA directed against SAM68 only slightly inhibited the use of the Bcl-x(S) 5′SS (41). Other RNA trans-factors, hnRNPs F/H, and the hnRNP F/H, have also been implicated in regulating the alternative splicing of Bcl-x (40, 42). Specifically, Revil and colleagues showed that downregulation of hnRNK expression by RNA interference resulted in an increase in the use of the Bcl-x(S) 5′SS (40), whereas the contrasting effect was observed for downregulation of hnRNPs F/H. In this regard, downregulation of hnRNK had no effect on the alternative splicing of Bcl-x in A549 or H520 cells, suggesting the existence of cell-type-specific mechanisms for NSCLC cells. Furthermore, the expression of hnRNK and the hnRNPs F/H was not affected by PKCζ downregulation, suggesting an alternative pathway for regulation of the Bcl-x 5′SS selection in NSCLCs.

In regard to an alternative pathway for the PI3K/PKCζ signaling to modulate the 5′SS selection of Bcl-x pre-mRNA, we then examined the expression of the RNA trans-factor, SAP155. Our laboratory has previously reported this RNA trans-factor functioning as a repressor of the Bcl-x(s) 5′SS (31), which was later confirmed by Moore and colleagues using a high-throughput screening approach (45). Indeed, this study provides a link between the expression of SAP155 to the activation/expression of the NSCLC proto-oncogene, PKCζ. As to how PKCζ regulates the expression of SAP155 is currently a conundrum, but PKCζ has been implicated in regulating the transcription of genes activated by collagenase via a STAT3- or c-fos–dependent mechanism (46). Currently, there are no known regulatory sequences for STAT3 or c-fos in the SAP155 promoter region; hence, further studies are necessary to determine the signaling link between PKCζ and SAP155. Also of note, our studies do not “rule-out” the possibility of alternative promoter choice or RNA polymerase II elongation in regulating the alternative splicing of Bcl-x pre-mRNA, although our data suggest a more “classical” regulation of 5′SS selection by RNA trans-factors (47, 48). For example, inhibition of the PI3K/aPKC pathway reduces the levels of SAP155 with concomitant loss of
the protein:RNA complex formation of SAP155 at CRCE-1. The loss of this complex has been previously reported by our laboratory to induce the activation of the Bcl-x(s) 5’SS (31), which was confirmed again in this study. Hence, the regulation of the Bcl-x 5’SS selection by the complex of SAP155 with CRCE-1 is the more likely mechanism.

The physiologic significance of the Bcl-x(L)/(s) mRNA ratio has been documented by many reports in the literature showing that the fate of the cell can be determined by the proportion of antiapoptotic Bcl-x(L) to proapoptotic Bcl-x(s) (9, 21, 22). Furthermore, the induction of proapoptotic Bcl-x(s) has also been shown to sensitize cells to apoptosis and loss of viability to chemotherapeutic agents (21, 22). Published findings from our laboratory corroborate these findings showing that treatment of A549 cells with concentrations of ceramide known to activate the Bcl-x(s) 5’SS also lowered the IC50 of the chemotherapeutic agent, daunorubicin (27). Taken together, these data suggest a link between signal transduction pathways mediating the 5’SS selection of Bcl-x pre-mRNA and the sensitivity of cells to apoptosis in response to chemotherapeutics. Specifically, SAP155 expression may be a key link, as we have previously shown that ceramide could not induce apoptosis or sensitize cells to daunorubicin in A549 cells above the extent of SAP155 downregulation by siRNA. Unfortunately, little is known about the PKCi signaling pathway to form a strong hypothesis as to how ceramide may be blocking signals mediating SAP155 expression. The activation of the Bcl-x(s) 5’SS is regulated by the serine/threonine protein phosphatase, PP1. Therefore, it is conceivable that a ceramide-activated protein phosphatase such as PP1 may dephosphorylate PKCi effectively “shutting-down” the prosurvival pathway and thus, SAP155 expression. Indeed, studies have linked the inhibition of serine/threonine phosphatases to activation of PKCi (49). In addition, Wang and colleagues have recently shown that overexpression of PKCi resulted in enhanced survival of PC12 cells treated with ceramide (50).

Outside of the realm of chemotherapy sensitivity, the alternative splicing of Bcl-x pre-mRNA may also have roles in oncogenesis. Recently, Finch and colleagues showed that Bcl-x(L) cooperated with c-Myc in oncogenic transformation in vivo (6). These findings correlate well with the observation that a large number of NSCLC tumors showed dysregulation of the alternative 5’SS selection of Bcl-x exon 2 to favor Bcl-x(L) expression. Furthermore, PKCi is a well-established oncogene for NSCLC as reported by Regala and colleagues (51). Therefore, PKCi may in one aspect act as an oncogene via simple removal of Bcl-x(s) with concomitant increase in Bcl-x(L) and promoting oncogenesis.

In conclusion, this study shows that the PI3K/PKC pathway regulates the alternative 5’SS selection of Bcl-x exon 2 in NSCLC cells. Second, this study provides data that the proto-oncogene PKCi regulates this distal mechanism via the expression of SAP155, an RNA trans-factor that regulates the 5’SS selection of Bcl-x pre-mRNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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