t-Darpp Promotes Cancer Cell Survival by Up-regulation of Bcl2 through Akt-Dependent Mechanism

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Abstract
t-Darpp is a cancer-related truncated isoform of Darpp-32 (dopamine and cyclic-AMP–regulated phosphoprotein of Mr 32,000). We detected overexpression of t-Darpp mRNA in two thirds of gastric cancers compared with normal samples (P = 0.004). Using 20 μmol/L ceramide treatment as a model for induction of apoptosis in AGS cancer cells, we found that expression of t-Darpp led to an increase in Bcl2 protein levels and blocked the activation of caspase-3 and caspase-9. The MitoCapture mitochondrial apoptosis and cytochrome c release assays indicated that t-Darpp expression enforces the mitochondrial transmembrane potential and protects against ceramide-induced apoptosis. Interestingly, the expression of t-Darpp in AGS cells led to ≥2-fold increase in Akt kinase activity with an increase in protein levels of p-Ser473 Akt and p-Ser9 GSK3β. These findings were further confirmed using tetracycline-inducible AGS cells stably expressing t-Darpp. We also showed transcriptional up-regulation of Bcl2 using the luciferase assay with Bcl2 reporter containing P1 full promoter, quantitative reverse transcription-PCR, and t-Darpp small interfering RNA. The Bcl2 promoter contains binding sites for cyclic AMP–responsive element binding protein CREB/ATF1 transcription factors and using the electrophoretic mobility shift assay with a CREB response element, we detected a stronger binding in t-Darpp–expressing cells. The t-Darpp expression led to an increase in expression and phosphorylation of CREB and ATF-1 transcription factors that were required for up-regulating Bcl2 levels. Indeed, knockdown of Akt, CREB, or ATF1 in t-Darpp–expressing cells reduced Bcl2 protein levels. In conclusion, the t-Darpp/Akt axis underscores a novel oncogenic potential of t-Darpp in gastric carcinogenesis and resistance to drug-induced apoptosis. [Cancer Res 2008;68(2):395–403]

Introduction

Gastric carcinoma is the second most common cause of cancer-related mortality worldwide, leading to more than 1 million deaths per year (1–3). Although a decline in distal gastric cancer has been observed over the past few decades in the Western world, a sharp increase in the incidence of proximal gastroesophageal junctional and esophageal adenocarcinomas was recently noted in the United States, as well as many other Western populations (4–6). Improvement in our currently limited diagnostic, preventive, and therapeutic approaches to gastric cancer is a pressing need to develop novel therapeutic alternatives and overcome the drug-resistant phenotype of the majority of these tumors. However, the critical molecular alterations and signaling pathways that drive gastric tumorigenesis remain largely uncharacterized.

A comprehensive molecular analysis of the 17q12 amplicon in gastric cancer has led to identification of dopamine and cyclic AMP (cAMP)–regulated phosphoprotein of Mr 32,000 (Darpp-32) as the critical target gene in the region (7, 8). We have identified a truncated isoform of Darpp-32, t-Darpp, as a novel gene that is expressed in cancer (8). t-Darpp is overexpressed in several common adenocarcinomas, such as those of the colon, esophagus, breast, and prostate (9–14). We have reported that overexpression of t-Darpp provides antiapoptotic potential to cancer cells in a p53-independent mechanism (15). However, the biological role and molecular signaling of t-Darpp in carcinogenesis remain largely unexplored.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway regulates fundamental cellular processes linked to tumorigenesis, including cell cycle progression; cell survival, adhesion, motility, and spreading; angiogenesis; glucose homeostasis; and cell and organ size control (16, 17). Recent reports have indicated that activation of Akt pathway is implicated in conferring resistance to conventional chemotherapy and multiple chemotherapeutic agents (5-fluorouracil, Adriamycin, mitomycin C, and cisplatinum) on cancer cells (18). Akt is hyperactivated in a wide range of human tumors as a result of constitutive activation of growth receptors, mutation of PI3K, and inactivation or loss of PTEN phosphatase (19). The majority of tumors with hyperactive Akt signaling have as few as 3 to 20% identifiable PI3K mutations (20–23). In gastric carcinomas, PI3K mutations are rare and tend to occur as isolated events in microsatellite instability tumors (24–26). Although Akt activation plays an important role in cancer cell survival, there is limited information regarding the regulation of this signaling cascade in gastric cancer.

The pro-survival Bcl2 family members, such as Bcl-xL and Bcl2, are overexpressed in several malignancies and promote tumor progression and resistance to therapy (27–30). The Bcl2 family proteins are pivotal regulators of apoptotic cell death that are considered as attractive targets for drug design (31). Bcl2 is mainly a mitochondrial membrane protein, and its overexpression inhibits cytochrome c release and activation of caspase-9. It also prevents cells from undergoing apoptosis in response to several stress stimuli (27, 32, 33). In a mouse model lymphoblastic leukemia, suppression of Bcl2 expression resulted in rapid loss of leukemic cells and significantly prolonged survival, indicating that Bcl2 may be a potential target for cancer therapy (34). In this study, we have investigated the role of t-Darpp in regulating Akt and bcl2 in gastric cancer cells.

Materials and Methods

Quantitative real-time reverse transcription-PCR. For quantitative real-time reverse transcription-PCR (qRT-PCR), mRNA was isolated from
14 normal gastric mucosa, 22 primary gastric cancer samples, and AGS cells stably expressing empty vector or t-Darpp, using the RNaseq kit (Qiagen, Gmbh). All human tissues were frozen samples and were dissected for optimal tissue content to obtain ≥70% cellular purity. The tissue samples were collected in accordance with institutional review board–approved protocols. Single-stranded cDNA was synthesized using the advantage enzyme and 20 μg/mL of puromycin (Sigma) for 24 h. GFP fluorescence was monitored using fluorescence microscopy. An optimal clone was selected to generate a tet-inducible AGS stable cell line for t-Darpp. The tet-responsive expression plasmid for t-Darpp was generated by PCR amplification of the full-length coding sequence of t-Darpp and cloning into BamHI and NotI sites of pTREpur2 vector (BD Biosciences Clontech). The rtTA-expressing (Tet-On) cells were transfected with pTRE-t-Darpp plasmid and selected in 0.8 μg/mL of puromycin (Sigma) for 2 weeks. Puromycin-resistant cell colonies were isolated with cloning rings, and stable clones with optimal t-Darpp expression after induction with 1 μg/mL of doxycycline (Sigma) were selected.

Mitochondrial apoptosis assay. Apoptosis was assessed using the MitoCapture mitochondrial apoptosis detection kit (Biovision) as per the manufacturer’s instructions. This assay is based on the disruption of the mitochondrial inner-membrane electrochemical potential using a fluorescent cationic dye. Briefly, AGS cultures (3 × 10⁴ cells per plate) expressing t-Darpp or empty vector were seeded onto 60-mm culture plates. Apoptosis was induced by treating cells with 20 μmol/L of acetyl ceramide or DMSO (vehicle) for 2 h. Vehicle-treated and drug-treated cells were stained with MitoCapture reagent and then analyzed immediately by flow cytometry. In healthy cells, MitoCapture dye accumulated in the mitochondria as aggregates (punctuate red fluorescence), and was detected in the FL2 channel. In apoptotic cells, the dye was dispersed throughout the cell as monomers (diffused green fluorescence) and detected in the FL1 channel. The percentage of apoptosis was determined in cells expressing t-Darpp versus nonexpressing cells with and without drug treatment.

Detection of cytochrome c release. AGS cells stably expressing t-Darpp or empty vector were cultured into eight-well slides (12 × 10⁵ per well). These cells were then treated with vehicle or 20 μmol/L of acetyl ceramide for 3 h at 37°C. After cell fixation with 4% paraformaldehyde for 1 h, cytochrome c was detected by immunofluorescence with mouse monoclonal anti-cytochrome c antibody (1:200 dilution, PharMingen) followed by secondary antimouse antibody conjugated to TRITC (1:100 dilution, Zymed Laboratories). In healthy cells, the mitochondrial cytochrome c staining (red fluorescence) was punctuate, whereas in apoptotic cells, it was bright and diffused, indicating release of cytochrome c from mitochondria into the cytoplasm. The ratio of cells that presented diffuse cytochrome c staining (indicating onset of apoptosis) to the total number of cells was averaged.

Figure 1. mRNA expression of t-Darpp and Bcl2 in primary gastric adenocarcinomas. The mRNA expression levels were quantified in 22 tumor samples compared with 14 normal gastric mucosa samples. The iCycler (Bio-Rad) was used for qRT-PCR and the expression of t-Darpp was compared with that of HPRRT1 gene, which had minimal variation in all normal and neoplastic gastric samples tested. X axis, samples; Y axis, fold overexpression.
The bottom right quadrant. A summary of the finding showing the percent of MitoCapturer fluorescence aggregates, was obtained by flow cytometry. The dot plot of the axis (FL1), which is the log of MitoCapture green fluorescent monomers, and the axis (FL2), which depicts MitoCapture red fluorescent aggregates, was obtained by flow cytometry. The apoptotic cells (typically FL1-positive and FL2-negative) are indicated in the bottom right quadrant. A summary of the finding showing the percent of apoptotic cells is shown in D and indicates that t-Darpp expression protects against ceramide-induced apoptosis (P = 0.001).

Figure 2. t-Darpp inhibits apoptosis induced by ceramide. AGS cells stably expressing empty vector or t-Darpp were treated with 20 μmol/L ceramide or DMSO for 2 h, and then stained with MitoCapture dye reagent (MitoCapture Apoptosis Detection Kit from Biovision). Cells were analyzed for apoptosis by flow cytometry (Becton Dickinson). In healthy cells, the MitoCapture dye formed red fluorescent aggregates that were detected in the FL2 channel. In apoptotic cells, the dye was dispersed in the cell as green fluorescent monomers, detected in the FL1 channel. The dot plot of the X axis (FL1), which is the log of MitoCapture green fluorescent monomers, and the Y axis (FL2), which depicts MitoCapture red fluorescent aggregates, was obtained by flow cytometry. The apoptotic cells (typically FL1-positive and FL2-negative) are indicated in the bottom right quadrant. A summary of the finding showing the percent of apoptotic cells is shown in D and indicates that t-Darpp expression protected against ceramide-induced apoptosis (P = 0.001).

from six different microscopic fields for more than 100 cells in each category.

Luciferase assays. A pcDNA3-based mammalian expression plasmid was generated for t-Darpp as described above. Luciferase activity using the Bcl2 luciferase reporter construct (LB 124; ref. 36), which comprises the full-length Bcl-2 P1 promoter (--3924 to --1286; a generous gift from Linda M. Boxer, Stanford University School of Medicine, Stanford, CA), was measured in AGS cells. The cells were cultured into 24-well plates and transiently cotransfected with (a) LB 124 and pcDNA3 vector; (b) LB 124 and t-Darpp; (c) LB 124, pcDNA3, and t-Darpp; or (d) LB 124, t-Darpp, and dominant-negative AKT (Akt1 T308A and S473A, Addgene plasmid 9030) by Fugene (Roche Diagnostics). The relative luciferase activity was determined at 72 h posttransfection after normalization for Renilla activity according to the supplier's guidelines (Promega).

Western blot analysis. Cultured cells were harvested by scraping in ice-cold PBS and spun down at 3,500 rpm in a bench top centrifuge for 10 min. Cell pellets were then lysed in radioimmunoprecipitation assay buffer containing 1% of Halt protease inhibitor cocktail (Pierce Biotechnology). Protein concentration was determined by standard Bradford assay, and total cell lysates were separated by SDS-PAGE. Gel loading was normalized for equal β-actin (10 μg protein per lane). Proteins were then transferred onto Hybond-P polyvinylidene difluoride membranes (Amer sham Biosciences), and the membranes were probed with each antibody. Mouse monoclonal anti–β-actin (C-2) and rabbit polyclonal anti–β-actin (H-62, Santa Cruz Biotechnology) recognized t-Darpp at --3924 to --1286; a generous gift from Linda M. Boxer, Stanford University School of Medicine, Stanford, CA), was measured in AGS cells. The cells were cultured into 24-well plates and transiently cotransfected with (a) LB 124 and pcDNA3 vector; (b) LB 124 and t-Darpp; (c) LB 124, pcDNA3, and t-Darpp; or (d) LB 124, t-Darpp, and dominant-negative AKT (Akt1 T308A and S473A, Addgene plasmid 9030) by Fugene (Roche Diagnostics). The relative luciferase activity was determined at 72 h posttransfection after normalization for Renilla activity according to the supplier's guidelines (Promega).

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Gene expression knockdown by small-interfering RNA. AGS cells stably expressing empty vector or t-Darpp were transfected with Akt small interfering RNA (siRNA; Cell Signaling Technology), control siRNA, t-Darpp siRNA, ATF-1 siRNA, or CREB-1 siRNA oligonucleotides using siRNA transfection reagent and transfection medium, following the manufacturer's instructions (Santa Cruz Biotechnology). For quantitative real-time RT-PCR of Bcl2, mRNA was isolated from AGS cells expressing t-Darpp and transfected with t-Darpp siRNA oligonucleotide (Santa Cruz Biotechnology).

Results

Frequent overexpression of t-Darpp and Bcl2 mRNA in gastric cancer. qRT-PCR showed overexpression of t-Darpp in 64% of primary gastric cancer samples compared with 14 normal gastric mucosa samples (P = 0.004). The expression of t-Darpp was almost undetectable in normal tissue samples (Fig. 1). Similarly, Bcl2 was overexpressed in 55% of tumors compared with normal samples (P = 0.01). There was a trend, not reaching statistical significance, between overexpression of t-Darpp and Bcl2 (P = 0.5), which is attributed to the relatively small sample size and the complex transcriptional regulation of Bcl2 in human tumors (Fig. 1).

Expression of t-Darpp protects mitochondrial transmembrane potential. To investigate the antipoptotic activity of t-Darpp, we used a drug-induced apoptosis model using acetyl ceramide to promote the disruption of the mitochondrial
transmembrane potential, as an early apoptotic event in AGS cells. In apoptotic cells, the mitochondrial membrane potential was dissipated; thus, the MitoCapture dye was dispersed in the cell as green fluorescent monomers detected in the FL1 channel. Cells expressing t-Darpp were considerably more resistant to apoptosis and showed a 7-fold reduction in apoptosis compared with control cells following treatment with 20 μmol/L ceramide ($P = 0.01$). Representative dot plots and the percentages of apoptotic cells based on FL1-positive (green fluorescence) and FL2-negative signals are depicted in Fig. 2.

Western blot analysis on protein extracts indicated that treatment of t-Darpp cells and control cells with 20 μmol/L ceramide for 24 h led to cleavage of procaspase-3, generating an active caspase-3 fragment (P17), and almost complete processing of procaspase-9 (Fig. 3A, lane 3), whereas t-Darpp–expressing cells showed no signs of activation of caspase-3 and caspase-9 (Fig. 3A, lane 6). A lower dose of 10 μmol/L ceramide for 24 h was not sufficient to induce apoptosis, confirming that 20 μmol/L was most appropriate for

Figure 3. t-Darpp blocks cytochrome c release and induces Akt kinase activity. AGS cells stably expressing t-Darpp or empty vector were treated with acetyl ceramide or DMSO (vehicle). A, protein extracts from cells treated for 24 h were subjected to Western blot analysis with anti–caspase-3 antibody, which detects the pro-form of caspase-3 protein (p35) and its active form (p17), and anti–caspase-9 antibody that recognizes only the pro-form of caspase-9. Activation of caspase-3 and caspase-9, as shown by cleavage of procaspase-3 and almost complete processing of procaspase-9, was detected in control cells treated with 20 μmol/L ceramide (lane 3). Under the same conditions, activation of caspase-3 and caspase-9 was blocked in t-Darpp–expressing cells (lane 6). Treatment with 10 μmol/L ceramide was not sufficient to activate caspase-3 in all cells (lanes 2 and 5) and is shown to confirm that the use of 20 μmol/L was optimal for the cytochrome c release experiment. The protein levels of both Bcl2 and t-Darpp were higher in ceramide or vehicle-treated AGS cells expressing t-Darpp (lanes 4–6) than control cells (lanes 1–3). B, cells treated with 20 μmol/L ceramide for 3 h were fixed with 4% paraformaldehyde. Cytochrome c was detected by anti–cytochrome c antibody and a secondary anti-mouse antibody conjugated to TRITC as depicted by red fluorescence. In healthy cells, cytochrome c staining was punctuated as shown for vehicle-treated cells and ceramide-treated t-Darpp–expressing cells. In apoptotic cells, cytochrome c release was indicated by diffused staining, which was also found for ceramide-treated control cells expressing empty vector. 4,6-Diamidino-2-phenylindole (blue fluorescence) was used as a nuclear counterstain. Cytochrome c results for 10 μmol/L ceramide are not shown as no effect was observed. C, the ratio of cells that presented diffused cytochrome c staining (indicating onset of apoptosis) to the total number of cells was averaged from six different microscopic fields for more than 100 cells in each category. Following treatment with 20 μmol/L ceramide, only 14.8% of t-Darpp–expressing cells had diffused cytochrome c staining as opposed to 82.7% of control cells ($P = 0.001$). D, protein extracts (15 μg) from AGS cells expressing t-Darpp or empty vector, and purified active Akt (6 ng), as a control, were subjected to an in vitro Akt kinase activity assay analysis. The relative Akt kinase activity in t-Darpp–expressing cells was more than 2-fold compared with control cells ($P = 0.01$).
t-Darpp Activates Akt Pathway and Up-regulates Bcl2

Figure 4. Phosphorylation of Akt is induced by t-Darpp. A, protein extracts from t-Darpp–expressing AGS cells or control cells were analyzed by immunoblotting for p-Ser473 Akt, Akt, p-Ser9 GSK3β, Bcl2, and t-Darpp. Gel loading was normalized for equal β-actin. Phosphorylation of Akt was higher in t-Darpp–expressing cells than empty vector control cells (Vect). This directly correlated with GSK3β phosphorylation and with both Bcl2 and t-Darpp protein levels. B, protein extracts from AGS cells expressing t-Darpp or empty vector and transfected with t-Darpp siRNA or control siRNA were subjected to Western blot analysis of p-Ser473 Akt, Akt, and t-Darpp. Knockdown of t-Darpp in cells expressing t-Darpp (lane 4) decreased Akt phosphorylation to a comparable level as control cells (lane 3). C, tetracycline-inducible AGS cells expressing t-Darpp were treated with doxycycline for 24 h to induce expression and cell lysates, at various time points after doxycycline removal, were analyzed as indicated in the figure. Protein extracts were analyzed by immunoblotting for p-Ser473 Akt, Akt, Bcl2, and t-Darpp. Gel loading was normalized for equal β-actin. As expected, t-Darpp protein level increased after 24 h induction of cells with doxycycline (lane 2) and remained high for 8 h after removal of doxycycline (lanes 3–5) and substantially decreased after 24 h as shown in lanes 6 to 9. Akt phosphorylation and Bcl2 protein levels were induced after doxycycline treatment and returned to base level at 96 h after its removal (lane 9). D, MKN45 and SNU1 gastric cancer cells were transfected with either control siRNA or t-Darpp siRNA to knockdown endogenous t-Darpp. Cell lysates were collected at 48 h for Western blot analysis. As shown, knockdown of t-Darpp led to a reduction in t-Darpp protein level with a notable decrease in pAKT and Bcl2 protein levels confirming the aforementioned results.

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our assays. Based on these results, we used 20 μmol/L ceramide for immunofluorescence analysis of cytochrome c (Fig. 3B). Immunofluorescence analysis, after treatment with 20 μmol/L ceramide for 3 h, showed that cytochrome c was released from mitochondria, indicating onset of apoptosis, as depicted by a diffuse red fluorescence in control cells expressing empty vector (82.7%; Fig. 3B and C). Conversely, the cytochrome c release was considerably blocked in cells expressing t-Darpp (14.8%; Fig. 3B and C). In healthy nonapoptotic cells, cytochrome c was blocked as shown by mitochondrial punctuate staining (Fig. 3B). Taken together, these results indicate that t-Darpp acts on the Bcl2 and the intrinsic apoptotic pathway mediated by the mitochondrion. Indeed, we noted that t-Darpp–expressing cells (Fig. 3A, lanes 4–6) consistently expressed Bcl2, whereas empty vector control cells (Fig. 3A, lanes 1–2) had no obvious expression of Bcl2 at the same exposure.

t-Darpp regulates Akt phosphorylation and kinase activity.

As Akt signaling pathway is one of the major cell survival pathways, we investigated the effects of t-Darpp on Akt phosphorylation and kinase activity. The in vitro Akt kinase assay results showed more than a 2-fold increase of Akt activity in AGS cells expressing t-Darpp compared with control cells (Fig. 3D). In addition, Western blot analysis indicated that phosphorylation of Akt at serine 473 was higher in AGS cells expressing t-Darpp than control cells (Fig. 4A). Similarly, phosphorylation of the Akt downstream target GSK3β was considerably higher in AGS cells expressing t-Darpp than control cells (Fig. 4A). Taken together, these data indicate that t-Darpp can regulate Akt phosphorylation and kinase activity in cancer cells. Using t-Darpp siRNA to knock down t-Darpp in AGS cells stably expressing t-Darpp led to a notable decrease in Akt phosphorylation (Fig. 4B, lane 4) compared with control siRNA (Fig. 4B, lane 2), confirming that increased level of p-Ser473 Akt was dependent on t-Darpp. Furthermore, using a tetracycline-inducible AGS stable cells expressing t-Darpp model, we showed t-Darpp protein level predetermine the levels of phosphorylation of Akt (Fig. 4C). Withdrawal of doxycycline to block t-Darpp expression led to a time-dependent decrease in t-Darpp and phospho-Akt protein levels as shown at time points (Fig. 4C, lanes 6–9). In addition, there was a remarkable induction in Bcl2 level following induction of t-Darpp and the Bcl2 protein levels returned to base levels 96 h postwithdrawal of doxycycline, suggesting that Bcl2 expression was regulated by t-Darpp. The knockdown of endogenous t-Darpp in MKN45 and SNU1 cells led to a notable reduction in pAkt and Bcl2 levels (Fig. 4D). Taken together, these data show that both exogenous and endogenous t-Darpp regulate the Bcl2 and pAkt protein levels.

t-Darpp expression leads to transcriptional up-regulation of Bcl2. Our results, as described above, indicated that t-Darpp mediated up-regulation of Bcl2 protein levels in AGS cells. Consistent with these findings, overexpression of t-Darpp in AGS
cells led to a considerable increase of Bcl2 protein level (Fig. 5A, lane 2), whereas a knockdown of t-Darpp resulted in a notable decrease of Bcl2 protein level (Fig. 5A, lane 3). To confirm that t-Darpp transcriptionally up-regulated Bcl2 expression, we performed real-time RT-PCR and luciferase assays in AGS cells. RT-PCR analysis indicated 5-fold increase of Bcl2 mRNA level in AGS cells expressing t-Darpp compared with control cells (Fig. 5B). Knockdown of t-Darpp expression with t-Darpp siRNA reduced considerably Bcl2 mRNA level in t-Darpp–expressing cells (Fig. 5B), indicating that Bcl2 expression was dependent on t-Darpp. Moreover, the relative luciferase activity of Bcl2 promoter was ~2.5-fold higher in t-Darpp–expressing cells than in control cells (Fig. 5C). Cotransfection with dominant-negative Akt and t-Darpp plasmids resulted in a considerable reduction of luciferase activity (Fig. 5C), indicating that Bcl2 expression was dependent on Akt.

**t-Darpp increases CREB/ATF-1-CRE binding activity.** The Bcl2 promoter contains binding sites for several transcription factors that include multiple binding sites for CREB. We investigated whether t-Darpp could modulate CREB/ATF-1 DNA-binding activity. The EMSA assay showed a stronger CREB/ATF-1-CRE binding in AGS cells expressing t-Darpp than in control cells (Fig. 6A, lanes 2 and 5). Supershift experiments using ATF-1 antibody, which also detects CREB, confirmed DNA-binding specificity as indicated by an upper shift of CREB/ATF-1-CRE complex signal (Fig. 6A, lanes 4 and 7). This complex signal was negated by the addition of unlabeled CREB probe in excess (Fig. 6A, lanes 3 and 6), thereby confirming specificity of the probe.

**Up-regulation of Bcl2 by t-Darpp requires Akt and CREB/ATF-1 factors.** The results as shown in Fig. 6A suggested that t-Darpp enhances binding of CREB/ATF1 to Bcl2 promoter. We therefore investigated the effects of t-Darpp on CREB/ATF1 and if this mechanism requires Akt. The results revealed an increase in total and phosphoprotein levels of CREB and ATF-1 in t-Darpp–expressing cells compared with empty vector control cells (Fig. 6B). This finding further confirmed that Bcl2 up-regulation by t-Darpp might be mediated through CREB/ATF-1 proteins. A further validation was obtained by using ATF1 siRNA and CREB siRNA. The results indicated that siRNA silencing of either ATF-1 (Fig. 6C, lane 2) or CREB (Fig. 6C, lane 3) expression led to down-regulation of Bcl2. Control siRNA oligonucleotide did not alter expression, thereby confirming siRNA specificity for both ATF-1 and CREB. These results, together with the aforementioned findings, led to the conclusion that t-Darpp–mediated Bcl2 regulation requires CREB/ATF-1 factors. As the Bcl2 luciferase results suggested that Bcl2 expression was dependent on Akt in AGS cells (Fig. 5E), we investigated whether knockdown of Akt could affect expression of CREB/ATF-1 transcription factors and Bcl2. The results showed that siRNA silencing of Akt in AGS cells expressing t-Darpp resulted in a dramatic decrease of Bcl2 protein and total and phosphoprotein levels of CREB/ATF-1 (Fig. 6D). Taken together, our results confirm that t-Darpp mediates the increase in Akt phosphorylation and kinase activity and this effect was required for the CREB/ATF1-mediated up-regulation of Bcl2.

**Discussion**

Using in vitro approaches, we showed that t-Darpp expression provides potent antiapoptotic function to cancer cells through up-regulation of Bcl2. One of our most important findings is the realization of the ability of t-Darpp to activate the Akt pathway, underscoring novel oncogenic functions of t-Darpp in cancer. t-Darpp was cloned as a novel truncated isoform of Darpp-32 that
maps to 17q12, a region that is frequently amplified in gastric cancer. Although Darrpp-32 is known as a major regulator of dopamine signaling in the brain (37, 38), there is no information about t-Darpp. We have found that t-Darpp is overexpressed in more than two thirds of gastric cancers compared with normal gastric mucosae \((P = 0.004)\). The fact that t-Darpp was identified as the critical target in the most commonly amplified region in gastric cancer (7, 8) has prompted us to investigate the potential oncogenicity of this protein. Our earlier results showed that t-Darpp expression provides antiapoptotic properties to gastric cancer cells without inhibiting the p53 transcription activity (15).

Disruption of mitochondrial transmembrane potential is an early apoptotic event that can be induced by drugs or stress. In this report, we have used ceramide as a model for induction of stress and apoptosis in cancer cells. Ceramide is known to induce apoptosis through generation of reactive-oxygen species and by affecting the mitochondrial membrane potential (reviewed by Mathias et al. in ref. 39). Ceramide analogues were reported to induce cytochrome \(c\) release from mitochondria (40), which is a critical step in the apoptotic pathway that involves activation of caspase-9. The expression of t-Darpp in AGS cells blocked the activation of caspase-3 and caspase-9 following ceramide treatment.

**Figure 6.** Involvement of CREB/ATF-1 factors and Akt in t-Darpp–mediated Bcl2 regulation. A, CREB/ATF-1 binding activity is enhanced by t-Darpp. Nuclear extracts (5 \(\mu\)g) from AGS cells expressing t-Darpp or empty vector were subjected to EMSA using a biotin-labeled probe containing a CRE (Panomics). DNA–protein binding reactions and detection were performed using LightShift Chemiluminescent EMSA kit (Pierce Biotechnology). CREB/ATF-1 DNA-binding activity was stronger in t-Darpp–expressing cells (lane 5) than in control cells (lane 2). Supershift experiments were done using ATF-1 antibody, which detects both CREB and ATF-1 proteins (lanes 4 and 7). B, protein extracts from AGS cells stably expressing t-Darpp or empty vector were analyzed by Western blot of t-Darpp, phospho-CREB (S133), phospho–ATF-1, CREB, ATF-1, and Bcl2. Protein levels of CREB, ATF-1, and Bcl2 were higher in t-Darpp–expressing cells compared with control cells. Similarly, phosphoproteins of both CREB and ATF-1 were also higher in t-Darpp–expressing cells than in control cells. C, protein extracts from AGS cells stably expressing t-Darpp and transfected with ATF-1 siRNA, CREB siRNA, or control siRNA oligonucleotides were subjected to Western blot analysis of CREB, ATF-1, and Bcl2. Anti-pCREB (serine 133) antibody, which also detected pATF-1, and anti–ATF-1 antibody, which detected both ATF-1 and CREB proteins, were used. Knockdown of ATF-1 (lane 2) and CREB (lane 3) resulted in a decrease of Bcl2 protein levels. D, protein extracts from AGS cells expressing t-Darpp and transfected with Akt siRNA or control siRNA were subjected to immunoblotting of Akt, phospho-CREB(S133), phospho–ATF-1, CREB, ATF-1, and Bcl2. Knockdown of Akt led to a significant decrease of phospho-CREB, CREB, phospho–ATF-1, ATF-1, and Bcl2. Gel loading was normalized for equal \(\beta\)-actin (10 \(\mu\)g protein per lane on 12% SDS-PAGE).
expressing t-Darpp and tetracycline-inducible AGS stable cells. In addition, using two independent cell models, AGS cells stably expressing t-Darpp. The luciferase assay using the full P1 Bcl2 promoter and qRT-PCR data indicated that t-Darpp regulates Bcl2 at the transcriptional level. The Bcl2 promoter consists of P1 (−3924 to −1286) and P2 (−1278 to −8) regulatory regions. The P1 promoter contains CRE, where both CREB and ATF family members bind and lead to transcriptional up-regulation of Bcl2 (36, 41, 42). In line with this finding, we showed that t-Darpp expression can increase Bcl2 promoter activity as indicated by enhanced CREB/ATF-1 binding to a CREB-specific probe on EMSA. The t-Darpp expression mediated up-regulation and phosphorylation of both CREB and ATF-1, whereas their silencing resulted in suppression of Bcl2. These findings indicate that t-Darpp regulates Bcl2 expression through a molecular mechanism that requires CREB and ATF-1 factors. The Bcl2 promoter contains binding sites for multiple transcription factors, and therefore the contribution of t-Darpp/Creb/Atf1 axis to this mechanism is possibly dependent on the molecular signature of cancer cells.

The Akt/protein kinase B pathway is a major regulator of cell survival and blocks apoptosis by growth factors against multiple apoptotic stimuli in many different cell types (43, 44). Several reports have emerged, indicating that Akt pathway activation is implicated in conferring resistance to conventional chemotherapy and multiple chemotherapeutic agents (5-fluorouracil, Adriamycin, mitomycin C, and cisplatinum) on cancer cells (18). Phosphorylated Akt (active form) expression may help to predict the clinical outcome of gastric cancer patients (45). Our data indicate that t-Darpp expression increases the kinase activity of Akt. In addition, using two independent cell models, AGS cells stably expressing t-Darpp and tetracycline-inducible AGS stable cells expressing t-Darpp, we showed a strong induction of phosphorylation and activation of Akt as shown by Western blot analyses of p-Ser473 Akt and p-Ser9 GSK3β, a known downstream target of Akt. We further validated this finding by knocking down exogenous t-Darpp stable expression (AGS) or endogenous expression (MKN45 and SNU1) that resulted in a dramatic loss of Akt phosphorylation without affecting the level of total Akt protein.

It is conceivable that t-Darpp mediates Akt activity and phosphorylation through regulating the upstream PI3K pathway. Signaling through PI3K begins with signals relaying to the internal cellular environment by transmembrane receptor tyrosine kinases (46). In addition, activation of PI3K through inactivation of the phosphatase and tensin homologue (PTEN) tumor-suppressor gene is a common mechanism in diverse cancer types (25). The knockdown of Akt in t-Darpp stably expressing cells led to a reduction in CREB/ATF1 and Bcl2 protein levels, compared with empty vector control, suggesting that the t-Darpp regulation of Bcl2 was mediated by CREB/ATF1 and dependent on Akt activity. Although the overall consensus is that CREB expression is constitutive and not subject to regulation, there are reports that indicate that CREB can be regulated by agents that activate the cAMP pathway (47). Akt potently induces serine 133 phosphorylation of CREB and promotes recruitment of CBP (48). Our results strongly indicate that the t-Darpp/Akt axis regulates the CREB/ATF-1 factors.

In summary, we showed frequent overexpression of t-Darpp in gastric cancer. The up-regulation of t-Darpp provided cancer cells with a drug-resistant phenotype and enhanced survival properties. The t-Darpp/Akt axis signaling underscores a novel oncogenic role for t-Darpp in cancer cell survival.

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