Darpp-32: a Novel Antiapoptotic Gene in Upper Gastrointestinal Carcinomas

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Abstract
We show the molecular mechanisms involved in Darpp-32 overexpression and its biological role in upper gastrointestinal adenocarcinomas (UGC). A tumor tissue array of 377 samples was developed and used to detect DARPP-32 DNA amplification and protein overexpression, which occurred in 32% and 60% of UGCs, respectively. Concomitant overexpression of mRNA for Darpp-32 and its truncated isoform t-Darpp was observed in 68% of tumors (P < 0.001). When Darpp-32 and t-Darpp were overexpressed in AGS and RKO gastrointestinal cells, up to a 4-fold reduction in the apoptosis rate was observed (terminal deoxynucleotidyl transferase–mediated nick-end labeling and Annexin V assays) in response to camptothecin, sodium butyrate, and ceramide. However, the introduction of mutations in phosphorylation sites abrogated this effect. Expression of Darpp-32 and t-Darpp preserved the mitochondrial transmembrane potential and was associated with increased levels of Bcl2 protein. A reversal of Bcl2 protein level was obtained using small interfering RNAs for Darpp-32 and t-Darpp. Luciferase assays using the p53 and p21 reporter plasmids and probing of immunoblots with antibodies specific for p53 transcriptional targets, such as Hdm2 and p21, indicated that neither Darpp-32 nor t-Darpp interfere with p53 function. Altogether, we show more frequent mRNA and protein overexpression of Darpp-32 than DNA amplification, suggesting that, in addition to amplification, transcriptional or posttranscriptional mechanisms may play an important role. The expression of Darpp-32 and t-Darpp is associated with a potent antiapoptotic advantage for cancer cells through a p53-independent mechanism that involves preservation of mitochondrial potential and increased Bcl2 levels.

Introduction
Upper gastrointestinal adenocarcinomas (UGC; i.e., adenocarcinomas of the stomach and esophagus) are poorly responsive to therapy and have an unfavorable outcome (1). It has been estimated that in the year 2000 alone, >1.3 million new cases of UGCs were identified and >984,000 people died from them. This combination of these cancers is therefore the most common form of incident cancer and the second most common cause of death from cancer in the world (2,3). UGCs are characterized by complex genetic changes and frequent genomic amplification at 17q and 20q; however, the critically important genes involved in the development of these tumors remain largely undefined (4).

Darpp-32 is a neuronal protein that plays a central role in the dopamine signaling pathway in the brain (specifically featured in the citation for the Nobel Prize in Physiology or Medicine in 2000; ref. 5). We have recently cloned DARPP-32 (AF464196) and its novel truncated isoform t-DARPP (AY070271) from adenocarcinoma of the stomach with 17q amplicon (6,7). In addition, we and others have recently documented the overexpression of Darpp-32 in a variety of common adenocarcinomas, such as those of the colon, esophagus, breast, and prostate (8,9).

Apoptosis (programmed cell death) is regulated by multiple signaling pathways and plays a critical role in cancer initiation and development. It is well established that some oncogenic mutations can disrupt apoptosis, leading to neoplasia and tumor progression (10). The BCL2 oncogene, which was first identified at the chromosomal breakpoint of chromosomal translocations t(8;14) (q24;q32) in a human leukemia cell line, promotes cell survival by blocking apoptosis (11). Genetic changes that affect apoptosis, such as overexpression of Bcl2 or loss of p53 function, can promote cancer development and are frequently found in cancer cells. The expression of Bcl2 regulates the release of proapoptotic mitochondrial proteins, including cytochrome c, and eventually, activation of the caspases. This mechanism is related to many of the effects of this protein involved and underscores a fundamental role for blocking apoptosis during tumor progression (12–14).

Drug resistance is a major problem that affects cancer therapy and can be induced by several mechanisms. In addition to drug inactivation (15,16) and altered drug transport systems (17,18), evasion of apoptosis plays a significant role (19). Overexpression and oncogenic mutations of prosurvival signaling molecules, such as epidermal growth factor receptor (20,21), Akt (also known as protein kinase B; ref. 22), nuclear factor-κB (23,24), and antiapoptotic Bcl2 family members (25,26), correlate with reduced response to chemotherapy.

The molecular mechanisms underlying Darpp-32 expression, as well as its biological role in cancer development and tumor progression, are yet to be elucidated. In this study, we investigated the molecular mechanisms underlying Darpp-32 overexpression in UGCs. Because of the frequent tumor chemoresistance and poor prognostic outcome for patients with UGCs, we explored the biological effect of Darpp-32 overexpression on apoptosis. We found that Darpp-32 and t-Darpp confer antiapoptotic effect against several drugs in a p53-independent fashion that involves an increase of Bcl2 protein level and preservation of mitochondrial potential.
Materials and Methods

**Tissue samples.** A total of 301 UGCs and 76 normal stomach paraffin-embedded tissue samples were available for the fluorescent *in situ* hybridization (FISH) and immunohistochemical analysis. In addition, 74 frozen tumor samples and 15 normal gastric epithelial samples were dissected for optimal tumor content (>70%) and used for RNA extraction, cDNA synthesis, and subsequent quantitative real-time reverse transcription-PCR (RT-PCR) assays. All tissue samples were collected in accordance with Institutional Review Board–approved protocols. Tissues were stained with H&E and representative regions were selected for inclusion in a tissue array. Tissue cores with a diameter of 0.6 mm were retrieved from the selected regions of the donor blocks and punched to the recipient block using a manual tissue array instrument (Beecher Instruments, Silver Spring, MD); samples were punched in duplicate. Control samples from normal epithelial specimens were punched in each sampled row. Sections (5 μm) were transferred to polylysine-coated slides (SuperFrostPlus, Menzel-Gläser, Braunschweig, Germany) and incubated at 37°C for 2 hours. The resulting tumor tissue array was used for FISH and immunohistochemical analysis. All tumors and normal gastric mucosal epithelial tissues were histologically verified. The adenocarcinomas collected ranged from well-differentiated (WD) to poorly differentiated (PD), stages 1 to IV, with a mix of intestinal and diffuse-type tumors.

**Fluorescent in situ hybridization.** Before hybridization, tumor tissue array sections were deparaffinized and pretreated in a microwave oven (10 minutes at 92°C in 0.01 mol/L citric acid). After rinsing in PBS, tissues mounted on slides were digested with pepsin solution (Digest-All 3, Zymed Laboratories, Inc., South San Francisco, CA) for 10 minutes at 37°C, rinsed in PBS, dehydrated in graded ethanol series, and air-dried. To investigate DARPP-32 copy number amplification, we obtained the BAC clone CTD-2091C10 (Research Genetics, Huntsville, AL) and sequenced it to verify that it contained the DARPP-32 gene. FISH analysis was employed to probe our tissue arrays. The DARPP-32 probe was labeled with biotin-14-ATP (Life Technologies, Invitrogen Corp., San Diego, CA) by nick translation, precipitated with herring sperm DNA (0.62 μg/μL, Sigma, St. Louis, MO) and human Cot-1 DNA (0.62 μg/μL, Life Technologies), and dissolved in hybridization buffer (50% formamide, 20% dextran sulfur, 2× SSC). The tissue arrays were denatured in 70% formamide/2× SSC at 68°C for 2 minutes. The hybridization was done at 37°C for 2 days. Posthybridization washes were done at 45°C using a series of solutions (pH 7): 50% formamide, 2× SSC, twice in 0.1× SSC, and finally in 4× SSC/0.2% Tween. Signal was detected with fluorescein avridin and fluorescein anti-avidin D (Vector Laboratories, Inc., Burlingame, CA). The slides were mounted with an antifading medium that contained 4′,6-diamino-2-phenylinole (DAPI) counterstain (Vector Laboratories). The tumor array preparations were surveyed with a Zeiss Axiophot fluorescence microscope (for the fluorescence-labeled arrays) and an Olympus BH-2 light microscope. A minimum of 50 nonoverlapping nuclei were scored from each case. FISH results were scored based on the number of signals per cell as follows: FISH score of 0 to 1 = 2 to 5 signals; a score of 2 = 6 to 9 signals; a score of 3 = 10 to 15 signals; and a score of 4 = >15 signals. Amplification was defined as ≥6 signals (score ≥2) in ≥50% of cancer cells, or when large gene copy number clusters were detected.

**Quantitative real-time reverse transcription-PCR.** For quantitative real-time RT-PCR, mRNA was isolated from 15 normal gastric mucosa samples and 74 primary UGCs, which included 22 distal adenocarcinomas (antrum and body), 52 proximal adenocarcinomas (GEJ and lower esophageal), using the RNeasy kit (Qiagen, GmbH, Hilden, Germany). Single-stranded cDNA was synthesized using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). Quantitative PCR was then done using an iCycler (Bio-Rad, Hercules, CA), with a threshold cycle number as determined using iCycler software version 3.0. Reactions were done in triplicate and threshold cycle numbers were averaged. Gene-specific primers for DARPP-32 and t-DARPP were designed and then obtained from GeneLink (Hawthorne, NY; sequences available upon request). Results were normalized to HPRT1, which had minimal variation in all normal and neoplastic samples tested and is considered a reliable and stable reference gene for real-time RT-PCR (27). The fold overexpression was then calculated according to the formula 2^({ΔCt}−{ΔCt})/(2^ΔCt), as described previously (7).

**Immunohistochemistry for Darpp-32.** An avidin-biotin immunoperoxidase assay was done after pretreatment in a microwave with citrate buffer for 20 minutes, and rabbit anti-Darpp-32 antibody (H-62; 1:200 dilution, Santa Cruz Biotechnology, Inc., California, CA) was applied at room temperature. The antibody had been raised against a recombinant protein corresponding to amino acids 134 to 195 at the COOH terminus of Darpp-32. The immunostaining for the gastric mucosa samples from healthy individuals lay between 0 and 1+. The immunoreactivity of the samples tested was scored as 0, absent; 1+, weak (1-33%); 2+, moderate (34-66%); 3+, strong (67-100%). Cases with 2+ or 3+ scores typically had moderate-to-strong cytoplasmic immunoreactivity. None of the normal samples had a FISH or immunohistochemistry score higher than 1.

**Cell culture, vectors, transfection, and generation of stable cell lines.** Colonic (RKO, ATCC No. CRL-2577) and gastric (AGS, ATCC No. CRL-1739) cancer cells were maintained in DMEM and F-12 (HAM) media (Invitrogen Life Technologies, Carlsbad, CA), respectively, with 10% fetal bovine serum (FBS), at 37°C in an atmosphere containing 5% CO2. The expression plasmid for FLAG-Darpp-32 was generated by PCR amplification of the full-length coding sequence of Darpp-32 cDNA and cloned in frame into a modified pcDNA3.Zeo (Invitrogen Life Technologies) plasmid containing an oligonucleotide corresponding to the FLAG peptide (DYKDDDKD) inserted into the HindIII and EcoRI sites. The expression plasmid for t-Darpp was generated by PCR amplification of the full-length coding sequence of t-Darpp cDNA and cloning into the HindIII and XhoI sites of pcDNA3.Zees. Darpp-32 (T34 — A or T75 — A) and t-Darpp (T75 — A) mutants were produced by site-directed mutagenesis using QuikChange kit (Stratagene, La Jolla, CA). AGS cells were transfected using LipofectAMINE (Invitrogen Life Technologies). Stably transfected AGS cells expressing Darpp-32, t-Darpp, or vector control [empty pcDNA3 (Zeo)] were therefore selected for by addition of 1 mg/mL zeocin (Invitrogen Life Technologies) to the growth medium. After 3 weeks of selection, several cell colonies were isolated using cloning rings and then transferred to fresh plates containing F-12 (HAM) medium, 10% FBS, and zeocin. These clones were propagated under selection and the protein expression of Darpp-32 and t-Darpp was determined by Western blot analysis.

**Aptosis assay (terminal deoxynucleotidyl transferase-mediated nick-end labeling).** RKO cells were seeded into 4-well chamber slides and transfected with 400 ng of pcDNA3-based expression plasmids per well, using Fugene according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). After 24 hours, nontreated and camptothecin-treated cells were stained by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL), according to the manufacturer’s instructions (Roche Diagnostics), and then visualized by FITC (green fluorescence). Darpp-32 and t-Darpp expression was determined by immunofluorescence staining in duplicate wells with Darpp-32 antibody (Santa Cruz Biotechnology) and donkey anti-rabbit IgG (HL)-rhodamine conjugate (Red; Jackson ImmunoResearch Laboratories, West Grove, PA). Transfection efficiency was reproducibly ~40% for all constructs, and evenly distributed in all wells. TUNEL-positive cells and Darpp-32- or t-Darpp-expressing cells (20 random fields at ×40, ×400 cells) were counted. The percentage of apoptosis was determined in Darpp-32- or t-Darpp-expressing cells versus nonexpressing cells after correction for background with vector alone.

**Aptosis assay (Annexin V).** AGS stables expressing Darpp-32, t-Darpp, or empty vector (Zeo) were seeded onto 60-mm culture plates. After 18 hours, nontreated and drug-treated (camptothecin, sodium butyrate, and ceramide) cells were stained with Annexin V/biotin, streptavidin/APC (BD Biosciences Pharmingen, San Diego, CA), and PI-treated AGS-zeo, and streptavidin/APC (BD Biosciences Pharmingen, San Diego, CA). After 24 hours, nontreated and camptothecin-treated cells were stained by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL), according to the manufacturer’s instructions (Roche Diagnostics), and then visualized by FITC (green fluorescence). Darpp-32 and t-Darpp expression was determined by immunofluorescence staining in duplicate wells with Darpp-32 antibody (Santa Cruz Biotechnology) and donkey anti-rabbit IgG (HL)-rhodamine conjugate (Red; Jackson ImmunoResearch Laboratories, West Grove, PA). Transfection efficiency was reproducibly ~40% for all constructs, and evenly distributed in all wells. TUNEL-positive cells and Darpp-32- or t-Darpp-expressing cells (20 random fields at ×40, ×400 cells) were counted. The percentage of apoptosis was determined in Darpp-32- or t-Darpp-expressing cells versus nonexpressing cells after correction for background with vector alone.
signal. FACS analysis was done within 1 hour after cell resuspension. A dot plot of the X-axis (FL1), being the log of Annexin V fluorescence, and the Y-axis (FL2), which reflects the PI fluorescence, was obtained by flow cytometry (Becton Dickinson, Mountain View, CA).

**Mitochondrial apoptosis assay.** This assay, which is based on disruption of the mitochondrial transmembrane potential (one of the critical events to occur following induction of apoptosis), was done using the MitoCapture mitochondrial apoptosis detection kit (BioVision, Mountain View, CA), as per manufacturer’s instructions. AGS stable cell lines (Darpp-32, t-Darpp, and empty vector control) were plated in 8-well chamber slides in duplicate wells. Apoptosis was induced by treating cells with 20 μmol/L camptothecin for 2 hours. Treated and untreated cells were stained with MitoCapture reagent and then observed under a fluorescence microscope using a band-pass filter that permits fluorescence from fluorescein and rhodamine. In healthy cells, MitoCapture dye accumulated in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, the dye does not aggregate in the mitochondria but remains in the cytoplasm and emits a green fluorescence.

**Table 1. FISH and immunohistochemical analysis of 257 UGCs on tumor tissue arrays**

<table>
<thead>
<tr>
<th>FISH score</th>
<th>Immunohistochemistry score</th>
<th>Total</th>
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<tr>
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<td>2</td>
<td>3</td>
</tr>
<tr>
<td>0-1, n = 176 (%)</td>
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<td>2, n = 54 (%)</td>
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<tr>
<td>4, n = 15 (%)</td>
<td>2 (13)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Total, n = 257 (%)</td>
<td>104 (41)</td>
<td>83 (32)</td>
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NOTE: FISH score, 0-1 = 2-5 signals, 2 = 6-9 signals, 3 = 10-15 signals, 4 = >15 signals.

*p < 0.01.

**Figure 1.** Genomic structure and localization of DARPP-32. A, genomic structure of DARPP-32 and t-DARPP. The mRNA of DARPP-32 is 1,983 bp, including the untranslated 3’ and 5’ ends, while t-DARPP is 1,502 bp. DARPP-32 and t-DARPP share identical sequence from exon 2 to the 3’ end. Exon 1 of t-DARPP is spliced from the intron 1 of DARPP-32. DARPP-32 encodes a protein of 204 amino acids, whereas t-DARPP encodes a 168-amino-acid protein. Darpp-32 contains four phosphorylation sites at T34, T75, Ser102, and Ser137, whereas t-Darpp lacks the T34 phosphorylation site of Darpp-32. B, genomic localization of DARPP-32 with FISH using BAC clone CTD-2019C10 (Research Genetics) as a probe for DARPP-32 on a normal metaphase spread. Arrows indicate the green FITC hybridization signals. Right, ideogram of chromosome 17 (left), together with the inverted DAPI banding (middle) and FISH hybridization signals on the two chromosome 17 (right). The FISH signals localize to chromosome band 17q12-q21 indicating the locus for DARPP-32.
content. Rabbit polyclonal antibodies against Darpp-32 (H-62, SC-11365, Santa Cruz Biotechnology) and phospho-Darpp-32 (T34) and (T75; Cell Signaling Technology, Beverly, MA) were obtained. Mouse monoclonal antibody against HDM2 (IF2) and p21Waf1 (Ab-1, OP64) were obtained from Oncogene Research Products (La Jolla, CA). Mouse monoclonal anti-actin antibody (C-2, SC-8432) was obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-Bcl2 antibody (610539) was received from BD Transduction Laboratories (Lexington, KY).

Darpp-32 and t-Darpp small interfering RNA. Cell lysates from AGS cells stably expressing empty vector, Darpp-32 or t-Darpp, with and without transfection of small interfering RNA (siRNA) oligonucleotides specific for Darpp-32 and t-Darpp (sc-35173), were subjected to immunoblot analysis for Darpp-32 and Bcl2. Transfection was done using siRNA transfection reagent (sc-29528) and Transfection medium (sc-36868), following the manufacturer’s instructions (Santa Cruz Biotechnology). Lysates were normalized for equal h-actin loading into the gel.

Results
Genomic structure and localization of DARPP-32. We have recently cloned DARPP-32 and its truncated isoform t-DARPP from primary gastric cancer (6, 7). The genomic structure of DARPP-32...
and t-DARPP is depicted in Fig. 1A. The mRNA for DARPP-32 and t-DARPP are 1,983 and 1,502 bp, respectively. DARPP-32 and t-DARPP share sequence identity from exon 2 to the 3' end. However, exon 1 of t-DARPP is spliced from intron 1 of DARPP-32. A protein of 204 amino acids is encoded by DARPP-32, whereas t-DARPP encodes a 168-amino-acid protein. Darpp-32 contains four phosphorylation sites, at T34, T75, S102, and S137, whereas t-Darpp lacks the T34 phosphorylation site of Darpp-32. To verify the chromosomal locus for DARPP-32, we did FISH analysis using the BAC clone CTD-2019C10 (Research Genetics) as a probe for DARPP-32 on a normal metaphase spread. A positive signal for the BAC clone CTD-2019C10 (Research Genetics) as a probe for DARPP-32 from FISH was indicated by the green FITC hybridization fluorescence depicted in Fig. 1B (arrows, left). Together with the inverted DAPI banding and FISH hybridization on the two chromosome 17, the FISH signals were found to localize to chromosome band 17q12-q21 indicating the locus for DARPP-32 (Fig. 1B, right).

DNA copy numbers, protein, and mRNA levels of DARPP-32 in upper gastrointestinal adenocarcinomas. To evaluate the DNA copy numbers and protein expression of DARPP-32, we did FISH and immunohistochemical analysis on identical replicates of the tumor tissue array. FISH and immunohistochemical staining in green and immunofluorescence with COOH-terminal Darpp-32-specific polyclonal antibody, which detects both Darpp-32 and t-Darpp (red fluorescence) combined picture (left), showing that Darpp-32- or t-Darpp-expressing cells (white arrows) are virtually protected from camptothecin-induced apoptosis. The DAPI (blue fluorescence) was used as nuclear counter stain to count all cells. The same experiment was done using phosphorylation mutants for Darpp-32 and t-Darpp and showed loss of the antipapoptotic potential (not depicted), as summarized in (C). B, RKO cells transfected with Darpp-32 and analyzed with TUNEL assay without camptothecin treatment indicate absence of green apoptotic signals, ruling out any effect of transfection on apoptosis. C, Western blot analysis following transient transfection with wild-type Darpp-32, t-Darpp, and phosphorylation mutants. pcDNA3 expression plasmids used (top). Antibodies used for blotting (right). The blot shows a strong specific band for Darpp-32 (~32 kDa) and t-Darpp (~29 kDa). Antibodies for phosph-T34 or -T75 Darpp-32 show that Darpp-32 had two phosphor bands, pT34 and pT75, whereas t-Darpp shows one phosphor band for pT75. The phosphorylation mutants do not show phosphorylation signal at the mutated site. t-Actin was used for normalization for equal loading. D, summary of the percentage of apoptosis in Darpp-32- and t-Darpp-expressing cells compared with pCDNA3-transfected cells, with and without 5 μmol/L camptothecin treatment for 18 hours. Results from mutants are shown and indicate loss of the antipapoptotic potential. The experiments were repeated three times. Columns, means; bars, ±SE.

Figure 3. DARPP-32 counteracts apoptosis induced by camptothecin (CPT). Darpp-32 and t-Darpp confer camptothecin drug resistance to wild-type p53 harboring tumor cells (RKO). RKO cells were transfected with Darpp-32-pcDNA3.1 (left) or t-Darpp-pcDNA3.1 (right) expression plasmids. Twenty-four hours after transfection, cells were treated with 5 μmol/L camptothecin for 18 hours or left untreated (not depicted). After fixation with paraformaldehyde, each well underwent both TUNEL staining in green and immunofluorescence with COOH-terminal Darpp-32-specific polyclonal antibody, which detects both Darpp-32 and t-Darpp (red fluorescence). DAPI was used as a nuclear counter stain (blue). Bottom, combined picture (red, green, and blue) showing that Darpp-32- or t-Darpp-expressing cells (white arrows) are virtually protected from camptothecin-induced apoptosis. The DAPI (blue fluorescence) was used as nuclear counter stain to count all cells. The same experiment was done using phosphorylation mutants for Darpp-32 and t-Darpp and showed loss of the antipapoptotic potential (not depicted), as summarized in (C). B, RKO cells transfected with Darpp-32 and analyzed with TUNEL assay without camptothecin treatment indicate absence of green apoptotic signals, ruling out any effect of transfection on apoptosis. C, Western blot analysis following transient transfection with wild-type Darpp-32, t-Darpp, and phosphorylation mutants. pcDNA3 expression plasmids used (top). Antibodies used for blotting (right). The blot shows a strong specific band for Darpp-32 (~32 kDa) and t-Darpp (~29 kDa). Antibodies for phosph-T34 or -T75 Darpp-32 show that Darpp-32 had two phosphor bands, pT34 and pT75, whereas t-Darpp shows one phosphor band for pT75. The phosphorylation mutants do not show phosphorylation signal at the mutated site. t-Actin was used for normalization for equal loading. D, summary of the percentage of apoptosis in Darpp-32- and t-Darpp-expressing cells compared with pCDNA3-transfected cells, with and without 5 μmol/L camptothecin treatment for 18 hours. Results from mutants are shown and indicate loss of the antipapoptotic potential. The experiments were repeated three times. Columns, means; bars, ±SE.
and immunohistochemistry in UGCs. In addition, we have observed that protein overexpression of Darpp-32 is more frequent in diffuse-type rather than intestinal-type UGC (P = 0.05).

To investigate whether DARPP-32 gene amplification causes commensurate mRNA gene expression, we set out to analyze the levels of mRNA expression. The expression of DARPP-32 and t-DARPP was compared with that of the HPRT1 gene, for which minimal variation was seen in all the normal and neoplastic tumor samples tested. The expression in each tumor sample was compared with the expression in 15 normal gastric mucosa samples (±SE = 5%). We used an arbitrary cutoff value of a 3-fold increase as the minimum considered to indicate overexpression. Overexpression was seen in 50 tumors (68%), along with concomitant overexpression of Darpp-32 and t-Darpp (P < 0.001; Fig. 2A and B). Therefore, the mRNA overexpression frequency is very close to the observed protein overexpression following immunohistochemical staining and is also higher than genomic amplification. This finding indicates that in addition to DNA amplification, transcriptional and/or posttranscriptional mechanism may play a role for Darpp-32 overexpression. FISH and mRNA data were available for 38 tumors. Tumors that had an average of more than five signals per cell (score ≥ 2) had a higher average fold mRNA expression for Darpp-32 or t-Darpp (>50-fold) compared with tumors without amplification (<30-fold). The data are summarized in Table 1 and Fig. 2.

**Darpp-32 counteracts apoptosis induced by camptothecin.** The antiapoptotic activity of Darpp-32 and t-Darpp was investigated in RKO cells that express wild-type p53. The results, following 18 hours of treatment with camptothecin, revealed a dramatic 4-fold reduction in apoptotic cell count in Darpp-32- or t-Darpp-expressing cells compared with control (Fig. 3A). The RKO cells transfected with Darpp-32 but not treated with camptothecin did not show any green fluorescence in the TUNEL assay, ruling out any effect of transfection on apoptosis (Fig. 3B). Western blot analysis following transient transfection with expression plasmids for Darpp-32, t-Darpp, or the T34 and T75 mutants confirmed the strong expression of these proteins and the lack of phosphorylation signal that correlated with the mutants tested (Fig. 3C). The same TUNEL experiment was done using phosphorylation mutants for Darpp-32 (T34 → A or T75 → A) and t-Darpp (T75 → A). For these variants, a complete loss of antiapoptotic protection against camptothecin was observed (Fig. 3D).

**Darpp-32 counters apoptosis induced by different drugs.** To further investigate the antiapoptotic effect of DARPP-32, several drugs were tested using Annexin V apoptosis assay. Overall, the results obtained for camptothecin, ceramide, and sodium butyrate in the Annexin V assay confirmed the strong antiapoptotic effect for Darpp-32 and t-Darpp observed in the TUNEL assay. The percentages of early apoptotic cells, Annexin V positive and PI negative, are summarized in Fig. 4 (bottom). These results indicate that Darpp-32 and t-Darpp provide an antiapoptotic advantage for gastrointestinal cells against several chemotherapeutic drugs that induce apoptosis through different mechanisms. However, the t-Darpp-expressing AGS cells were considerably more resistant to apoptosis than Darpp-32, following ceramide treatment.

**Darpp-32 preserves mitochondrial transmembrane potential.** In healthy cells, the MittoCapture dye accumulated in the mitochondria in a similar manner to untreated cells (Fig. 5, left); the same was seen in Darpp-32 and t-Darpp camptothecin-treated cells (Fig. 5, right). In apoptotic cells, the dye did not aggregate in the mitochondria, as seen following camptothecin treatment of cells transfected with empty vector (Zeo) cells (Fig. 5, top right). This indicates that both Darpp-32 and t-Darpp protected the mitochondrial transmembrane potential in AGS cells following treatment with camptothecin.

**Darpp-32 and t-Darpp abrogate apoptosis in a p53-independent mechanism.** As expected, cotransfection of ΔNp73 and p53 dramatically suppressed the transcriptional activity of wild-type p53, as measured by PG13-luc and p21-luc reporters. In contrast, cotransfection of p53 with either Darpp-32 or t-Darpp did not suppress the p53 activity (Fig. 6A and B). Western blot analysis shows that transfection of Darpp-32 or t-Darpp does not change...
Bcl2 was therefore up-regulated by both Darpp-32 and t-Darpp in Darpp-32 and t-Darpp stable cell lines (Fig. 6). The expression of Bcl2, the antiapoptotic protein, was not detected in the vector control cells (Fig. 6). Similar results were obtained upon transient transfections. On the other hand, the expression of Bcl2 in apoptotic cells, the dye does not aggregate in the mitochondria. This was seen as absence of red fluorescence, as shown following camptothecin treatment in empty vector (Zeo)–expressing cells. A recent study has shown that esophageal squamous cell carcinomas frequently overexpress Darpp-32 but not t-Darpp (9). In a related study, amplification and overexpression of ERBB2, which coincides at the same locus as DARPP-32 (17q12-21), was found in ~10% of both diffuse-type and intestinal-type gastric cancers (31). The frequent gene amplification and high mRNA and protein expression levels of Darpp-32 suggest that it plays an important role in tumor development and progression of UGCs.

Darpp-32 is known as a neurosignaling molecule that plays a crucial role in dopamine signaling in the brain (32, 33). To date, the biological function of Darpp-32 in normal tissues and in cancer remains unknown. In this study, we have shown that Darpp-32 and t-Darpp proteins have a potential antiapoptotic role and provide drug resistance with a survival advantage to neoplastic gastrointestinal cells. Apoptosis, or programmed cell death, is an essential physiologic process that is involved in the elimination of infected, damaged, or unwanted cells (34–37). Deregulation of apoptosis is an important step in cancer development, with aberrant cancer cells escaping cell death and acquiring additional neoplastic features. This feature renders several cancers, including UGCs, resistant to cancer therapy (25, 38, 39). These independent methods (i.e., TUNEL, Annexin V, and mitochondrial potential assays) revealed a consistent dramatic reduction in apoptosis in cells expressing either Darpp-32 or t-Darpp. Phosphorylation mutants of Darpp-32 (T34 → A or T75 → A) and t-Darpp (T75 → A) did not confer any antiapoptotic protection against camptothecin. This finding shows that the T34 and T75 phosphorylation sites may be critical to maintain the full antiapoptotic potential of both Darpp-32 and t-Darpp. The antiapoptotic advantage was observed in gastric cancer cells expressing either Darpp-32 or t-Darpp following treatment with camptothecin, ceramide, or sodium butyrate. However, following induction of apoptosis with ceramide, we observed that the t-Darpp-expressing cells were considerably more resistant to apoptosis than Darpp-32 expressing cells. This may be a drug-specific effect, because Darpp-32 and t-Darpp conferred cytogenetic localization of the gene encoding Darpp-32 to chromosome band 17q12-q21. Our study shows that overexpression of Darpp-32, at the mRNA and protein level, in UGCs is more frequent than genomic DNA amplification. Using tumor tissue arrays, we detected gene amplification of DARPP-32 in approximately one third of UGCs, whereas overexpression of Darpp-32 at the mRNA and protein levels was detected in more than two thirds of tumors. Therefore, Darpp-32 overexpression was not limited to genomic amplification. The similar high frequency of Darpp-32 overexpression at both mRNA and protein level suggests that transcriptional and/or posttranscriptional regulatory mechanisms may be involved. Nevertheless, tumor samples with a high level of genomic DARPP-32 DNA amplification (>10 signals per cell) showed consistently higher immunohistochemical staining (3+) than any other tumor (P < 0.01). This indicates that amplification, although not the only mechanism, can play a role in inducing high levels of Darpp-32 expression. Interestingly, protein overexpression of Darpp-32 was more frequent in diffuse-type UGC (P < 0.005). Diffuse-type tumors are known to be more aggressive with a poor outcome (1).

Interestingly, we have detected concomitant mRNA overexpression of Darpp-32 and t-Darpp (P < 0.001). The Darpp-32 and t-Darpp were frequently overexpressed in both distal and proximal gastroesophageal adenocarcinomas. We have observed a similar trend for Darpp-32 and t-Darpp overexpression in a small number of common adenocarcinomas, including breast, colon, lung, and prostate (8). However, a recent study has shown that esophageal squamous cell carcinomas frequently overexpress Darpp-32 but not t-Darpp (9). In a related study, amplification and overexpression of ERBB2, which coincides at the same locus as DARPP-32 (17q12-q21), was found in ~10% of both diffuse-type and intestinal-type gastric cancers (31). The frequent gene amplification and high mRNA and protein expression levels of Darpp-32 suggest that it plays an important role in tumor development and progression of UGCs.

Discussion

We have earlier cloned Darpp-32 and a novel truncated isoform, t-Darpp, in gastric cancer (7). In this study, we verified the
similar drug resistance against both camptothecin and sodium butyrate. It has to be noted that Darpp-32 has two main phosphorylation sites (T34 and T75), whereas t-Darpp lacks the T34 (40); therefore, differences can be expected.

These drugs are known to act upon different signaling pathways. Ceramide activates protein phosphatases such as PP1 and PP2A, through which it can indirectly inhibit pro-growth signaling kinases such as PKC isoforms and Akt (41). On the other hand, camptothecin is a well-established cancer drug that inhibits topoisomerase I and blocks DNA replication in neoplastic cells (42). Camptothecin treatment results in up-regulation and activation of p53 and down-regulation of Bcl2 and Bcl-xl (43, 44). The sodium butyrate is a histone deacetylase inhibitor that induces the loss of the mitochondrial transmembrane potential and the release of cytochrome c into the cytoplasm, as well as activating both caspase 9 and caspase 3 (45). Sodium butyrate can also induce apoptosis in a p53-independent mechanism through the Fas signaling pathway (46). Our results that Darpp-32 and t-Darpp can provide resistance to these different drugs and act upon different signaling cascades suggest that Darpp-32 proteins may be related to the drug resistance phenotype in UGCs.

Figure 6. DARPP-32 counteracts apoptosis in a p53-independent mechanism. A-B, luciferase activity using the PG13 and p21 promoter luciferase constructs in p53 null H1299 cells. △Np73 was used as a positive control that mediates suppression of the wild-type p53/TAp73-responsive reporter construct PG13-luciferase. Luciferase activity is normalized for Renilla luciferase activity. Coexpressed △Np73 caused a dramatic suppression of the transcriptional activity of wild-type p53 and p21. The cotransfection of p53 with Darpp-32 or t-Darpp did not suppress the p53 or the p21 activity. Columns, averages of three independent experiments; bars, ± SE. C, immunoblots of H1299 cells transfected with p53 or △Np73, either alone or in combination with Darpp-32 or t-Darpp. Transfections were done in parallel with A-B. Samples were normalized to β-actin before loading. The transfection of empty vector alone shows low base levels of Hdm2 and p21, which remained similar after transfection of Darpp-32 or t-Darpp alone. The transfection of p53 induced transactivation of the endogenous target genes p21 and Hdm2 (lane 2). Cotransfection of p53 with Darpp-32 or t-Darpp did not change the levels of Hdm2 or p21, whereas cotransfection of p53 with △Np73 significantly reduced the levels of Hdm2 and p21 compared with p53 alone. D, immunoblots of AGS cells stably expressing empty vector (Zeo), Darpp-32, or t-Darpp, with and without transfection of siRNA oligonucleotide specific for Darpp-32 (Santa Cruz Biotechnology). Samples were normalized to β-actin before loading. The Darpp-32 and t-Darpp cells show high levels of Bcl2 that is not detected in the vector control. Transfection with a siRNA oligonucleotide leads to complete loss of the Bcl2 in AGS cells stably expressing either Darpp-32 or t-Darpp.
Darrp-32 and t-Darrp was conferred through a p53-independent mechanism. These findings also may explain that apoptosis by three different anticancer drugs was counteracted by Darrp-32 and t-Darrp.

The preservation of the mitochondrial transmembrane potential by Darrp-32 or t-Darrp following induction of apoptosis with camptothecin, determined independently the strong antiapoptotic potential of Darrp-32 and t-Darrp. Disruption of the mitochondrial transmembrane potential is one of the early events to occur following induction of apoptosis (47–49). Taken together, these results suggested that the antiapoptotic mechanism employed by Darrp-32 and t-Darrp may involve a mitochondrial-related molecule downstream of p53. In this report, we showed that expression of Bcl2 was up-regulated by Darrp-32 and t-Darrp expression. Expression knockdown of Darrp-32 or t-Darrp by siRNA confirmed that the levels of Bcl2 are specifically regulated by Darrp-32 and t-Darrp. Bcl2 inhibits drug-induced apoptosis by preventing cytochrome c release (50) and subsequent abrogation of the activation of caspases (51–54). The interactions between antiapoptotic Bcl2 family members (such as Bcl-XL and Mcl1) and proapoptotic proteins (such as Bad and Bax) determine whether apoptosis occurs (55). Thus, we have identified a novel antiapoptotic signaling pathway by which Darrp-32 proteins counter apoptosis.

In conclusion, our results indicate that tumor samples with high-level DNA amplification have consistently high immunohistochemical staining for Darrp-32. However, mRNA and protein overexpression of Darrp-32 were more frequent than genomic DNA amplification in UGCs. Our findings underscore both a biological function and a potential oncogenic role for Darrp-32 and t-Darrp in UGCs. Overexpression of Darrp-32 and t-Darrp is associated with a potent antiapoptotic advantage against several known drugs. This pathway involves both Bcl2 up-regulation and preservation of the mitochondrial transmembrane potential in a p53-independent manner. Further studies are ongoing to understand the signaling mechanism of Darrp-32 proteins in cancer.

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