

# Tissue Transglutaminase Inhibits Autophagy in Pancreatic Cancer Cells

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## Abstract

Elevated expression of tissue transglutaminase (TG2) in cancer cells has been implicated in the development of drug resistance and metastatic phenotypes. However, the role and the mechanisms that regulate TG2 expression remain elusive. Here, we provide evidence that protein kinase C $\delta$  (PKC $\delta$ ) regulates TG2 expression, which in turn inhibits autophagy, a type II programmed cell death, in pancreatic cancer cells that are frequently insensitive to standard chemotherapeutic agents. Rottlerin, a PKC $\delta$ -specific inhibitor, and PKC $\delta$  small interfering RNA (siRNA) down-regulated the expression of TG2 mRNA and protein and induced growth inhibition without inducing apoptosis in pancreatic cancer cells. Inhibition of PKC $\delta$  by rottlerin or knockdown of TG2 protein by a TG2-specific siRNA resulted in a marked increase in autophagy shown by presence of autophagic vacuoles in the cytoplasm, formation of the acidic vesicular organelles, membrane association of microtubule-associated protein 1 light chain 3 (LC3) with autophagosomes, and a marked induction of LC3-II protein, important hallmarks of autophagy, and by electron microscopy. Furthermore, inhibition of TG2 by rottlerin or by the siRNA led to accumulation of green fluorescent protein (GFP)-LC3-II in autophagosomes in pancreatic cancer cells transfected with GFP-LC3 (*GFP-ATG8*) expression vector. Knockdown of Beclin-1, a specific autophagy-promoting protein and the product of *Becn1* (*ATG6*), inhibited rottlerin-induced and TG2 siRNA-induced autophagy, indicating that Beclin-1 is required for this process. These results revealed that PKC $\delta$  plays a critical role in the expression of TG2, which in turn regulates autophagy. In conclusion, these results suggest a novel mechanism of regulation of TG2 and TG2-mediated autophagy in pancreatic cancer cells. (Mol Cancer Res 2007;5(3):241–9)

## Introduction

Tissue transglutaminase (TG2, EC 2.3.2.13) is a unique member of the transglutaminase family of enzymes. In addition to catalyzing Ca<sup>2+</sup>-dependent posttranslational modification of proteins, it can catalyze calcium-independent hydrolysis of GTP, the protein disulfide isomerase reaction, and serine/threonine kinase activity (1–4). The ability of TG2 to hydrolyze GTP enables it to serve as a signaling molecule and activates a downstream target, phospholipase C (5). Transamidation by TG2 can both facilitate and inhibit apoptosis, whereas the GTP-bound form of the enzyme generally protects cells against death (6). Although predominantly a cytosolic protein, TG2 can be secreted outside the cell, where it regulates cell-matrix interactions (7). It can also translocate to the nucleus, where it associates with pRb, p53, and histones to regulate certain cellular functions (8). TG2 also can be expressed on the cell membrane in association with  $\beta$  members of the integrin family of proteins and serve as a coreceptor for integrin-mediated cell survival signaling (9).

We and others have reported that the basal expression of TG2 in several tumor cells and tumor cell lines is elevated when they become resistant to chemotherapeutic drugs (10). Indeed, inhibition of TG2 by antisense or small interfering RNA (siRNA) has been shown to render cancer cells sensitive to chemotherapeutic drugs (11). Tumor cells from metastatic sites and cell lines with metastatic potential also have been found to express high basal levels of TG2 (10). Elevated expression of TG2 in pancreatic cancer cells has been detected by conventional methods and cDNA microarrays (12, 13). In addition, our recent findings suggest that TG2 expression plays a role in activation of nuclear factor- $\kappa$ B and the development of drug resistance and metastatic phenotypes in cancer cells (14, 15). However, no direct link between TG2, drug resistance, and metastasis has been established, and the molecular pathways that result in constitutive expression of TG2 in cancer cells remain elusive.

Protein kinase C (PKC) plays a central role in signal transduction pathways that mediate the action of growth factors, tumor promoters, and cellular oncogenes (16). The tumor promoter phorbol ester results in the activation of PKC and can either promote or inhibit the growth of human pancreatic cancer cells (17). Similarly, phorbol ester can induce TG2 expression in various cell types. Depending on the cell type, PKC $\delta$  can function as a tumor suppressor, proapoptotic factor, or antiapoptotic factor and can regulate cell proliferation and cell survival functions (18). Like the expression of TG2, expression of the PKC $\delta$  isoform is associated with the metastatic phenotype in some cancers (19).

Received 7/27/06; revised 1/8/07; accepted 1/22/07.

**Grant support:** National Cancer Institute grants U54 RFA CA096300 (G. Lopez-Berestein) and CA92115 (K. Mehta).

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**Note:** U. Akar and B. Ozpolat are cofirst authors and contributed equally to this work.

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doi:10.1158/1541-7786.MCR-06-0229

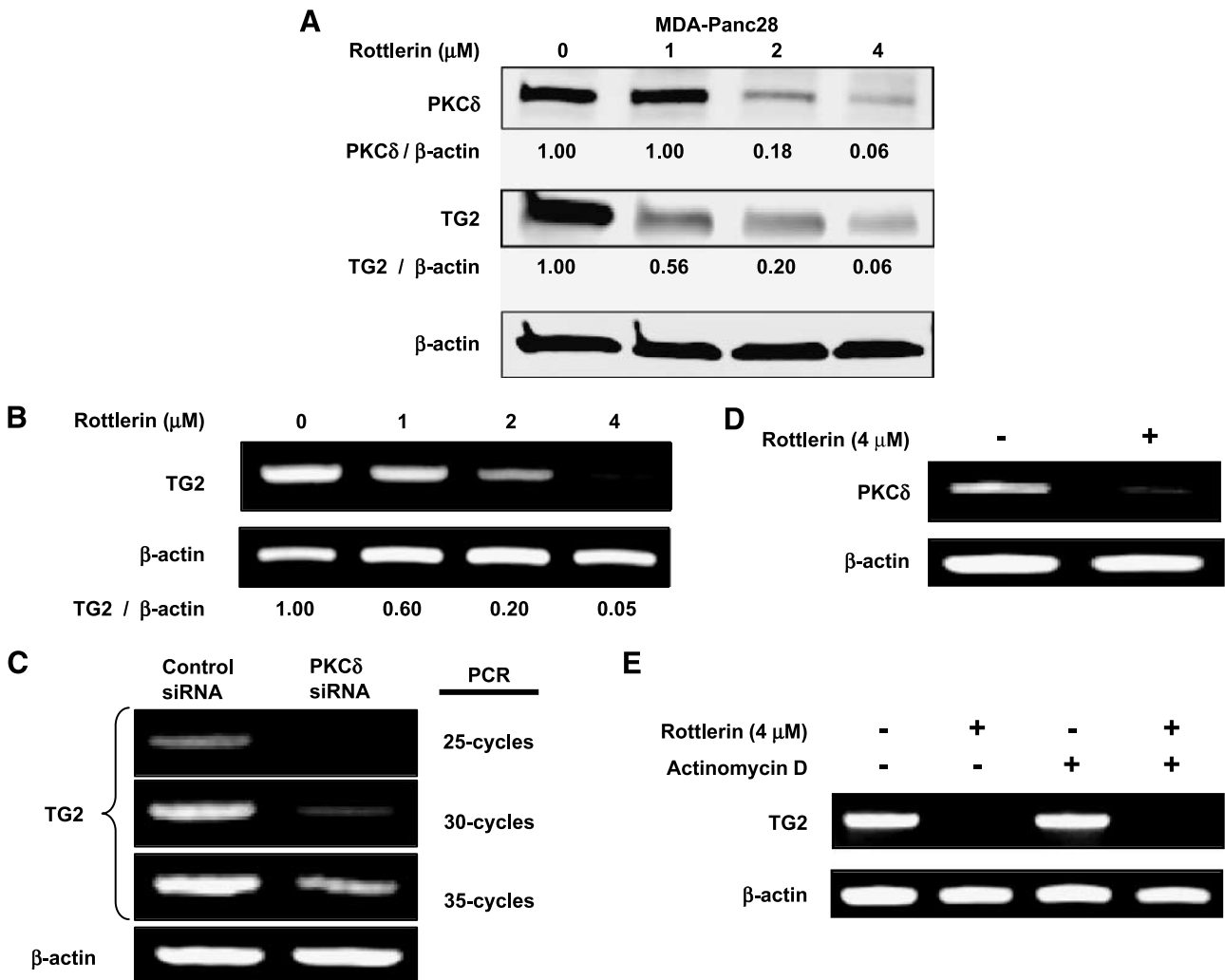
In this study, we investigated the relationship between PKC $\delta$  and TG2 in pancreatic carcinoma cells, which are frequently insensitive to standard chemotherapeutic agents. Understanding this relationship may suggest strategies for overcoming tumor cell resistance in this disease. Here, we present *in vitro* evidence that PKC $\delta$  plays an important role in regulation of TG2 expression in pancreatic ductal carcinoma cells and helps protect cells from autophagy, thus contributing to resistance to treatment.

## Results

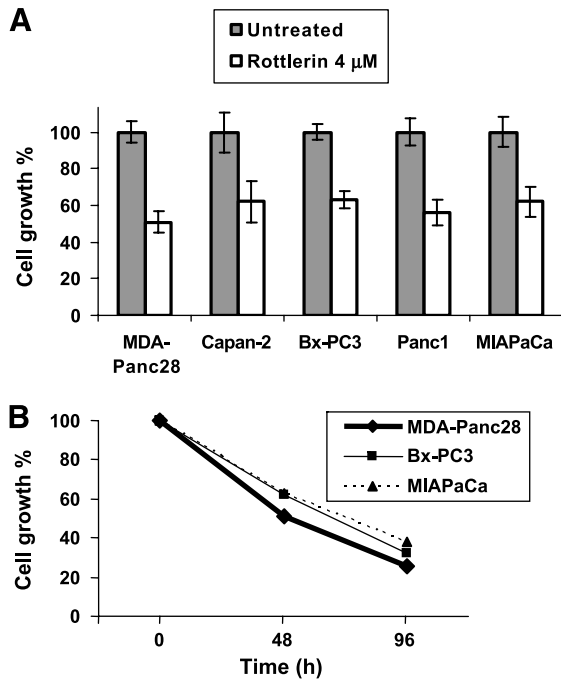
### *Inhibition of PKC $\delta$ Results in Down-Regulation of TG2*

As a first step to elucidate the relationship between PKC $\delta$  and TG2 expression in pancreatic cancer cells, we determined the effect of a PKC $\delta$ -specific inhibitor, rottlerin, on constitutive

expression of TG2 in MDA-Panc28 pancreatic ductal carcinoma cells (Fig. 1A). Treatment of MDA-Panc28 cells with 2  $\mu\text{mol/L}$  rottlerin inhibited PKC $\delta$  protein expression by 72% and treatment with 4  $\mu\text{mol/L}$  rottlerin inhibited expression by 94%. Notably, rottlerin also caused a dose-dependent inhibition in the TG2 protein expression of the cells. As little as 1  $\mu\text{mol/L}$  rottlerin inhibited TG2 expression by 44%, with maximum inhibition (94%) seen at the 4  $\mu\text{mol/L}$  dose after 48 h of treatment. Reverse transcription-PCR (RT-PCR) analysis of treated and untreated cells confirmed the inhibitory effect of rottlerin on TG2 and PKC $\delta$  expression at the level of transcription (Fig. 1B and D). We also knocked down PKC $\delta$  using siRNA and found that PKC $\delta$  siRNA inhibited TG2 mRNA expression detected by semiquantitative RT-PCR assay in pancreatic



**FIGURE 1.** Rottlerin inhibits both PKC $\delta$  and TG2 in MDA-Panc28 cells. **A.** Cells at 80% confluence were treated with 1, 2, or 4  $\mu\text{mol/L}$  of rottlerin. After 48 h of treatment, the cells were lysed and cell lysates were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane to determine PKC $\delta$  and TG2 protein expression by Western blot analysis. The membrane was stripped and reprobed with an anti- $\beta$ -actin antibody to confirm equal loading of protein in each lane. Total RNA was extracted, and transcript levels of TG2 (**B** and **C**) and PKC $\delta$  (**D**) were determined by RT-PCR analysis as described in Materials and Methods. Relative expression levels were determined by densitometry compared with  $\beta$ -actin. Experiments were repeated at least three times. **C.** For siRNA-mediated knockdown experiments, Panc28 cells transfected with nonsilencing control or PKC $\delta$ -specific siRNA were used and TG2 mRNA expression was detected by semiquantitative RT-PCR assay in pancreatic cancer cells. **E.** To determine if rottlerin interfered with stability of TG2 mRNA, the cells were pretreated with actinomycin D for 4 h. Panc28 cells were treated with rottlerin (4  $\mu\text{mol/L}$ ), actinomycin D (5  $\mu\text{g/mL}$ ), or both. Total RNA was extracted and expression of TG2 mRNA was detected with semiquantitative RT-PCR as described in Materials and Methods.



**FIGURE 2.** Growth inhibition by rottlerin in pancreatic cancer cells. **A.** Different pancreatic cancer cell lines were treated with rottlerin (4 μmol/L) for 48 h, and viable cells were detected by proliferation assay using Alamar Blue assay. Columns, growth inhibition after normalizing untreated cells to 100%. **B.** Growth of the indicated cell lines after treatment with rottlerin (4 μmol/L) for 48 and 96 h.

cancer cells (Fig. 1C), confirming that PKC $\delta$  regulates TG2 mRNA expression. Rottlerin has been shown to block PKC $\delta$  specifically at concentrations between 3 and 6 μmol/L (20). However, at higher concentrations, it can also inhibit other isoforms of PKC. Based on these observations, we conclude that the effects of rottlerin on cell growth and TG2 expression are mediated by the selective inhibition of PKC $\delta$ . No reduction in the kinetics of message stability in the presence of actinomycin D was observed (Fig. 1E).

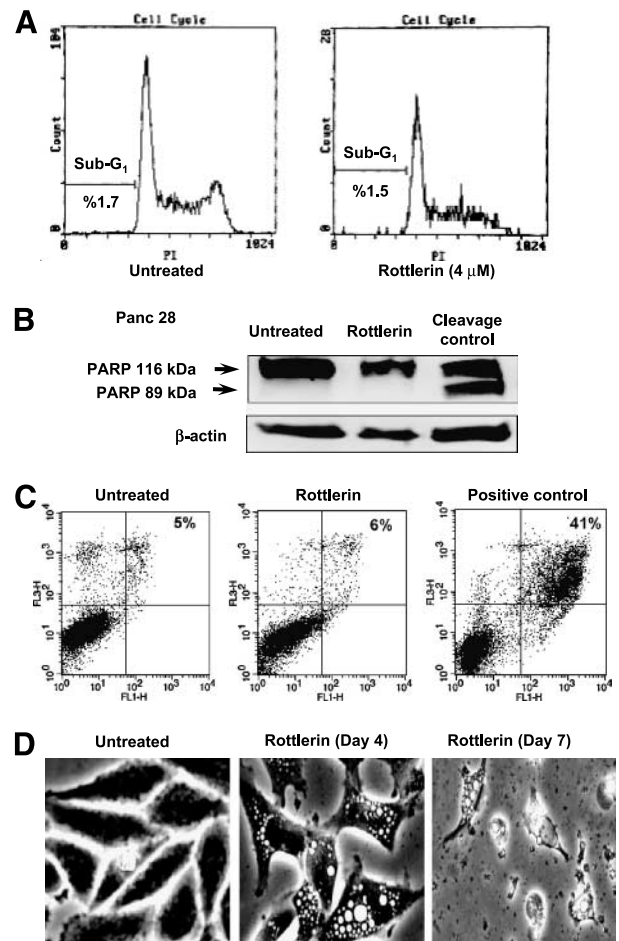
*Rottlerin Induces Inhibition of Cell Growth in Pancreatic Cancer Cells*

We next determined the growth-inhibitory effect of rottlerin in several pancreatic cancer cell lines, including Bx-PC3, MIAPaCa, MDA-Panc28, Panc1, and Capan-2 (Fig. 2A). Treatment of cells with rottlerin (4 μmol/L for 48 h) induced growth inhibition in all five cell lines tested. We also determined the time course of rottlerin-induced growth inhibition in MDA-Panc28, Bx-PC3, and MIAPaCa cell lines and found a time-dependent inhibition in cell growth of all three (Fig. 2B).

*Rottlerin-Induced Growth Inhibition Is Independent of Apoptosis*

To determine the mechanism of rottlerin-induced cell growth inhibition, we determined the extent of apoptosis induced in response to rottlerin in MDA-Panc28 cells. Under optimal conditions (4 μmol/L rottlerin treatment), although rottlerin

induced a 49% inhibition in cell growth, it failed to promote any apoptosis in MDA-Panc28 cells as revealed by the failure of cells to accumulate in the sub-G<sub>1</sub> phase (Fig. 3A). Moreover, rottlerin-treated cells failed to show any poly(ADP-ribose) polymerase cleavage (Fig. 3B) and Annexin V positivity at 96 h (Fig. 3C) and 7 days (data not shown), further supporting the contention that rottlerin-induced inhibition of cell growth in pancreatic cancer cells was independent of apoptosis. Morphologic changes induced by rottlerin in MDA-Panc28 cells are shown in Fig. 3D. After 48 h of treatment, cells seemed to lose cell-to-cell contact and the cytoplasmic vacuoles started to become apparent. After 96 days of treatment, extensive vacuolization with some cell damage started to become evident

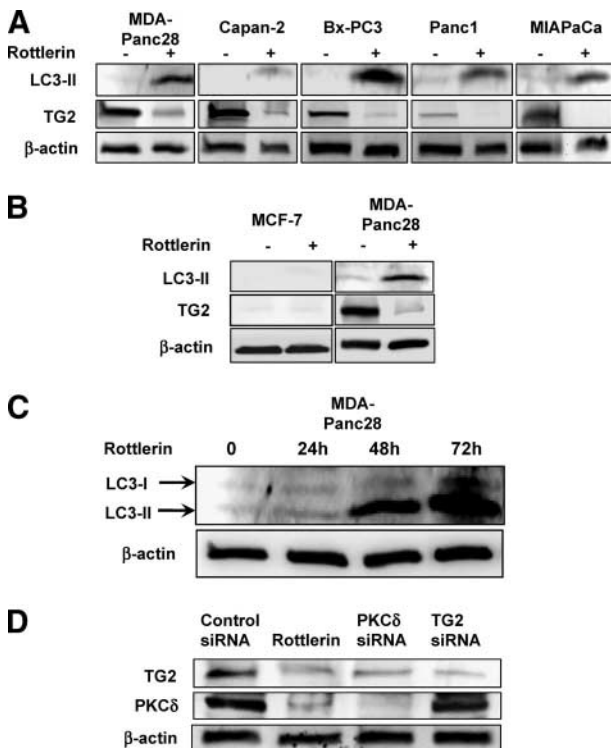


**FIGURE 3.** Rottlerin did not induce apoptosis. **A.** FACS analysis of the sub-G<sub>1</sub> population in MDA-Panc28 cells showed that treatment with 4 μmol/L rottlerin for 48 h did not cause cells to accumulate in this phase, indicating a lack of apoptosis. **B.** Rottlerin did not induce poly(ADP-ribose) polymerase (PARP) cleavage by Western blot analysis. Poly(ADP-ribose) polymerase cleavage-positive cell lysate was used as a positive control. **C.** Rottlerin treatment (96 h; middle) did not induce Annexin V detected by FACS analysis in Panc28 cells. Annexin V was not detected 7 d after rottlerin treatment (data not shown). Right, N-(4-hydroxyphenyl)retinamide-treated cells were used as positive control for Annexin V expression. **D.** Morphologic changes after treatment with rottlerin. Microphotographs were taken using a phase-contrast microscope. Magnification, ×300. Left, untreated cells at 48 h of treatment; middle, cells treated for 96 h; right, cells treated for 7 d (168 h).

(Fig. 3D, *middle*), and at day 7 (168 h), most cells became rounded and detached from plastic surfaces and lost cellular integrity and clear indication of cellular damage was observed compared with untreated controls (Fig. 3C, *left*).

#### Down-Regulation of TG2 Induces Autophagy

We next determined the effect of rottlerin-induced down-regulation of TG2 on accumulation of microtubule-associated protein 1 light chain 3 (LC3)-II protein, a hallmark of autophagy. LC3, the homologue of the yeast *Apg8/Aut7p* gene, localizes on the autophagosomal membrane during autophagy (21-23). Results shown in Fig. 4A showed a significant increase in endogenous LC3-II accumulation in MDA-Panc28, Capan-2, Bx-PC3, Panc1, and MIAPaCa cells treated with rottlerin at a concentration of 4  $\mu\text{mol/L}$  for 48 h. In all pancreatic cancer cell lines tested, the rottlerin-induced increase in LC3-II protein was associated with a parallel decrease in TG2 expression (Fig. 4A). Therefore, we next elucidated the possible involvement of TG2 in rottlerin-induced autophagy. First, we determined whether rottlerin treatment induces autophagy in the TG2-deficient breast cancer cells MCF-7 (10). Treatment of MCF-7 cells with rottlerin under optimal conditions failed to produce any increase in LC3-II accumulation (Fig. 4B), indicating that the induction of autophagy by rottlerin involved TG2. Figure 4C represents time-dependent induction of LC3-II protein (the lower band) expression but not LC3-I in Panc28 cells.



**FIGURE 4.** Induction of autophagy in pancreatic cancer cells. **A.** A panel of pancreatic cancer cell lines, including MDA-Panc28, Capan-2, Bx-PC3, Panc1, and MIAPaCa cells, was treated with rottlerin (4  $\mu\text{mol/L}$ ) for 48 h for the detection of LC3-II and TG2 expression by Western blot. **B.** MCF-7 cell line was also tested for LC3-II and TG2 expression by Western blot. **C** and **D.** Expression of TG2 in MDA-Panc28 cells was determined on exposure to different treatments.

Another important finding was that knockdown of PKC $\delta$  expression by a specific siRNA in MDA-Panc28 cells led to the down-regulation of TG2 protein (Fig. 4D), further showing that PKC $\delta$  regulates TG2 expression. We next determined whether TG2 mediates PKC $\delta$ -induced inhibition of autophagy. To determine whether TG2 is directly involved in the regulation of autophagy in MDA-Panc28 cells, we knocked down TG2 expression with siRNA. Figure 4C shows that TG2 siRNA specifically down-regulates TG2 protein expression. We found that the inhibition of TG2 expression by rottlerin or by TG2 siRNA in MDA-Panc28 cells resulted in formation of autophagic vacuoles by phase-contrast microscopy (Fig. 5A, *left*), marked increase in the number of acidic vesicular organelles detected by acridine orange staining (Fig. 5A, *middle*), and accumulation of green fluorescent protein (GFP)-LC3-II protein in autophagosomes in GFP-LC3 plasmid-transfected MDA-Panc28 cells (Fig. 5A, *right*). When autophagy is induced, LC3-II, a cleaved product of LC3, specifically localizes to the membrane of autophagosomes (21, 22, 24). Therefore, accumulation of GFP-LC3 in the vacuoles following rottlerin and TG2 siRNA treatments indicates formation of autophagosomes and induction of autophagy in the cells. In control siRNA-treated cells, none of the changes were observed by any of the assays. Fluorescence microscopy (Fig. 5A, *middle*) and flow cytometry (Fig. 5B and C) of rottlerin-treated or TG2 siRNA-transfected MDA-Panc28 cells stained with acridine orange revealed massive accumulation of acidic vesicular organelles representing formation of autophagosomes. Quantification of acidic vesicular organelles by flow cytometry revealed that the percentage of red fluorescein-positive cells in rottlerin-treated cells (64%) and TG2 knockdown cells (43%) was significantly higher than in the control cells (4%; Fig. 5B and C). Knockdown of Beclin-1, the product of autophagy-promoting gene *Becn1* (*Atg6*), inhibits rottlerin-induced or TG2 siRNA-induced autophagy as indicated by reduction in acridine orange positivity (Fig. 5B and C) and expression of LC3-II (Fig. 5D) in pancreatic cancer cells.

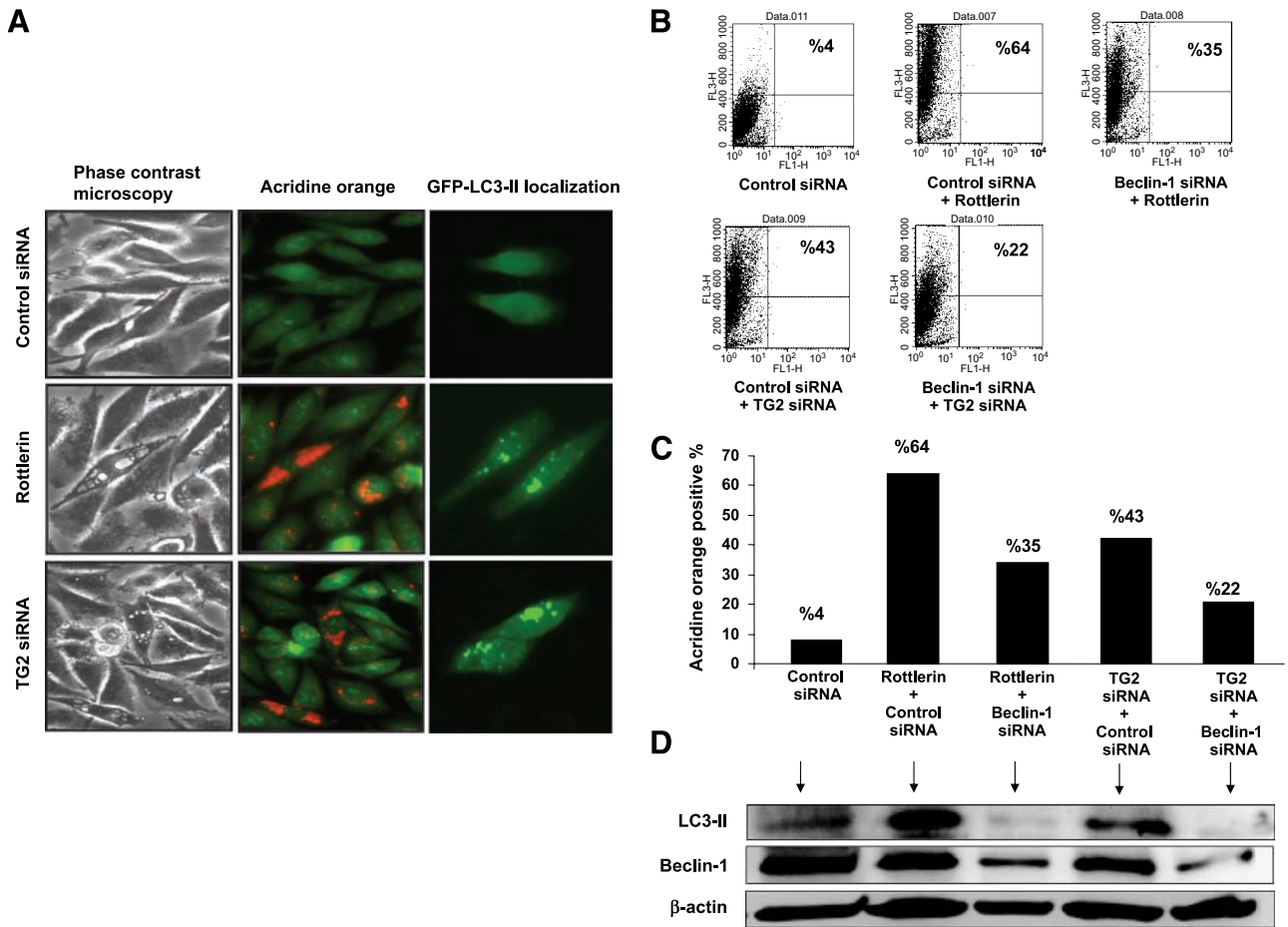
We further analyzed induction of autophagy by transmission electron microscopy, which clearly showed formation of autophagosomes containing cellular organelles after rottlerin or TG2 siRNA treatments in pancreatic cancer cells but not in control siRNA-treated cells (Fig. 6). Rottlerin-treated or TG2 siRNA-treated cells show formation of autophagic vesicles, merging of autophagic vesicles with lysosomes and mitochondria and cellular organelles, and lysed cellular content in the autophagosomes, indicating activity of lysosomal function and degradation.

These results further supported the observations that inhibition of TG2, either directly by siRNA transfection or indirectly by inhibition of PKC $\delta$  with rottlerin, induces the autophagic death in pancreatic cancer cells. Overall, these results suggest that PKC $\delta$  regulates autophagy through expression of TG2, and PKC $\delta$ -induced increase in TG2 expression confers protection to the pancreatic cancer cells against autophagy.

#### PKC $\delta$ /TG2-Mediated Autophagy Is Independent of the Mammalian Target of Rapamycin Pathway

Because mammalian target of rapamycin (mTOR) has been shown to regulate autophagy, we sought to determine whether





**FIGURE 5.** A. Detection of autophagolysosomes. MDA-Panc28 cells were treated with either control siRNA, rottlerin, or TG2 siRNA for 48 h. Left, photomicrographs show morphologic changes of the cells by the phase-contrast microscopy; middle, acridine orange staining of the acidic vesicular organelles by fluorescent microscopy; right, localization LC3-II at autophagosomes in cells after transfection with GFP-LC3 plasmid. B and C. Quantitative detection of acidic vesicular organelles by acridine orange staining in cells using FACS analysis. Cells transfected with either control siRNA, rottlerin, or TG2 siRNA had increased positivity. Beclin-1 siRNA inhibited induction of acridine orange-positive cells after rottlerin or TG2 siRNA treatments. Green fluorescence intensity indicates cytoplasm and nucleus, whereas red color intensity shows acidic vesicular organelles. D. Knockdown of Beclin-1, the product of autophagy-promoting gene *Becn1* (*Atg6*), inhibits rottlerin-induced or TG2 siRNA-induced LC3-II expression detected by Western blot analysis in pancreatic cancer cells.

rottlerin induces autophagy by inhibiting mTOR as well as TG2. We found that rottlerin treatment down-regulated phosphorylated mTOR (Fig. 7A) and phosphorylated p70S6K, a downstream target of mTOR, in MDA-Panc28 cells (data not shown). To further determine whether rottlerin-induced down-regulation of mTOR was a cause of autophagy, we investigated the effect of rapamycin, a specific inhibitor of mTOR, in MDA-Panc28 cells. Rapamycin failed to induce autophagy in these cells (Fig. 7B), but down-regulation of TG2 by a TG2-specific siRNA induced autophagy as observed in the previous experiments, indicating that rottlerin-induced autophagy is not mediated by mTOR.

**Discussion**

The present study provides first evidence that TG2 expression can regulate autophagy in pancreatic cancer cells. Our data show that PKC $\delta$  plays a critical role in the expression of TG2 and that increased expression of TG2 plays an important role in

preventing pancreatic cancer cells from undergoing autophagy (Fig. 8).

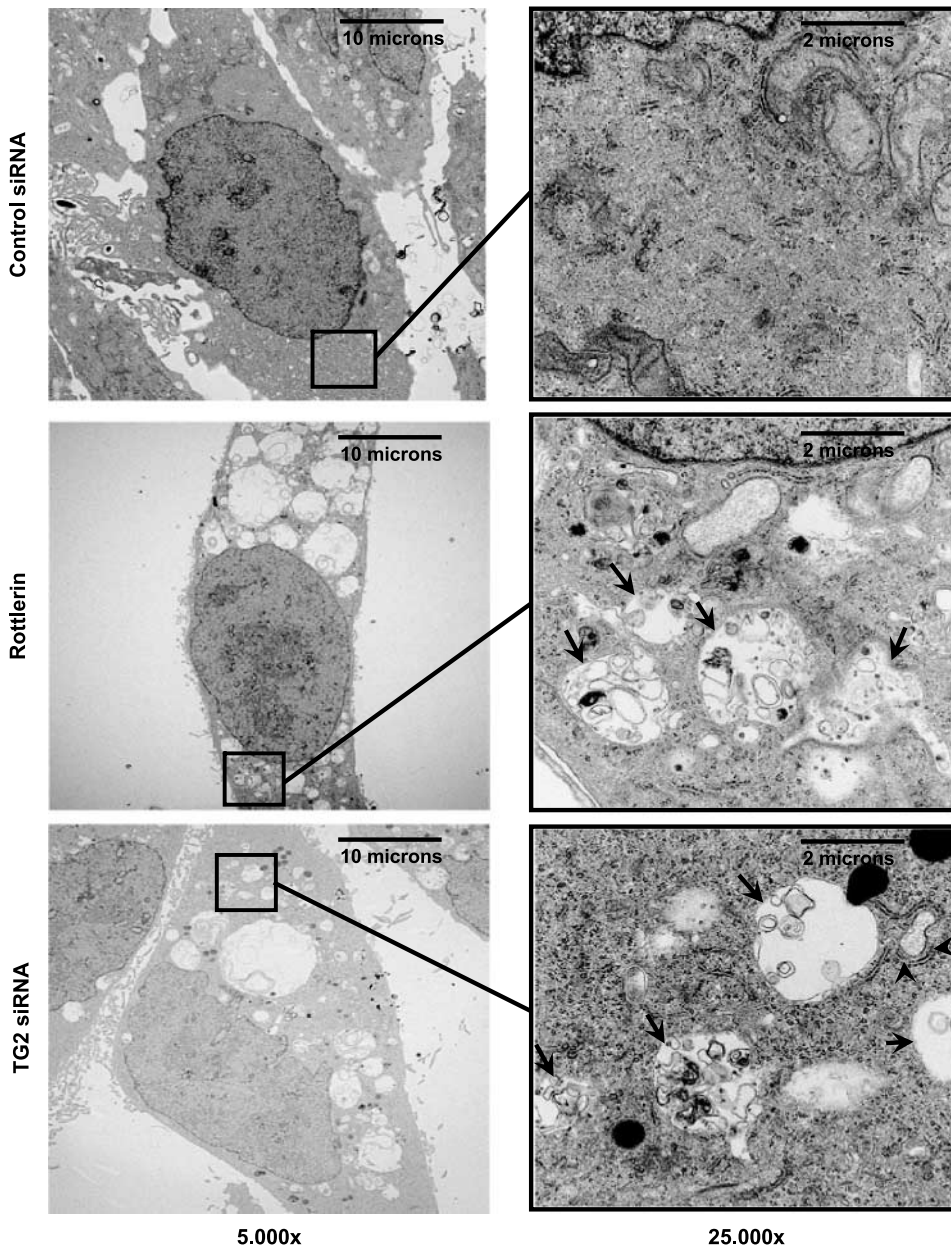
Apoptosis and autophagy are both highly regulated forms of programmed cell death and play crucial roles in such physiologic processes as the development, homeostasis, and elimination of unwanted cells. Autophagy is characterized by an increase in the number of autophagosomes, vesicles that surround such cellular organelles as Golgi complexes, polyribosomes, and endoplasmic reticulum (25). Subsequently, autophagosomes merge with lysosomes and digest the organelles, leading to cell death (26). In contrast to apoptosis, autophagic cell death is caspase independent and does not involve classic DNA laddering (25). Cancer cells in general tend to undergo less autophagy than their normal counterparts, supporting the contention that defective autophagic cell death plays a role in the process of carcinogenesis (27). In fact, studies of carcinogen-induced pancreatic cancer in animal models have shown that pancreatic adenocarcinoma cells have lower autophagic capacity than premalignant cells

(24). However, the mechanisms underlying the inhibition of autophagy in cancer cells have not been well characterized.

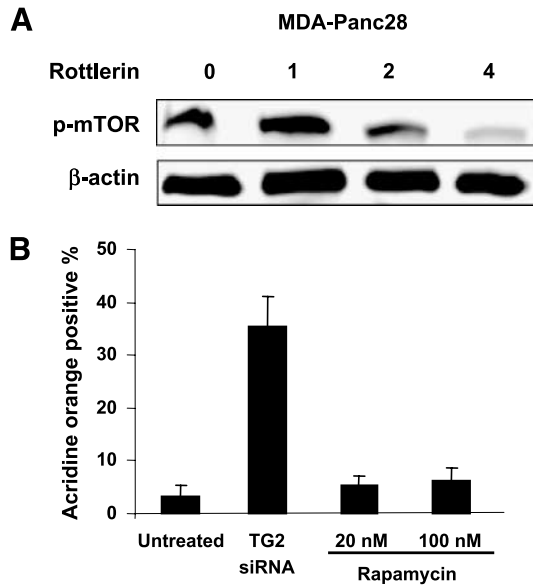
The data presented here suggest that down-regulation of PKC $\delta$  by specific inhibitor rottlerin led to autophagy of pancreatic cancer cells. These results suggest a clear link between PKC $\delta$  and autophagy. Notably, down-regulation of PKC $\delta$  by either rottlerin or siRNA was accompanied by a parallel decrease in TG2 expression and the induction of autophagy, suggesting a possible link between PKC $\delta$ , TG2 expression, and autophagy (Fig. 8). Indeed, our earlier studies showed that elevated TG2 expression in a variety of tumor cells and tumor cell lines contributes to the development of resistance to apoptosis (28). Our current data, showing that inhibition of TG2 expression by siRNA resulted

in autophagy in pancreatic cells, may help explain these earlier findings.

The phosphatidylinositol 3-kinase-Akt-mTOR pathway, which is activated in many cancer types, has been shown to suppress autophagy in cancer cells (29). Rapamycin, an inhibitor of mTOR, induces autophagy (30, 31). In the present study, we also found that inhibition of PKC $\delta$  by rottlerin was associated with down-regulation of mTOR protein in MDA-Panc28 cells during the induction of autophagy. However, inhibition of mTOR by rapamycin did not result in autophagy, suggesting that the mTOR pathway, at least in the cells studied here, is not involved in autophagy. For instance, Beclin-1 protein functions as a stimulator of autophagy in breast cancer cells, and its inactivation in mice (Becn1<sup>+/-</sup>) was found to



**FIGURE 6.** Electron micrographs showing the ultrastructure of Panc28 cells treated with rottlerin (4  $\mu$ mol/L) or TG2 siRNA for 96 h. Numerous autophagic vacuoles (arrows) were observed in the rottlerin-treated and TG2 siRNA-treated cells.



**FIGURE 7.** Rottlerin inhibits mTOR activity in the MDA-Panc28 cancer cell line but does not induce autophagy via the mTOR pathway. **A.** Phosphorylated mTOR (*p-mTOR*) was detected after 48 h of treatment with various doses of rottlerin, and cells were subjected to Western blot analysis. Phosphorylation of mTOR was inhibited in a dose-dependent manner. **B.** MDA-Panc28 cells were treated with a specific mTOR inhibitor, rapamycin (at either 20 or 100 nmol/L), or TG2 siRNA or were left untreated. FACS analysis of cells stained with acridine orange to detect acidic vesicular organelles showed that rapamycin had no effect on autophagy, indicating that cell death was not induced via the mTOR pathway.

markedly increase the incidence of tumors, including lung cancer, hepatocellular carcinoma, and lymphoma (32, 33). To determine whether Beclin-1 plays a role in autophagy that is induced by down-regulation of TG2 by rottlerin and TG2 siRNA, we knocked down Beclin-1 expression. Our data showed that knockdown of Beclin-1 inhibited rottlerin-induced and TG2 siRNA-induced autophagy (Fig. 5B and C), indicating that Beclin-1 mediates the autophagy observed in MDA-Panc28 cancer cells.

Results from previous studies suggest that TG2 can exert both proapoptotic and antiapoptotic effects depending on the cell type (6, 34). TG2 expression is up-regulated in various types of cancer cells, and it has been implicated in resistance to stress-induced apoptosis (28). However, in some cell lines, among them leukemia, cervical adenocarcinoma, and neuroblastoma cell lines, TG2 expression has been shown to facilitate the induction of apoptosis (35). Inhibition of TG2 by stable transfection with antisense or transient transfection with siRNA has been shown to restore sensitivity of cancer cells to chemotherapeutic drugs (9, 11). These observations suggest that TG2 expression contributes to cellular resistance to chemotherapy (9). Furthermore, lymph node metastases from breast cancers were found to express significantly higher levels of TG2 than the primary tumors, suggesting that TG2 is involved in increasing the metastatic potential of breast cancer cells (10).

The prognosis of patients with pancreatic cancer remains extremely poor. In part, this poor outlook may relate to molecular abnormalities that stimulate pancreatic tumorigenesis

and that also contribute to reducing sensitivity of cancer cells to standard treatments, such as chemotherapy and radiotherapy. Previously published data and those from the present study suggest that defective regulation of autophagy and its activation play important roles in tumorigenesis and the induction of cancer cell death, respectively. Targeting of PKC, TG2, or both may be an effective and novel approach to therapy in patients with pancreatic cancer. Thus, identifying molecules that induce autophagy may be valuable in the development of novel agents against this devastating disease.

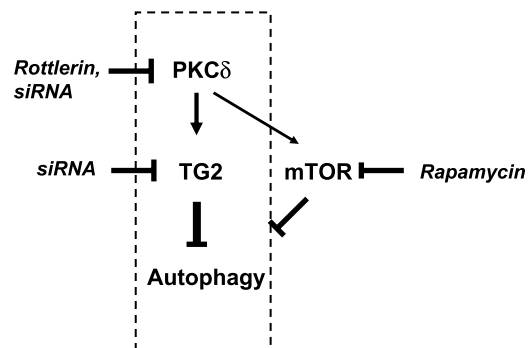
### Materials and Methods

#### Cell Lines, Culture Conditions, and Reagents

Pancreatic cell lines MDA-Panc28, Capan-2, Bx-PC3, Panc1, and MIAPaCa were provided by Dr. Shrikant Reddy (M. D. Anderson Cancer Center, Houston, TX). MDA-Panc28 and Bx-PC3 cell lines were maintained in RPMI 1640 supplemented with 10% FCS. Panc1 and MIAPaCa cell lines and Capan-2 cells were maintained in DMEM and McCoy's 5A, respectively. All media were supplemented with 10% FCS. For the cell proliferation experiments, cells were seeded at a density of  $1 \times 10^5$  to  $2 \times 10^5$  in T-25 tissue culture flasks. Twenty-four hours after the seeding, cells were treated with various concentrations of rottlerin for the indicated periods. Adherent cells were collected by trypsinization, and cell numbers were determined using a Neubauer cell counting chamber. All experiments were replicated at least twice.

#### Western Blot Analysis

After treatment, the cells were collected and centrifuged, and whole-cell lysates were obtained using a lysis buffer. Total protein concentration was determined using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). Rottlerin and mTOR inhibitor (rapamycin) were purchased from Calbiochem (La Jolla, CA). Aliquots containing 30  $\mu$ g of total protein from each sample were subjected to SDS-PAGE with a 4% to 20% gradient and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBS-Tween 20, probed with primary antibodies diluted in



**FIGURE 8.** Model for the role and regulation of TG2 by PKC $\delta$  in pancreatic cancer cells. PKC $\delta$  plays a role in the constitutive expression of TG2. Expression of TG2 plays an important role in preventing pancreatic cancer cells from undergoing autophagy. Activation of mTOR pathway inhibits autophagy in some tumor cell lines but not in pancreatic cancer cells. Dashed line, events taking place in pancreatic cancer cells.



TBS-Tween 20 containing 2.5% dry milk, and incubated at 4°C overnight. We used primary antibodies against PKC $\delta$  and phosphorylated mTOR (Ser<sup>2448</sup>) from Cell Signaling Technology (Beverly, MA) and TG2 antibody from NeoMarkers (Fremont, CA). Anti-LC3 antibody against a synthetic peptide corresponding to the NH<sub>2</sub>-terminal 14 amino acids of the isoform B of human LC3 and an additional cysteine (PSEKTFKQRRTFEQC) was prepared by immunization of a rabbit and affinity purified on an immobilized peptide-Sepharose column (Covance, Denver, PA; refs. 36, 37). After being washed, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Amersham Life Science, Cleveland, OH). Mouse anti- $\beta$ -actin and donkey anti-mouse secondary antibodies were purchased from Sigma Chemical (St. Louis, MO) so that  $\beta$ -actin expression could be monitored to ensure equal loading of proteins. Chemiluminescent detection was done with ChemiGlow (Alpha Innotech, San Leandro, CA) detection reagents. The blots were visualized with a FluorChem 8900 imager and quantified by a densitometer using the Alpha Imager application program (Alpha Innotech). All experiments were independently repeated at least twice.

#### RNA Isolation and RT-PCR Analysis

Cells were collected for isolation of total RNA. Total cellular RNA was isolated with Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA), and cDNA was obtained from 5  $\mu$ g of total RNA using a SuperScript II reverse transcriptase kit (Invitrogen/Life Technologies). Briefly, 5  $\mu$ L of the total 20  $\mu$ L of reverse-transcribed product were used for PCR in 1 $\times$  PCR buffer containing 1.5 mmol/L MgCl<sub>2</sub>, 250  $\mu$ mol/L deoxynucleotide triphosphates, 0.5 unit of Taq polymerase (Invitrogen/Life Technologies), and 100 ng of TG2 primer (primer I, 5'-TAGGCCAGTGTGGTCTTCGCC-3'; primer II, 5'-GGTC-CAGGGTTAGGTTGAGCAGG-3') or PKC $\delta$  primers (primer I, 5'-CGAAGAGTTCATCCTCATCATA and primer II, 5'-TTTC-TCACCCACCTCATCTG) or  $\beta$ -actin-specific primer (Sigma-Genosys, Houston, TX). Semiquantitative RT-PCRs were done using different cycles of PCR (26, 30, 36) to find optimum PCR cycle that falls in the linear range of amplification. For the TG2 and PKC experiments, 30 PCR cycles were used. The reaction products were analyzed on a 2% agarose gel containing ethidium bromide, and cDNA synthesis was verified by detection of the  $\beta$ -actin transcript, which was used as an internal control.

#### Evaluation of Acidic Vesicular Organelles

To detect and quantify acidic vesicular organelles, cells were stained with acridine orange as described previously (38). The number of acridine orange-positive cells was determined by fluorescence-activated cell sorting (FACS) analysis. Cell morphology was examined using phase-contrast and fluorescence microscopy (Nikon, Melville, NY) with the cells remaining in their culture flasks.

#### Transfections with siRNA and GFP-LC3 Plasmid

Exponentially growing untreated MDA-Panc28 cells were plated 24 h before transfection. Plated cells were transfected with double-stranded siRNA targeting TG2 mRNA (Qiagen,

Venlo, the Netherlands), PKC $\delta$  (Santa Cruz Biotechnology, Santa Cruz, CA), and Beclin-1 (Santa Cruz Biotechnology) and control (nonsilencing) siRNA (Qiagen) and/or the GFP-LC3 fusion vector (22, 38) using the Qiagen transfection reagent according to the manufacturer's protocol. Two siRNA sequences targeting TG2 were designed using siRNA-designing software (Qiagen). PKC $\delta$  siRNA was obtained from Santa Cruz Biotechnology. Untransfected cells and nonsilencing control siRNA-transfected cells were used as negative controls. After treatment, the cells were harvested for Western blot analysis or FACS analysis to determine whether autophagy had occurred.

#### Flow Cytometric Analysis of Apoptosis

One of the earliest changes of apoptosis is that the membrane phospholipid PS translocates from the inner to the outer leaflet of the membrane. Thus, PS is exposed to the external membrane and can be detected using PS-binding protein, such as Annexin V (39). To provide a comparative assay of apoptosis by Annexin V labeling, tumor cells ( $1 \times 10^6$ ) treated with rottlerin, TG2 siRNA, or control siRNA for 4 days were harvested, washed, fixed with ice-cold 70% ethanol (50 min, 4°C), and resuspended in binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub>]. FITC-Annexin V (50  $\mu$ L; R&D Systems, Minneapolis, MN) was added and incubated for 15 min in the dark at room temperature before flow cytometric analysis.

#### Transmission Electron Microscopy

Panc28 cells were grown on six-well plates, treated with rottlerin, TG2 siRNA, or control siRNA, fixed for 2 h with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4), and postfixed in 1% OsO<sub>4</sub> in the same buffer and then subjected to the electron microscopic analysis as described previously (40). Representative areas were chosen for ultrathin sectioning and viewed with a Hitachi 7600 electron microscope (Japan).

#### Acknowledgments

We thank Kenneth Dunner, Jr. for his assistance about electron microscopy.

#### References

- Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* 2003;4:140–56.
- Mhaouty-Kodja S. Gho/tissue transglutaminase 2: an emerging G protein in signal transduction. *Biol Cell* 2004;96:363–7.
- Hasegawa G, Suwa M, Ichikawa Y, et al. A novel function of tissue-type transglutaminase: protein disulphide isomerase. *Biochem J* 2003;373:793–803.
- Mishra S, Murphy LJ. The p53 oncoprotein is a substrate for tissue transglutaminase kinase activity. *Biochem Biophys Res Commun* 2006;339:726–30.
- Kang SK, Kim DK, Damron DS, Baek KJ, Im MJ. Modulation of intracellular Ca(2+) via  $\alpha$ (1B)-adrenoreceptor signaling molecules, G $\alpha$ (h) (transglutaminase II) and phospholipase C- $\delta$ 1. *Biochem Biophys Res Commun* 2002;293:383–90.
- Fesus L, Szondy Z. Transglutaminase 2 in the balance of cell death and survival. *FEBS Lett* 2005;579:3297–302.
- Zemskov EA, Janiak A, Hang J, Waghay A, Belkin AM. The role of tissue transglutaminase in cell-matrix interactions. *Front Biosci* 2006;11:1057–76.
- Oliverio S, Amendola A, Di Sano F, et al. Tissue transglutaminase-dependent posttranslational modification of the retinoblastoma gene product in promonocytic cells undergoing apoptosis. *Mol Cell Biol* 1997;17:6040–8.
- Herman JF, Mangala LS, Mehta K. Implications of increased tissue



- transglutaminase (TG2) expression in drug-resistant breast cancer (MCF-7) cells. *Oncogene* 2006;25:3049–58.
10. Mehta K, Fok J, Miller FR, Koul D, Sahin AA. Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. *Clin Cancer Res* 2004;10:8068–76.
  11. Han JA, Park SC. Reduction of transglutaminase 2 expression is associated with an induction of drug sensitivity in the PC-14 human lung cancer cell line. *J Cancer Res Clin Oncol* 1999;125:89–95.
  12. Elsasser HP, MacDonald R, Dienst M, Kern HF. Characterization of a transglutaminase expressed in human pancreatic adenocarcinoma cells. *Eur J Cell Biol* 1993;61:321–8.
  13. Iacobuzio-Donahue CA, Ashfaq R, Maitra A, et al. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res* 2003;63:8614–22.
  14. Mann AP, Verma A, Sethi G, et al. Overexpression of tissue transglutaminase leads to constitutive activation of nuclear factor- $\kappa$ B in cancer cells: delineation of a novel pathway. *Cancer Res* 2006;66:8788–95.
  15. Verma A, Wang H, Manavathi B, et al. Increased expression of tissue transglutaminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis. *Cancer Res* 2006;66:10525–33.
  16. Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. *Cancer Lett* 2006;235:1–10.
  17. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ. Fatty acid and phorbol ester-mediated interference of mitogenic signaling via novel protein kinase C isoforms in pancreatic  $\beta$ -cells (INS-1). *J Mol Endocrinol* 2003;30:271–86.
  18. Jackson DN, Foster DA. The enigmatic protein kinase C $\delta$ : complex roles in cell proliferation and survival. *FASEB J* 2004;18:627–36.
  19. La Porta CA, Di Dio A, Porro D, Comolli R. Overexpression of novel protein kinase C $\delta$  in BL6 murine melanoma cells inhibits the proliferative capacity *in vitro* but enhances the metastatic potential *in vivo*. *Melanoma Res* 2000;10:93–102.
  20. Gschwendt M, Muller HJ, Kielbassa K, et al. Rottlerin, a novel protein kinase inhibitor. *Biochem Biophys Res Commun* 1994;199:93–8.
  21. Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *EMBO J* 2000;19:5720–8.
  22. Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, Kondo S. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res* 2004;64:4286–93.
  23. Kirisako T, Baba M, Ishihara N, et al. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol* 1999;147:435–46.
  24. Toth S, Nagy K, Palfia Z, Rez G. Cellular autophagic capacity changes during azaserine-induced tumour progression in the rat pancreas. Up-regulation in all premalignant stages and down-regulation with loss of cycloheximide sensitivity of segregation along with malignant transformation. *Cell Tissue Res* 2002;309:409–16.
  25. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005;5:726–34.
  26. Kroemer G, Jaattela M. Lysosomes and autophagy in cell death control. *Nat Rev Cancer* 2005;5:886–97.
  27. Edinger AL, Thompson CB. Defective autophagy leads to cancer. *Cancer Cell* 2003;4:422–4.
  28. Mehta K, Fok JY, Mangala LS. Tissue transglutaminase: from biological glue to cell survival cues. *Front Biosci* 2006;11:173–85.
  29. Arico S, Petiot A, Bauvy C, et al. The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 2001;276:35243–6.
  30. Takeuchi H, Kondo Y, Fujiwara K, et al. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. *Cancer Res* 2005;65:3336–46.
  31. Paglin S, Lee NY, Nakar C, et al. Rapamycin-sensitive pathway regulates mitochondrial membrane potential, autophagy, and survival in irradiated MCF-7 cells. *Cancer Res* 2005;65:11061–70.
  32. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;402:672–6.
  33. Pattingre S, Tassa A, Qu X, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005;122:927–39.
  34. Sarang Z, Molnar P, Nemeth T. Tissue transglutaminase (TG2) acting as G protein protects hepatocytes against Fas-mediated cell death in mice. *Hepatology* 2005;42:578–87.
  35. Oliverio S, Amendola A, Rodolfo C, Spinedi A, Piacentini M. Inhibition of “tissue” transglutaminase increases cell survival by preventing apoptosis. *J Biol Chem* 1999;274:34123–8.
  36. He H, Dang Y, Dai F, et al. Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B. *J Biol Chem* 2003;278:29278–87.
  37. Tanida I, Ueno T, Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met<sup>121</sup> to expose Gly<sup>120</sup> for lipidation and targeting to autophagosomal membranes. *J Biol Chem* 2004;279:47704–10.
  38. Daido S, Yamamoto A, Fujiwara K, Sawaya R, Kondo S, Kondo Y. Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. *Cancer Res* 2005;65:4368–75.
  39. Lu L, Osmond DG. Apoptosis during B lymphopoiesis in mouse bone marrow. *J Immunol* 1997;158:5136–45.
  40. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004;11:448–57.