(−)−Epigallocatechin Gallate Overcomes Resistance to Etoposide-Induced Cell Death by Targeting the Molecular Chaperone Glucose-Regulated Protein 78

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Abstract

Many beneficial properties have been attributed to (−)−epigallocatechin gallate (EGCG), including chemopreventive, anticarcinogenic, and antioxidant actions. In this study, we investigated the effects of EGCG on the function of glucose-regulated protein 78 (GRP78), which is associated with the multidrug resistance phenotype of many types of cancer cells. Our investigation was directed at elucidating the mechanism of the EGCG and GRP78 interaction and providing evidence about whether EGCG modulates the activity of anticancer drugs through the inhibition of GRP78 function. We found that EGCG directly interacted with GRP78 at the ATP-binding site of protein and regulated its function by competing with ATP binding, resulting in the inhibition of ATPase activity. EGCG binding caused the conversion of GRP78 from its active monomer to the inactive dimer and oligomer forms. Further, we showed that EGCG interfered with the formation of the antiapoptotic GRP78-caspase-7 complex, which resulted in an increased etoposide-induced apoptosis in cancer cells. We also showed that EGCG significantly suppressed the transformed phenotype of breast cancer cells treated with etoposide. Overall, these results strongly suggested that EGCG could prevent the antiapoptotic effect of GRP78, which usually suppresses the caspase-mediated cell death pathways in drug-treated cancer cells, contributing to the development of drug resistance. (Cancer Res 2006; 66(18): 9260-9)

Introduction

The resistance of cancer cells to chemotherapeutic drugs remains a major obstacle for effective cancer treatment. The complexity of drug resistance in human cancer strongly suggests that the mechanisms of drug resistance are likely to involve multiple signaling pathways that differ within various cancer types. One important mechanism in cancer involves the aberrant induction of the chaperone protein glucose-regulated protein 78 (GRP78), which is correlated with resistance to chemotherapeutic agents, including doxorubicin, etoposide, and Adriamycin (1–3). Overexpression, antisense, and ribozyme approaches in cell culture systems have directly shown that GRP78 can protect cells against apoptotic death by preventing the formation of a functional apoptosome (3–5). GRP78 also complexes with caspase-7 to form an antiapoptotic complex (2, 6). Whereas GRP78 overexpression can limit damage in normal tissue and organs exposed to endoplasmic reticulum (ER) stress, the antiapoptotic function of GRP78 also predicts that the aberrant induction of GRP78 in neoplastic cells can lead to cancer progression and drug resistance (3, 7). In human cancers, including breast (8), gastric (9), and hepatocellular carcinomas (10), enhanced GRP78 expression has been correlated with greater malignancy and with the development of anticancer drug resistance (2, 11). A GRP78 mutant defective in ATP hydrolysis, which is required for GRP78 chaperone activity, was reported to lose its protective function against apoptosis (12). GRP78 is believed to confer resistance to etoposide-induced apoptosis based on the discovery that GRP78 exists as a transmembrane protein that physically interacts with caspase-7 to prevent apoptosis, a function that is dependent on the GRP78 ATP-binding domain (2).

Although GRP78 is constitutively expressed in many cell types, its synthesis can be induced by a variety of stressful conditions, including glucose deprivation, treatment with Ca^{2+} ionophores, oxidative stress, and hypoxia (13, 14). Solid tumors usually contain regions that are exposed to glucose deprivation and hypoxia because of poor vascularization, resulting in acidosis and alterations in cell metabolism (15). Elevated levels of GRP78 seem to be responsible for maintaining the viability of cells that are subjected to stresses. For example, induction of stress proteins in tumor cells has been shown to protect them against cell death and immune attack and also confer drug resistance (16–18). Thus, the induction of GRP78 activity can be an effective defense mechanism to enhance cancer cell survival.

(−)−Epigallocatechin gallate (EGCG) is a major component in green tea, and its inhibitory activity against tumorigenesis has been shown (19–22). The mechanisms of action of EGCG explaining its anticancer effects are not very well understood but are being intensively investigated. Previous studies by other investigators have suggested that EGCG is synergistically cytotoxic to human cancer cells through modulating P-glycoprotein and the estrogen receptor (23, 24). On the other hand, the identification of proteins interacting directly with EGCG is a key step in understanding the molecular mechanisms of the anticancer effects of EGCG. Several proteins that can directly bind with EGCG have been identified, including fibronectin, fibrinogen, histidine-rich glycoprotein, laminin (25), Fas (26), matrix metalloproteinase (MMP)-2 and MMP-9 (27), Bcl-2 (28), the 67-kDa laminin receptor (29), and vimentin (30). In the present study, we provide new evidence showing that EGCG prevents the antiapoptotic role of GRP78, a function that is associated with the suppression of GRP78 of the caspase-mediated cell death pathways in drug-treated cells, contributing to the development of drug resistance.

Note: S.P. Ermakova and B.S. Kang contributed equally to this work.

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Materials and Methods

Materials. All media were obtained from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA). CNBr-Sepharose 4B was purchased from Amersham Biosciences (Piscataway, NJ). [^{3}H]EGCG (13 Ci/mmol in ethanol containing 8 mg/mL ascorbic acid) was a gift from Dr. Yukihiko Hara (Food Research Laboratory, Mitsui Norin Co. Ltd., Fujieda, Shizuoka, Japan). Recombinant GRP78 (rGRP78) was purchased from StressGen (Victoria, British Columbia, Canada), and the goat GRP78 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Etoposide was purchased from Calbiochem (San Diego, CA), thapsigargin was from LC Laboratories (Woburn, MA), and procaspase-7 (human recombinant) was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). EGCG, (−)−epicatechin (EC), (−)−epicatechin-3-gallate (ECG), and (−)−epigallocatechin (EGC) were generous gifts from Dr. Chi-Tang Ho (Rutgers University, Piscataway, NJ).

Cell culture. The JB6 Cl41 mouse epidermal cell line was grown in MEM supplemented with 5% FBS. The stable T24/83 human bladder carcinoma cell line overexpressing human GRP78 (T24/83-GRP78) or transfected with the empty expression vector pcDNA3.1 (T24/83-pcDNA) was kindly provided by Dr. Richard C. Austin (McMaster University and Hamilton Health Sciences, Hamilton, Ontario, Canada; ref. 31). These cell lines were maintained in 199 medium supplemented with 10% FBS containing 1% penicillin and 200 μg/mL G418. The MDA-MB-231 human breast cancer cell line was maintained in DMEM supplemented with 10% FBS. The T-47D human breast cancer cell line was maintained in RPMI 1640 adjusted to contain 0.2 unit/mL of bovine insulin and supplemented with 10% FBS.

Affinity chromatography, two-dimensional gel electrophoresis, and matrix-assisted laser desorption/ionization-time of flight analysis. EGCG was first coupled to the CNBr-activated Sepharose 4B matrix, and the binding between GRP78 and EGCG was examined by affinity chromatography according to the manufacturer's instructions, which corresponded to a method described previously (30). Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis were done as reported previously (30).

Construction of GRP78 plasmids. The eukaryotic expression plasmid pcDNA3 for human GRP78 was generously provided by Dr. Richard C. Austin. A cDNA encoding GRP78 was generated by PCR and subcloned into the *Bam*HI/*Xho*I sites of the pGEX-5X-1 vector (Amersham Biosciences) to produce the glutathione *S*-transferase (GST)-GRP78 fusion protein. The serial truncated deletion mutants of GRP78 were generated from pGEX-5X-1-GRP78 wild-type and also inserted in-frame into the *Bam*HI/*Xho*I sites of pGEX-5X-1.

Bacterial expression of GST fusion proteins. For expression of the full-length and deletion mutants of GRP78, the appropriate plasmids (pGEX-5X-1-GRP78 and deletion mutants for GST-GRP78) were expressed in *Escherichia coli* BL21. rGRP78 protein was purified with a protein refolding kit (Novagen, San Diego, CA), GSTrap FF (Amersham Biosciences), after digesting GST-GRP78 with Factor Xa.

***In vitro* EGCG-Sepharose 4B and ATP-agarose 4B pull-down assays.** rGRP78 (2 μg) or a JB6 Cl41 cellular supernatant fraction (600 μg) was incubated with EGCG-Sepharose 4B beads (100 μL, 50% slurry) in reaction buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 2 μg/mL bovine serum albumin, 0.02 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1× protease inhibitors]. Reduced GST-GRP78 or GST-GRP78 deletion mutants (2 μg) were incubated with EGCG-Sepharose 4B or ATP-agarose 4B beads in reaction buffer. After incubation with gentle rocking overnight at 4°C, the beads were washed five times with washing buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 0.02 mmol/L PMSF] and proteins bound to the beads were analyzed by immunoblotting with the appropriate antibodies.

Physical binding and K_d measurement. GRP78-binding assays were carried out as described (30) with some modifications. For analyzing the concentration-dependent uptake, 39 pmol/L to 20 mmol/L concentrations of EGCG were applied.

Immunoblotting and immunoprecipitation. Cells were disrupted in lysis buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 0.02 mmol/L PMSF, 1× protease inhibitors]. The proteins were resolved by SDS-PAGE (32) or nondenaturing gel electrophoresis and then subjected to Western blot analysis (33). The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4°C. Protein bands were visualized by the chemiluminescence detection kit (Amersham Biosciences) after hybridization with the horseradish peroxidase–conjugated secondary antibody. Immunoprecipitations were carried out as described previously (2).

ATPase assay. The ATPase activity of GRP78 was analyzed by measuring the free Pi liberated during ATP hydrolysis using the method of Seals et al. (34) with some modifications. Assays to determine inhibition of ATPase activity were done in the presence of GRP78 (3 μmol/L) and EGCG (1, 5, or 10 μmol/L) or in the presence of EGCG analogues (10 μmol/L).

Conversion of GRP78. Assays were done at 25°C in a 25 μL mixture containing reaction buffer [20 mmol/L HEPES (pH 7.0), rGRP78 (0.5 μg), different doses of ATP (1, 10, or 30 μmol/L), EGCG (1, 5, or 10 μmol/L), or both (30 μmol/L ATP plus 1, 5, or 10 μmol/L EGCG)]. After incubation for 30 minutes, 50 μL of nondenaturing sample buffer were added to stop the reaction and nondenaturing gel electrophoresis was used for detection of the different migration patterns of GRP78. JB6 Cl41 cells were treated with different concentrations of EGCG (1, 5, or 10 μmol/L), and proteins were separated under nondenaturing conditions for detection of the conformational conversion of GRP78.

Caspase-7-binding assay. For the GST pull-down assay, 2 μg GST, GST-GRP78, or GST-GRP78 deletion mutants were incubated overnight at 4°C with 0.1 μg of recombinant procaspase-7 in a 500 μL reaction mixture containing buffer (see *In vitro* pull-down assay) and different concentrations of EGCG (0, 1, or 2 μmol/L). After incubation with gentle rocking overnight at 4°C, the beads were washed five times with washing buffer (see *In vitro* pull-down assay) and proteins bound to the beads were analyzed by immunoblotting with a caspase-7 antibody.

Annexin V staining and fluorescence-activated cell sorting analysis. Apoptosis was evaluated using the Annexin V-FITC Apoptosis Detection kit from MBL International Corp. (Watertown, MA). Cells were harvested with 0.025% trypsin plus 5 mmol/L EDTA in PBS, and 2.5% FBS in PBS was added as soon as the cells were released from the dish. Then, the cells were transferred to a centrifuge tube, washed with PBS, and incubated for 5 minutes at room temperature with Annexin V-FITC plus propidium iodide following the protocol included in the kit. Cells were analyzed on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), placing the FITC signal in FL1 and the propidium iodide signal in FL2. Intact cells were gated in the forward/side scatter plot to exclude small debris.

Soft agar clonogenic assay. Soft agar assays were done on human breast adenocarcinoma cells MDA-MB-231 and T-47D. Cells (5×10^5) were plated in 10-cm dishes following cultured in DMEM with 10% FBS at 37°C. After incubation for 24 hours, the cells were pretreated with EGCG (10 μmol/L) for 2 hours and then further treated with etoposide (0.1 μmol/L) or etoposide with EGCG (10 μmol/L) for 48 hours. The cells were collected by trypsinization and subjected for soft agar clonogenic assay. The cell colonies were scored using a microscope and the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD) as described by Colburn et al. (35).

Data analysis. All figures shown in this article are representative of at least three independent experiments with similar results. Statistical differences were evaluated using the Student's *t* test and considered significant at $P \leq 0.05$.

Results

EGCG interacts with GRP78. To identify novel proteins that interact with EGCG, we did affinity chromatography using EGCG-Sepharose 4B beads and JB6 Cl41 cell lysate proteins. Fractions containing proteins bound with EGCG were analyzed by two-dimensional electrophoresis (Fig. 1*A*), and MALDI-TOF mass spectrometry was used to identify proteins that directly bind with

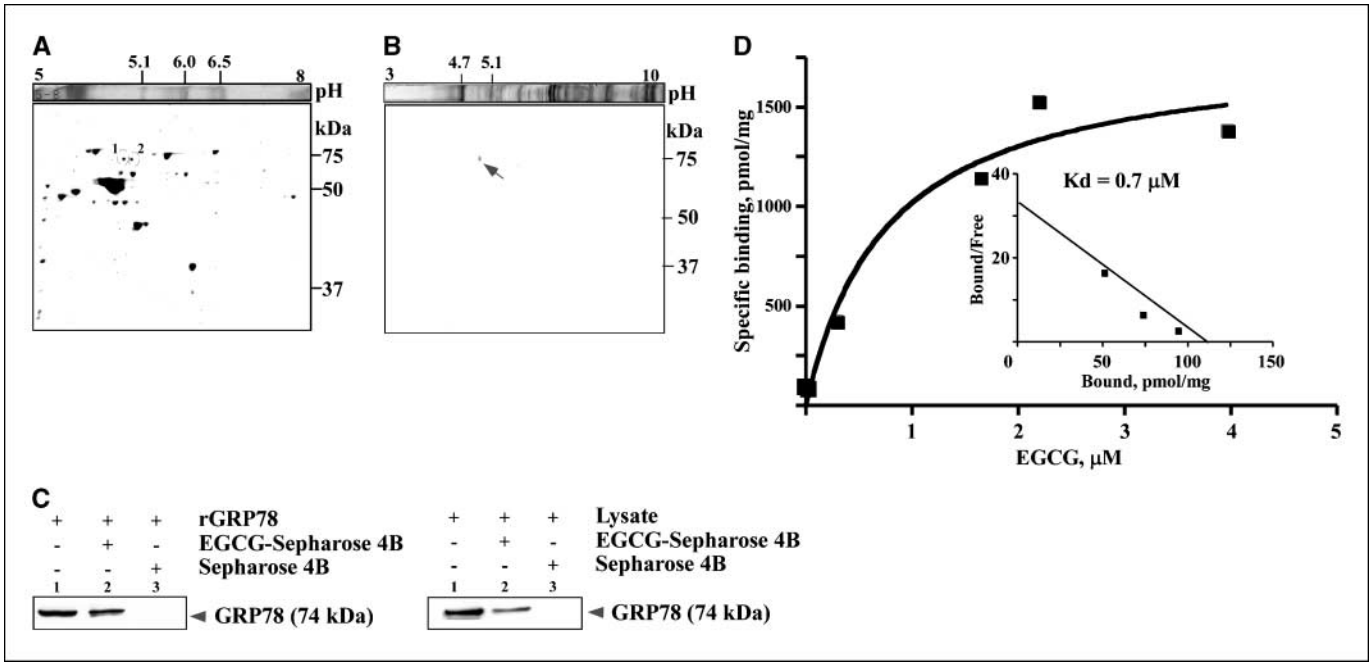

Figure 1. *In vitro* and *in vivo* identification of EGCG-bound GRP78. *A,* cell lysates were precipitated by EGCG-Sepharose 4B affinity chromatography and then analyzed by two-dimensional electrophoresis. *Dotted ring,* numbered spots pertaining to GRP78. Numbered protein spots were digested with trypsin, and the resulting peptides were analyzed by mass spectroscopy. *B,* proteins precipitated by EGCG-Sepharose 4B affinity chromatography were analyzed by two-dimensional electrophoresis followed by SDS-PAGE and Western blot using an antibody against GRP78. *Arrow,* GRP78-EGCG bound complexes. *C, left,* GRP78-EGCG binding *in vitro* was confirmed by immunoblotting using an antibody against GRP78. *Lane 1,* input control, GRP78 protein standard; *lane 2,* rGRP78 was pulled down using EGCG-Sepharose 4B affinity beads; *lane 3,* control, Sepharose 4B was used to pull down rGRP78 as described in Materials and Methods. *Right,* GRP78-EGCG binding *in vivo* was confirmed by immunoblotting using an antibody against GRP78. *Lane 1,* input control, whole-cell lysate from JB6 Cl41 cells; *lane 2,* whole-cell lysate from JB6 Cl41 cells was precipitated by EGCG-Sepharose 4B affinity chromatography as described in Materials and Methods; *lane 3,* control, a lysate of JB6 cells was precipitated with Sepharose 4B beads as described in Materials and Methods. *D,* specific binding assay of GRP78 and EGCG. The K_d (dissociation kinetic value) of the EGCG-GRP78 interaction was obtained by using a GST-GRP78 affinity binding assay as described in Materials and Methods.

EGCG. A search of the National Center for Biotechnology Information database revealed that two spots corresponded to GRP78 (Fig. 1*A, circled spots*).

The interaction of GRP78 and EGCG was assessed in an EGCG-Sepharose 4B affinity chromatography pull-down experiment and with subsequent detection by immunoblotting with anti-GRP78 (Fig. 1*B, arrow*). Further results also indicated not only *in vitro* binding of EGCG with rGRP78 (Fig. 1*C, left, lane 2*) but also *in vivo* binding of EGCG and GRP78 in JB6 Cl41 cell lysates (Fig. 1*C, right, lane 2*).

To characterize the physical binding between EGCG and GRP78, we determined the binding affinity (K_d) of this complex using a GST pull-down assay with radiolabeled EGCG. The K_d value of GRP78 and EGCG was found to be 0.7 μmol/L (Fig. 1*D*). Taken together, these data suggested that the interaction between EGCG and GRP78 was a direct and specific interaction.

Identification of the EGCG-binding site of GRP78. The GRP78 molecule contains an ATPase domain at the NH_2-terminal region, which catalyzes the hydrolysis of ATP to ADP, and a peptide-binding domain at the COOH-terminal region, which binds polypeptides with specific heptapeptide sequences (36, 37). To determine the specific binding region of GRP78 for EGCG, we first generated two GRP78 deletion constructs, an NH_2-terminal (GRP78-N) and COOH-terminal (GRP78-C) deletion mutants (Fig. 2*A*), and evaluated the binding affinity of the two GRP78 domains using the EGCG-Sepharose 4B pull-down assay. Results indicated that the GST-GRP78-N domain (amino acids 1-405) containing the ATP-binding site (amino acids 175-201; refs. 2, 12) interacted efficiently with EGCG, but the GST-GRP78-C (amino acids 406-654) containing the peptide-binding domain was not detected in the EGCG-Sepharose 4B pull-down assay (Fig. 2*B*).

The specific region of interaction between EGCG and GST-GRP78 was confirmed by the use of serial truncated GST-GRP78 fusion proteins from the NH_2-terminal region. We tested the ability of each NH_2-terminal truncated GST-GRP78 fusion protein to interact with EGCG using an EGCG-Sepharose 4B pull-down assay. The domains containing the NH_2-terminal ATP-binding region (residues 80-654, 160-654, and 211-654) interacted efficiently with EGCG (Fig. 2*C, top*). Then, to determine whether ATP binds to a region similar to that which binds EGCG, we examined the binding of ATP and each GST-GRP78 truncated protein by using an ATP-agarose 4B pull-down assay. These binding studies indicated that EGCG bound to the same NH_2-terminal regions of GST-GRP78 as did ATP, which is the area containing the ATPase activity (Fig. 2*C, middle*).

Because EGCG binds with the ATPase catalytic domain of GRP78, it might compete with ATP for binding with free GRP78. rGRP78 and EGCG-Sepharose 4B beads or ATP-agarose 4B beads were used to determine whether EGCG could inhibit the binding of ATP to GRP78. Results confirmed that ATP competed with EGCG for binding with GRP78 in a concentration-dependent manner because the binding of EGCG with GRP78 decreased with increasing amounts of ATP (Fig. 2*D, left*). Likewise, the binding of ATP with GRP78 also decreased with increasing amounts of EGCG (Fig. 2*D, right*). These results further supported the idea that EGCG binds to the ATPase catalytic domain of GRP78.

EGCG inhibits the ATPase activity of GRP78 and modulates GRP78 through conformational conversion. ATP binding and hydrolysis are essential for the chaperone activity of all HSP70 proteins, suggesting that the protective effect of GRP78 may be dependent on a functional ATP-binding domain (2, 38). To determine whether rGRP78 protein maintained ATPase activity, we measured ATPase activity for rGRP78 within a range of rGRP78 concentrations from 0.5 to 3 μmol/L. The results indicated that rGRP78 maintained ATPase activity (Fig. 3*A, left*). In a second set of experiments, we determined the effect of EGCG on rGRP78 ATPase activity and results showed that EGCG (5 or 10 μmol/L) had a significant inhibitory effect (56% and 61%, respectively; Fig. 3*A, middle*). The next question to be answered was whether the EGCG inhibition of ATPase activity of GRP78 was related to the presence of the gallate group. Therefore, we investigated the effect of EGCG analogues, including EC, ECG, and EGC, on ATPase activity of GRP78. The results indicated that, besides EGCG, ECG, which contains the gallate group, also inhibited ATPase activity in a manner similar to EGCG (64%; Fig. 3*A, right*). Compared with EGCG and ECG, other analogues, EC and EGC, showed a weaker

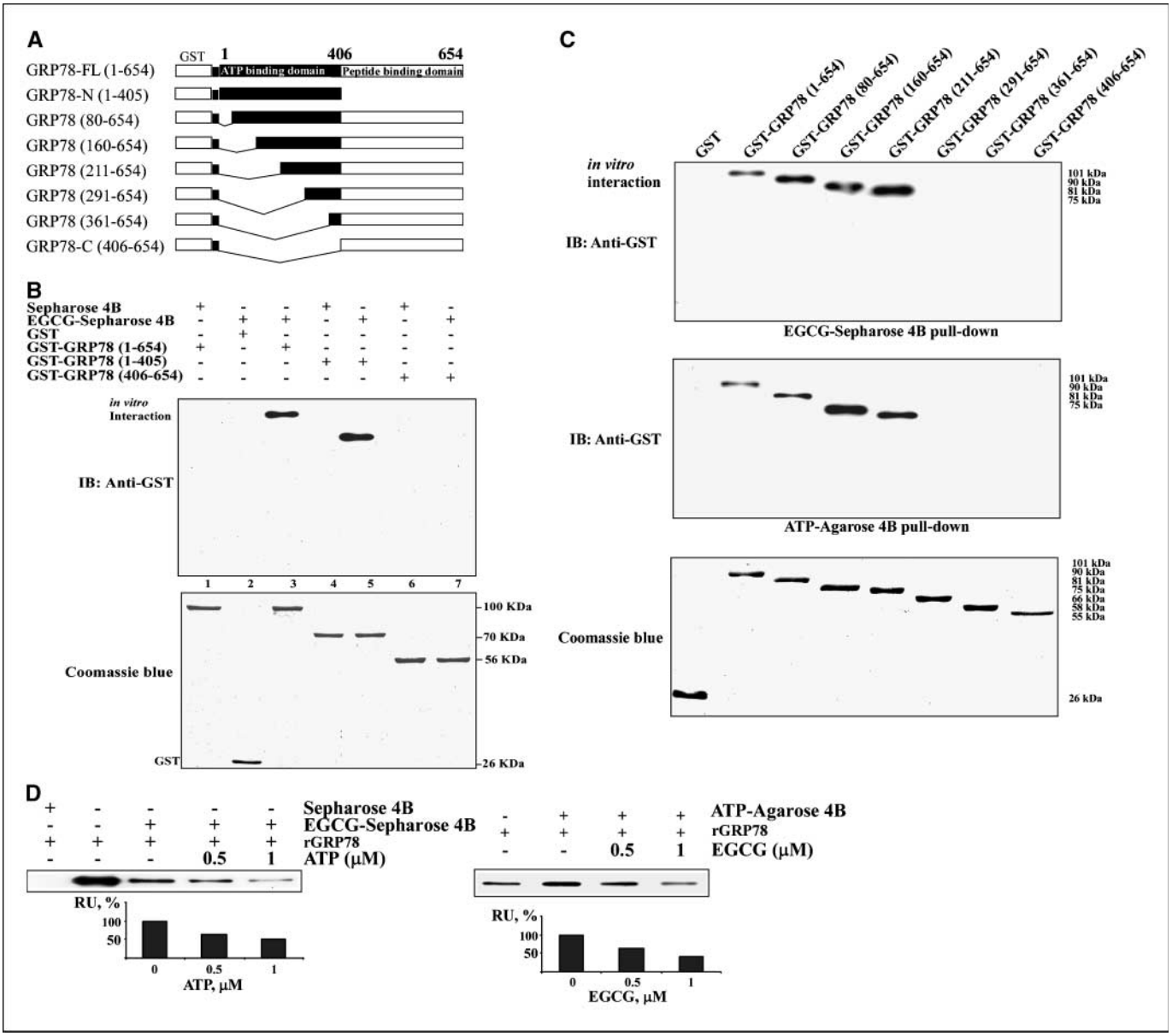

Figure 2. EGCG-binding site of GRP78. *A,* deletion mutants of GRP78. *B, in vitro* interactions of EGCG with full-length GST-GRP78 (*GRP78-FL*; amino acids 1-654), NH_2-terminal GST-GRP78 (amino acids 1-405), or COOH-terminal GST-GRP78 (amino acids 406-654). GST proteins were incubated with EGCG-Sepharose 4B beads overnight at 4°C. Precipitates were analyzed by immunoblotting (*IB*) using a GST antibody (*top*) and Coomassie blue staining (*bottom*). *C, in vitro* interactions of EGCG or ATP with various deletion mutants of GRP78. Various GST-GRP78 deletion mutant proteins were incubated with EGCG-Sepharose 4B or ATP-agarose 4B beads. Precipitates were analyzed by immunoblotting using a GST antibody (*top* and *middle*) and Coomassie blue staining (*bottom*). *D,* rGRP78 was incubated with EGCG-Sepharose 4B beads overnight at 4°C. ATP (0.5 or 1 μmol/L) was added to the reaction volume and incubated for 30 minutes at 25°C. Precipitates were analyzed by Western blot analysis using a GRP78 antibody (*left, top*) and quantified using the ImageJ software program (NIH, Bethesda, MD; *left, bottom*). EGCG (0.5 or 1 μmol/L) was incubated with rGRP78 for 4 hours at 4°C, and then 50 μL of ATP-agarose 4B were added to the reaction volume and incubated overnight at 4°C. Precipitates were analyzed by Western blot using a GRP78 antibody (*right, top*) and quantified using the ImageJ software program (*right, bottom*).

inhibition of GRP78 ATPase activity (38% and 53%, respectively; Fig. 3*A, right*). These results indicated that the gallate moiety might be important for binding with GRP78 and/or inhibiting GRP78 ATPase activity.

In mammalian cells, GRP78 exists in interconvertible oligomeric and monomeric forms (39). The monomeric form is the biologically active species that can bind to unfolded or unassembled proteins (39). The oligomeric forms of GRP78 are converted to the active monomeric form by binding with ATP *in vitro* (40) and *in vivo* (41). We first used different doses of ATP to examine the conversion state of rGRP78. Nondenatured gel electrophoresis was used to analyze the migration of monomeric, dimeric, and oligomeric forms of rGRP78, and results confirmed that ATP effectively converted rGRP78 dimers to monomers (Fig. 3*B, left*). We then investigated the effect of EGCG on the state of oligomerization of rGRP78, and results indicated that the addition of EGCG induced the conversion of the active monomeric form of rGRP78 to the inactive dimeric form of rGRP78 (Fig. 3*B, right*). This effect was dependent on the concentration of EGCG because the monomeric form of rGRP78 was completely converted to the dimeric form as EGCG increased to 10 μmol/L. To further investigate the effect of EGCG on the conversion of the monomeric form induced by ATP, we conducted a competition assay. Results indicated that, in the presence of EGCG (10 μmol/L), the dissociation of dimers to monomers was almost totally prevented (Fig. 3*C, left, lane 5*). This observation prompted us to investigate the effect of EGCG analogues on the conformational conversion of GRP78. Results indicated that, besides EGCG, only ECG had similar effects on the state of oligomerization of GRP78 (Fig. 3*C, right, lane 4*). Both EGCG and ECG contain the gallate group, further confirming that

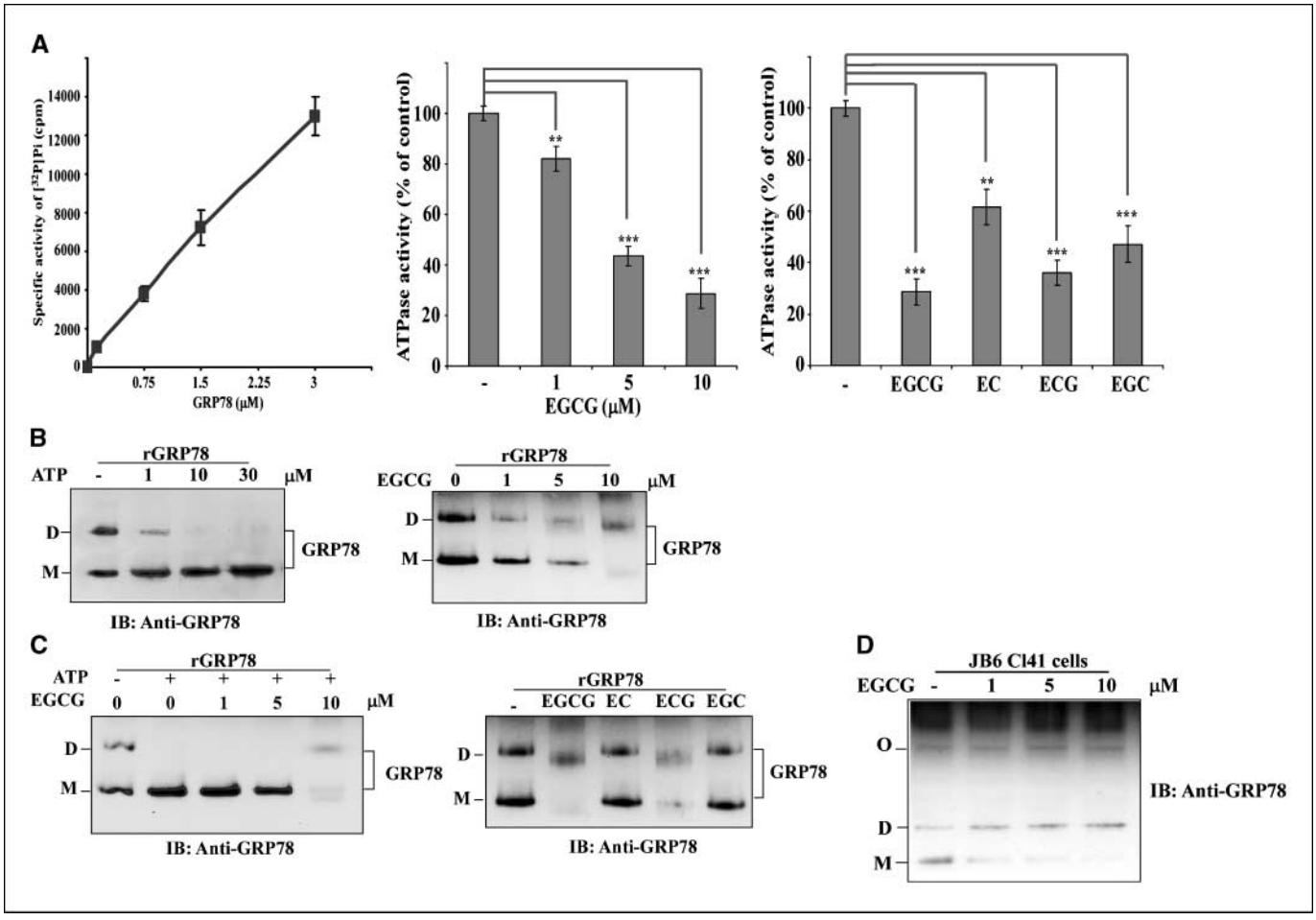

Figure 3. Effect of EGCG and its analogues on GRP78 ATPase activity and conformation of GRP78. *A,* ATPase activity of GRP78 was assayed under standard conditions as described in Materials and Methods using 1 mmol/L ATP at pH 7.5 and 37°C for 1 hour. Aliquots were removed from the supernatant fraction and counted in a liquid scintillation counter to determine the amount of [^{32}P]Pi released. *Left,* ATP hydrolysis was calculated from measuring [^{32}P]Pi activity and standardized by subtracting a blank reaction mixture containing no GRP78. *Middle,* inhibition of GRP78 (3 μmol/L) ATPase activity by different concentrations of EGCG (1, 5, or 10 μmol/L). Inhibition of GRP78 (3 μmol/L) ATPase activity by analogues of EGCG. *Right,* assay was done using 10 μmol/L EGCG, EC, ECG, or EGC. *Points,* mean of three independent experiments; *bars,* SD. *Asterisk,* significant decrease in ATPase activity in EGCG or analogue-treated samples compared with untreated control samples. **, $P < 0.005$; ***, $P < 0.0001$. *B, in vitro* effect of ATP concentration on the conversion of the GRP78 dimer to a monomer. rGRP78 (0.5 μg) and different doses of ATP (1, 10, or 30 μmol/L) were incubated for 30 minutes at 25°C in a 25 μL reaction mixture [20 mmol/L HEPES (pH 7.0)]. The analysis for GRP78 conformation changes was done using nondenaturing gel electrophoresis (4-15% Tris-HCl gradient gels; Bio-Rad, Hercules, CA). *Left,* for detection of GRP78, an antibody against GRP78 was used. *In vitro* effect of EGCG on the oligomerization states of GRP78. *Right,* assay was done using different doses of EGCG (1, 5, or 10 μmol/L) without ATP. *C,* effect of EGCG on the ATP-induced conformational changes of GRP78. rGRP78 (0.5 μg) was incubated with ATP (30 μmol/L) and different doses of EGCG (1, 5, or 10 μmol/L). *Left,* proteins were separated on nondenaturing gels, and subsequent detection was done by immunoblotting with anti-GRP78. *In vitro* effect of EGCG analogues on the oligomerization state of GRP78. *Right,* rGRP78 (0.5 μg) was incubated with 10 μmol/L each of EGCG, EC, ECG, or EGC. *D, in vivo* effect of EGCG on the oligomerization states of GRP78. JB6 Cl41 cells were treated with different doses of EGCG (1, 5, or 10 μmol/L) for 24 hours and analyzed by immunoblotting with the GRP78 antibody. *M,* monomer; *D,* dimer; *O,* oligomer.

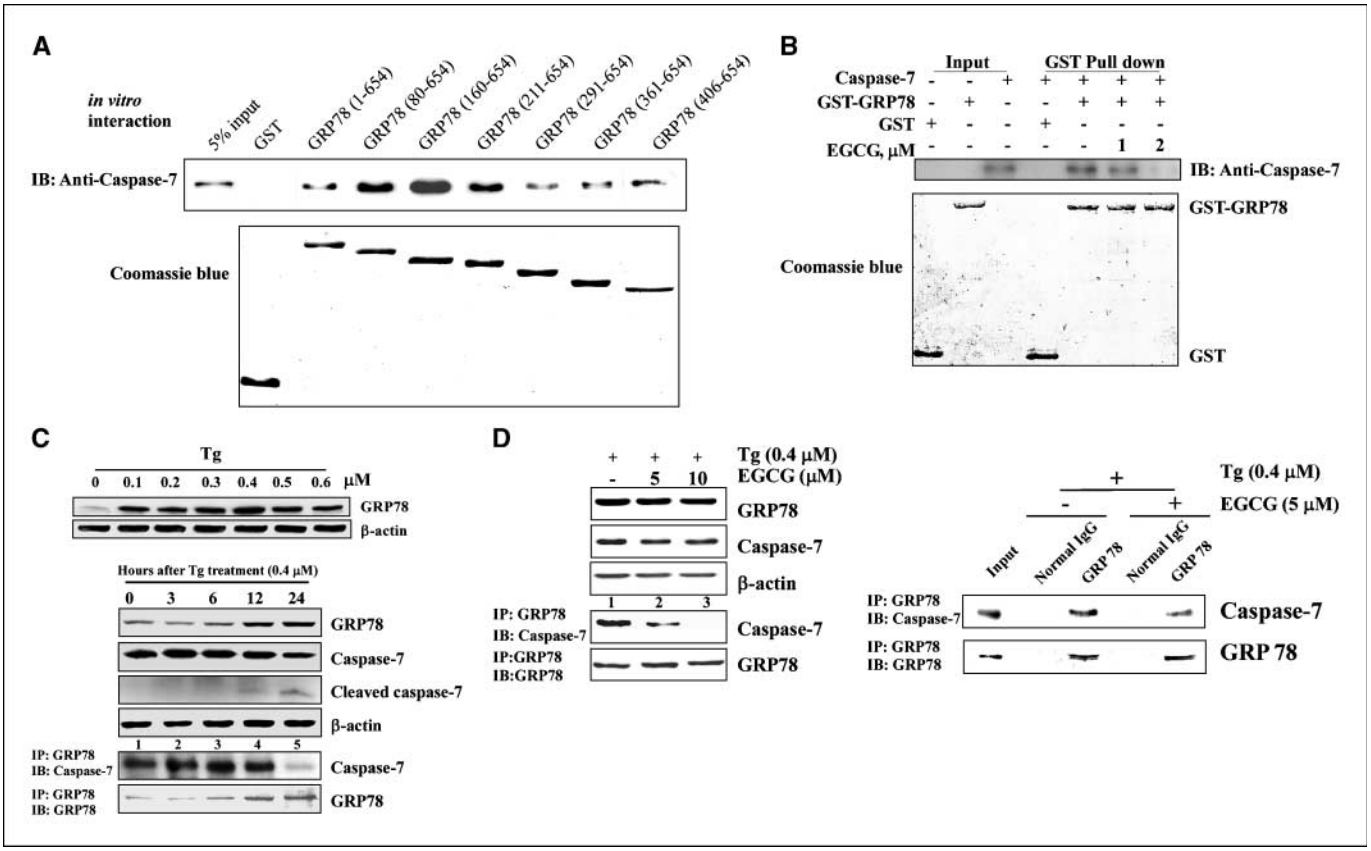

Figure 4. Effect of EGCG on complex formation between GRP78 and caspase-7. *A, in vitro* interactions of caspase-7 with full-length and various deletion mutants of GRP78. Procaspase-7 (0.1 μg) was incubated overnight at 4°C with GST-GRP78 deletion mutants immobilized on Sepharose 4B beads. Precipitates were analyzed by immunoblotting using anti-caspase-7 (*top*) or Coomassie blue staining (*bottom*). *B, in vitro* effect of EGCG on the interaction of GRP78 with procaspase-7. EGCG was incubated for 3 hours at 4°C with full-length GST-GRP78 immobilized on Sepharose 4B beads. Procaspase-7 (0.1 μg) was then added to the reaction mixture, and incubation was continued overnight at 4°C. Precipitates were analyzed by immunoblotting using anti-caspase-7 (*top*) or Coomassie blue staining (*bottom*). *C,* thapsigargin (*Tg*) induces expression of GRP78. *Top,* JB6 Cl41 cells were treated for 24 hours with increasing concentrations of thapsigargin (0-0.6 μmol/L). Total protein lysates were prepared from these cells, protein samples (30 μg) were subjected to SDS-PAGE, and subsequent detection was done by immunoblotting with anti-GRP78. β-Actin was used as an internal control to monitor equal protein sample loading. *Bottom,* time course of thapsigargin-induced GRP78 expression and the interaction of caspase-7 with GRP78. JB6 Cl41 cells were treated with 0.4 μmol/L thapsigargin and harvested at the indicated times. *D, in vivo* regulation of GRP78 binding with caspase-7 by EGCG. JB6 cells were treated or not treated for 3 hours with 5 or 10 μmol/L EGCG for dose response (*left*) or with 5 μmol/L EGCG for normalization with IgG (*right*) and then treated with 0.4 μmol/L thapsigargin for another 6 hours. Total protein lysates were used for immunoprecipitation (*IP*) with a GRP78 antibody.

this moiety might be critical for interacting with GRP78. We next determined whether similar effects might be observed *in vivo* by using a JB6 Cl41 cell lysate treated with EGCG. Results showed that the dimeric and/or oligomeric form of GRP78 increased after treatment with increasing concentrations of EGCG (Fig. 3*D*). For detecting endogenous GRP78, the whole-cell lysate was separated on a nondenaturing gel and subsequent detection was done by immunoblotting with anti-GRP78. Data indicated that GRP78 could exist as monomeric, dimeric, and oligomeric forms in cells. EGCG induced the conversion of the monomeric form to dimeric and oligomeric forms in a dose-dependent manner. Thus, EGCG could also induce a conformational change of GRP78, suggesting that EGCG might be inhibiting the activity of GRP78 *in vivo*.

EGCG can prevent the formation of the inactive GRP78 and caspase-7 complex. One of the interesting questions arising from these studies was whether EGCG binding could block the antiapoptotic function of GRP78 (2, 6). Based on the results of GRP78 binding and inhibition of ATPase activity assays, EGCG might block the association of the GRP78-caspase-7 inhibitory complex. This is based on the finding that the mechanism of ER stress-induced apoptosis involves caspase-7 and requires both the association and dissociation of the inhibitory complex of GRP78 and caspase-7 (2, 6, 42). Thus, to confirm that GRP78 and caspase-7 directly interact physically, NH_2-terminal truncated GST-GRP78 fusion proteins were evaluated for their ability to associate with recombinant procaspase-7. Previously, residues 175 to 201 of the GRP78 NH_2-terminal ATP-binding domain are important for binding caspase-7 (2). Our results also indicated that caspase-7 bound to the NH_2-terminal domain of GRP78 (Fig. 4*A*), which contains the same regions for binding EGCG (Fig. 2*B* and *C*). These results strongly suggested that the binding region of caspase-7 and EGCG is similar. Thus, EGCG might prevent the formation of a complex between caspase-7 and GRP78.

To directly test this possibility, we further investigated the effect of EGCG on the binding of caspase-7 and GRP78 *in vitro* and *in vivo*. For the *in vitro* test, we used the GST pull-down assay with recombinant procaspase-7 and GST-GRP78. Different doses of EGCG were added to the reaction for pretreatment of GRP78, and results showed that the binding of procaspase-7 to GRP78 decreased with increasing concentrations of EGCG (Fig. 4*B*). Then,

to confirm the interaction of GRP78 and caspase-7 *in vivo*, a JB6 Cl41 cell lysate was used for immunoprecipitation with anti-GRP78. Given that conditions of cellular stress, such as heat shock or ER stress, are usually responsible for up-regulation of chaperone proteins, we used increasing concentrations of thapsigargin, an inhibitor of intracellular calcium pumps, for induction of GRP78 (Fig. 4*C, top*). The results indicated that GRP78 interacted with caspase-7 (Fig. 4*C, bottom*), which has been reported for *in vivo* association before and following stress conditions (2, 6). On the other hand, prolonged treatment with thapsigargin (24 hours) could also activate caspase-7 as shown by detection of cleaved caspase-7 (Fig. 4*C, bottom, lane 5*), which agreed well with a previous report (6).

To further confirm whether EGCG prevents the association of GRP78 and caspase-7, we pretreated JB6 Cl41 cells with different doses of EGCG followed by treatment with thapsigargin. The results showed that as little as 5 μmol/L EGCG could suppress the binding of GRP78 with caspase-7 and 10 μmol/L EGCG totally prevented the formation of the GRP78 and caspase-7 complex (Fig. 4*D, left, middle bottom*).

EGCG prevents the protective function of GRP78 against etoposide-induced cell death. One of the possible mechanisms for the antiapoptotic effects of GRP78 has been suggested that GRP78 overexpression can cause the formation of many inhibitory complexes, which would suppress the activation of caspase-7 (2). This could lead to increased cancer progression and drug resistance (3). Therefore, to verify whether GRP78 affects caspase-7-mediated cell death induced by etoposide, we used human bladder carcinoma T24/83 cells stably transfected with mock empty vector pcDNA3.1 (T24/83-pcDNA) or with GRP78 (T24/83-GRP78). Activated caspase-7 was detected in both etoposide-treated T24/83-pcDNA and T24/83-GRP78 cells (Fig. 5*A*). On the other hand, T24/83-GRP78 cells a showed a weaker etoposide-induced caspase-7 activation compared with T24/83-pcDNA cells (Fig. 5*A, lane 7* versus *lane 3*). EGCG treatment alone did not induce caspase-7 activation in either T24/83-pcDNA or T24/83-GRP78 cells (Fig. 5*A lanes 2* and *6*). On the other hand, cotreatment of cells with EGCG and etoposide induced activation of caspase-7 in either T24/83-pcDNA or T24/83-GRP78 cells but more strongly in the T24/83-GRP78 cells (Fig. 5*A lane 8* versus *lane 4*).

To further determine whether EGCG can prevent the anti-apoptotic formation of the GRP78-caspase-7 inhibitory complex to suppress apoptosis, T24/83-pcDNA and T24/83-GRP78 cells were treated with etoposide (20 μmol/L), EGCG (10 μmol/L), or EGCG plus etoposide. Apoptosis was assessed by flow cytometry analysis as described in Materials and Methods. Treatment of T24/83-pcDNA cells with etoposide resulted in a 26.3% apoptotic cells (Fig. 5*B, top*), whereas the percentage of apoptotic T24/83-GRP78 cells increased to only 8.9% (Fig. 5*B, bottom*). On the other hand, the percentage of apoptotic cells in etoposide/EGCG-treated T24/83-pcDNA cells was similar to etoposide-treated cells (28.2%; Fig. 5*B, top*). However, treatment of T24/83-GRP78 cells with EGCG and etoposide increased the percentage of apoptotic cells to 14.1% (Fig. 5*B, bottom*). These results indicated that, compared with T24/83-pcDNA cells, T24/83-GRP78 cells were more resistant to etoposide. However, treatment with EGCG in combination with etoposide seemed, at least partially, to overcome the resistance of T24/83-GRP78 cells to etoposide-induced apoptosis (2).

To verify that EGCG can facilitate etoposide-induced apoptosis in other malignant cancer cell lines, we used human breast adenocarcinoma MDA-MB-231 and T-47D cells to study the effect of EGCG on the activation of caspase-7. Results indicated that etoposide could induce caspase-7 activation in either MDA-MB-231 (Fig. 5*C, left*) or T-47D cells (Fig. 5*C, right*), but 10 μmol/L EGCG alone had no effect on the activation of caspase-7. However, treatment of cells with both etoposide and EGCG induced a dose-dependent increase in the activation of caspase-7. These results suggested that EGCG could enhance etoposide-induced caspase-7 activation, which strongly indicated that EGCG might prevent the antiapoptotic effects of GRP78. To further test this idea, we next examined the effect of EGCG on etoposide-induced apoptosis using flow cytometry analysis. EGCG treatment of etoposide-treated MDA-MB-231 cells increased the etoposide-induced apoptosis from 17.7% to 22.0% and 28.6%, respectively (Fig. 5*D, top*). For T-47D cells, the percentage of apoptosis increased from 8.8% to 13.5% and 15.2%, respectively (Fig. 5*D, bottom*). These results are consistent with the idea that EGCG prevents the formation of the inhibitory complex comprising endogenous GRP78 and caspase-7, especially following treatment with etoposide. Overall, these results strongly indicated that EGCG could prevent the protective effect of GRP78, in which it suppresses the caspase-mediated cell death pathways in drug-treated cells, contributing to the development of drug resistance.

EGCG sensitizes human breast cancer cells to etoposide. Etoposide has been shown to induce apoptosis in a variety of tumor cell lines (43, 44). It is an important cancer chemotherapeutic agent with clinical activity against a broad range of human malignancies (45). To evaluate the effect of EGCG in etoposide-inhibited colony formation, we carried out soft agar clonogenic assays using human breast cancer cell lines MDA-MB-231 and T-47D (Fig. 6*A* and *B*). Results indicated that treatment of human breast cancer cells with etoposide and EGCG together significantly reduced colony number as well as colony size compared with cells treated with either etoposide or EGCG alone (Fig. 6*A* and *B*), which had little effect on colony number or size. Taken together, these data indicate that EGCG can assist in preventing the drug resistance of MDA-MB-231 and T-47D breast cancer cells against etoposide.

Discussion

EGCG is the major polyphenol component of green tea and the most active single anticancer factor found in tea (27). Several reports have shown that tea components target specific cell signaling pathways, which are responsible for regulating cell proliferation and cell transformation (21, 46). Although several mechanisms explaining the anticancer activities of EGCG have been reported in cell culture studies, the mechanism of EGCG action is not yet fully understood.

In the present study, using affinity chromatography, two-dimensional electrophoresis, and MALDI-TOF analysis, we are the first to show that EGCG binds with the molecular chaperone GRP78, which is one of the major regulatory proteins in the ER. GRP78 normally functions to facilitate the proper folding or maintenance of proteins in a folded competent state and also prevents protein folding intermediates from aggregating (36, 47). GRP78 is also involved in the translocation of newly synthesized precursors across the ER membrane (48). We have provided clear evidence that EGCG directly binds with GRP78, which was subsequently confirmed by both *in vitro* and *in vivo* binding assays.

The association of EGCG with GRP78 was found to be markedly ATP dependent, and our observations support the idea that the GRP78-binding region for EGCG and ATP is the same. This suggested that EGCG specifically inhibits GRP78 ATPase activity, which is essential for the chaperone function of the HSP70 family (38, 48, 49). In most HSP70 proteins, ATP hydrolysis is the rate-limiting step in the ATPase cycle that consists of alterations between the ATP state of low affinity and

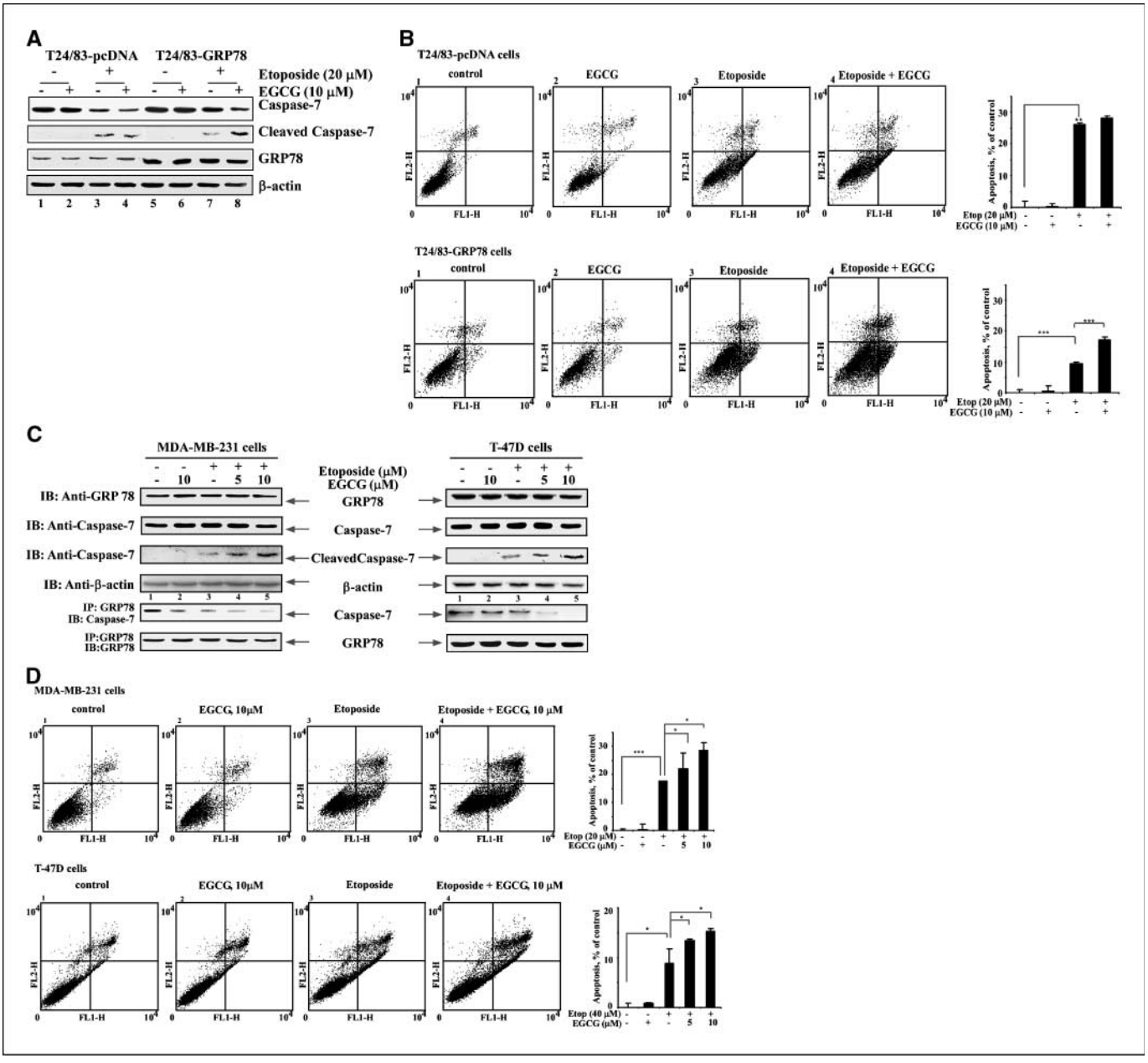

Figure 5. Effect of EGCG on etoposide-induced apoptosis in cancer cells. *A,* effect of EGCG on etoposide-induced activation of caspase-7 in GRP78-overexpressing cells. T24/83-pcDNA and T24/83-GRP78-transfected cells were either treated or not treated with 10 μmol/L EGCG in serum-free medium for 3 hours and then treated or not treated with 20 μmol/L etoposide for an additional 5 hours. Then, 24 hours after drug treatment, total protein lysates were prepared. The protein samples (50 μg) were subjected to SDS-PAGE and followed by detection with immunoblotting using antibodies against GRP78, caspase-7, or β-actin. Caspase-7 (35 kDa) and the cleaved caspase-7 (20 kDa) proteins. *B, top* and *bottom,* fluorescence-activated cell sorting (FACS) analysis of apoptotic cells after the addition of etoposide (*Etop*; 20 μmol/L) in the presence or absence of EGCG (10 μmol/L). At 24 hours following drug treatment, the T24/83-pcDNA (*top*) or T24/83-GRP78 (*bottom*) cells were labeled with Annexin V-FITC and propidium iodide. The distribution pattern of live and apoptotic cells was determined by FACS analysis. *Bottom left quadrants,* viable cells are those displaying low Annexin or no Annexin and propidium iodide staining; *bottom right quadrants,* early-stage apoptotic cells are represented by high Annexin and low propidium iodide staining; *top right quadrants,* late-stage apoptotic cells are represented by high Annexin and high propidium iodide staining; *top left quadrants,* necrosis is represented by cells with high propidium iodide and low Annexin staining. The percentage of apoptosis in EGCG-treated cells compared with untreated cells is representative of at least three independent experiments. *Columns,* mean of three independent experiments; *bars,* SD. *Asterisk,* significant enhancement of etoposide-induced apoptosis by EGCG. **, $P < 0.005$; ***, $P < 0.0001$. *C,* effect of EGCG on etoposide-induced caspase-7 activation in MDA-MB-231 (*left*) or T-47D (*right*) breast cancer cells. MDA-MB-231 and T-47D cells were either treated or not treated with 10 μmol/L EGCG in serum-free medium for 3 hours and then treated or not treated with 20 μmol/L (MDA-MB-231 cells) or 40 μmol/L (T-47D cells) etoposide for 48 hours. The analysis of proteins (50 μg) was as for (*A*). *D,* flow cytometry analysis of apoptosis of MDA-MB-231 cells (*top*) or T-47D cells (*bottom*) was done as for (*B*). The percentage of apoptosis is representative of at least three independent experiments. *Columns,* mean; *bars,* SD. *Asterisk,* significant enhancement of etoposide-induced apoptosis by EGCG. *, $P < 0.05$; ***, $P < 0.0001$.

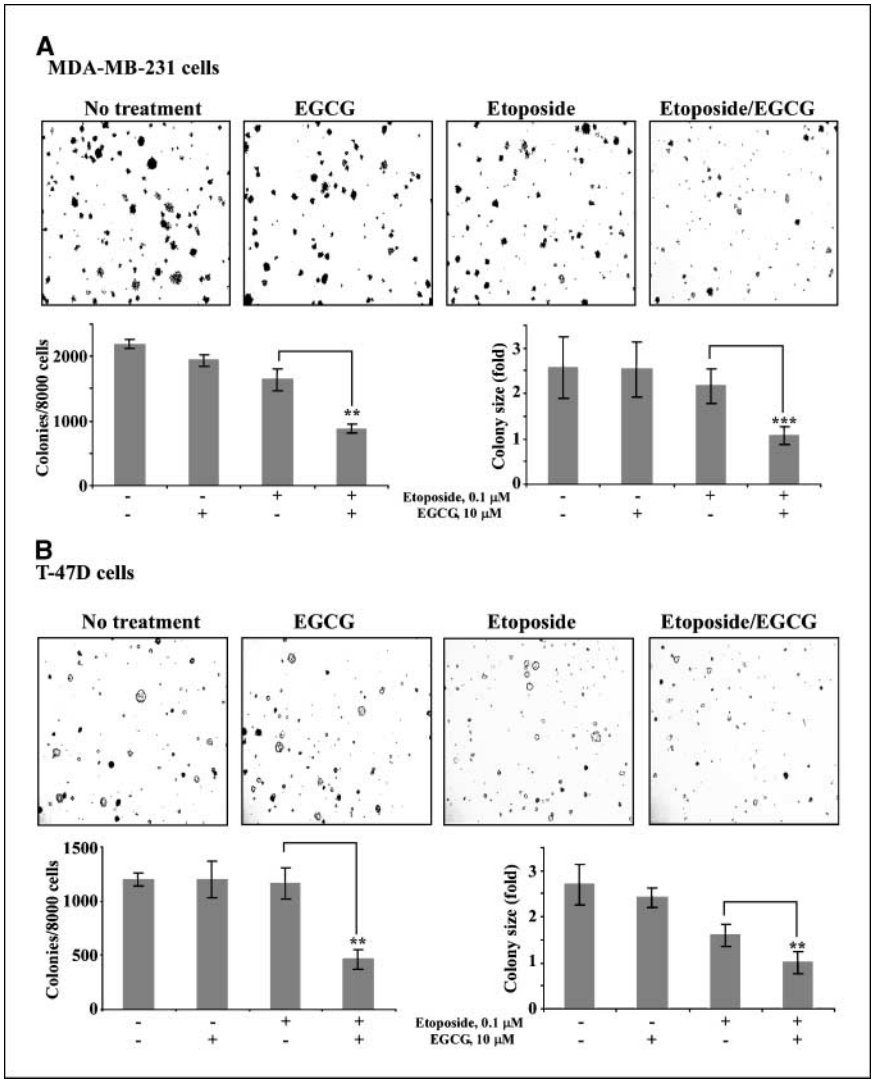

Figure 6. Effect of EGCG on phenotype expression (colony formation) of human breast cancer cells. MDA-MB-231 (*A*) or T-47D (*B*) human breast cancer cells were either treated or not treated with 10 μmol/L EGCG in serum-free medium for 3 hours and then treated or not treated with 0.1 μmol/L etoposide for 48 hours. Cells were then subjected to a soft agar assay. Cells (8×10^3/mL) were grown in 1 mL of 0.3% basal medium Eagle's agar containing 10% FBS. The cultures were maintained at 37°C in 5% CO_2 atmosphere for 3 weeks. The cultures were photographed (*A* and *B, top*), and the average colony number (*A* and *B, bottom left*) was calculated from three separate experiments. Colony sizes (*A* and *B, bottom right*) were calculated using the ImageJ software program and compared between cells treated with etoposide and etoposide with EGCG. *Columns,* mean; *bars,* SD. *Asterisk,* significant suppression of colony number and size induced by EGCG. **, $P < 0.005$; ***, $P < 0.001$.

fast exchange rates for substrates and the ADP state of high affinity and low exchange rates for substrates (38). The GRP78 protein exists in multiple forms, including ATP-mediated monomeric, dimeric, and oligomeric forms (39, 40, 50). The GRP78 monomeric species reflect maximal activity, whereas the various dimeric or oligomeric species are less active or inactive. Our *in vitro* and *in vivo* results showed that EGCG could convert GRP78 from the active monomeric to the inactive dimeric or oligomeric form. In addition to EGCG, the ECG analogue is also capable of inhibiting ATPase activity and inducing the conversion of the GRP78 protein from the monomeric to the oligomeric form. These data strongly indicated that the gallate group might be critical for binding with GRP78 and inhibiting GRP78 ATPase activity.

Based on these results, we hypothesized that EGCG might be involved in the regulation of GRP78 function in cells. Heat shock proteins have a cytoprotective role and inhibit the apoptotic response. Previous studies by other investigators have shown that GRP78 and a related protein, GRP94, can protect cells against cell death (2, 6, 42). Whereas the mechanisms explaining the antiapoptotic effect of GRP78 are still under investigation, recent studies have revealed that GRP78 can inhibit ER-induced apoptosis through the direct binding and subsequent inhibition of caspase-7 activation (2, 6). GRP78 may be an ideal drug target because of its function as a chaperone for key molecules involved in apoptosis. Our results showed that EGCG prevents the formation of the complex between GRP78 and caspase-7, therefore suppressing the antiapoptotic effect of GRP78.

The protective effect of GRP78 against apoptosis caused by ER disturbance induced by pharmacologic agents has been established (2, 3). Herein, we examined the effect of EGCG on etoposide-induced apoptosis in GRP78-overexpressing T24/83 bladder carcinoma cells and MDA-MB-231 and T-47D breast cancer cells. Results indicated that EGCG sensitized these cells to etoposide-induced caspase-7 activation and apoptosis. A tumor cell may escape from caspase-mediated apoptosis by overexpressing GRP78, and increased GRP78 expression has been observed in many human cancer cell types (7, 51). For drug resistance of cancer cells involving induction of GRP78, we examined the preventive effect of EGCG on clonogenecity of the breast cancer cells in soft agar and results indicated that EGCG sensitized breast cancer cells to etoposide. The human breast cancer cells treated with etoposide and EGCG together

had a significant reduction in their ability to expressed their transformed phenotype. These studies imply that GRP78 is a general contributor to tumor growth and drug resistance in human cancer and therefore is a potential drug target. Based on our results, we propose a new mechanism of EGCG in cancer prevention and chemotherapy that occurs through its suppression of the antiapoptotic function of GRP78. From a therapeutic standpoint, suppression of GRP78 functions in cancer cells by EGCG could represent a novel approach to cancer chemotherapy.

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