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Curcumin sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through CHOP-independent DR5 upregulation

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Death receptor DR5 (DR5/TRAIL-R2) is an apoptosis-inducing membrane receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In this study, we showed that curcumin, a plant product containing the phenolic phytochemical, is a potent enhancer of TRAIL-induced apoptosis through upregulation of DR5 expression. Both treatment with DR5/Fc chimeric protein and silencing of DR5 expression using small interfering RNA (siRNA) attenuated curcumin plus TRAIL-induced apoptosis, showing that the critical role of DR5 in this cell death. Curcumin also induced the expression of a potential pro-apoptotic gene, C/EBP homologous protein (CHOP), both at its mRNA and protein levels. However, suppression of *CHOP* expression by small interfering RNA did not abrogate the curcumin-mediated induction of DR5 and the cell death induced by curcumin plus TRAIL, demonstrating that *CHOP* is not involved in curcumin-induced DR5 upregulation. Taken together, the present study demonstrates that curcumin enhances TRAIL-induced apoptosis by CHOP-independent upregulation of DR5.

Introduction

Tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand (TRAIL), also known as Apo2L, is a cytokine of the TNF family which is capable of inducing apoptotic cell death in a variety of cancer cell types, while having only negligible effects on normal cells (1–3). TRAIL binds to four different types of membrane-bound death receptors (DR4, DR5, DcR1 and DcR2). Both DR4 and DR5 contain a conserved cytoplasmic region called the 'death domain' that is required for TRAIL-induced apoptosis (4–6). TRAIL also binds to decoy receptors DcR1 and DcR2 that sequester the ligand but are unable to initiate an apoptotic signal (5,7). Recent studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL (8–10). TRAIL resistance has been attributed to the loss of TRAIL receptors, upregulation of TRAIL decoy receptors, enhanced expression of cFLIP (cellular Flice-inhibitory protein) or alternations in expression of the Bcl-2 family proteins (11–14). However, TRAIL-resistant cancer cells can be sensitized by the combined treatment with chemotherapeutic drugs and TRAIL *in vitro*, indicating that combination therapy may overcome TRAIL resistance in cancer cells. Therefore, both the understanding of the molecular mechanisms of TRAIL resistance and the development of the strategies to sensitize these cancer cells to undergo TRAIL-mediated apoptosis are important issues for effective cancer therapy.

Recently, the use of phytochemicals as anti-cancer agent has gained high importance. Curcumin is a major component of the *Curcuma* species, which possesses anti-proliferative activity against tumor cells *in vitro* (15). Moreover, curcumin exhibits anti-carcinogenic activity *in vivo,* as indicated by its inhibition of tumor promotion induced by various carcinogens or phorbol esters (16–18). We have already reported that curcumin induces apoptosis in human renal carcinoma cells and leukemia cells (19,20). Renal cell carcinoma remains one of the most drug-resistant malignancies in humans and is a frequent cause of cancer mortality (21), necessitating the development of novel therapeutic strategies against this type of cancer.

In this study, we investigated the sensitizing effect of curcumin on TRAIL-mediated apoptosis in human renal cancer cells. We found that curcumin treatment results in the upregulation of both DR5 and CHOP, also known as growth arrest and DNA damage-inducible gene 153 (*GADD153*) (22). For the first time, we present here the clear evidences that curcumin-induced DR5 upregulation but not CHOP upregulation is critical for its stimulating activity of TRAIL-mediated apoptosis.

Abbreviations: CHOP, C/EBP homologous protein; DcR, decoy receptor; DEVD-*p*NA, Asp-Glu-Val-Asp-chromophore *p*-nitroanilide; DR, death receptor; ER, endoplasmic reticulum; FACS, fluorescent activated cell sorting; FLIP, Flice-inhibitory protein; GFP, green fluorescent protein; IAP, inhibitor of apoptosis; NAC, *N*-acetyl-cysteine; PLC-γ1, phospholipase C-γ1; ROS, reactive oxygen species; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; siRNA, small interfering RNA; XIAP, X-linked inhibitor of apoptosis.

Materials and methods

Cells and materials

Caki, HCT116, HT29, Hep G2 and Hep 3B cells were obtained from the American Type Culture Collection (ATCC Manassas, VA). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml gentamicin. Primary culture of human mesangial cells (Cryo NHMC) and its corresponding growth medium (CC-3146 MsGM) were purchased from Clonetics (San Diego, CA). Recombinant human TRAIL/Apo2 ligand (the non-tagged 19 kDa protein, amino acid 114–281) was purchased from KOMA Biotech (Seoul, Korea). CHOP siRNA, -ERK, anti-FADD, anti-Bcl-2, anti-PLC-γ1, anti-caspase 3, anti-caspase 12, anti-DR4, anti-DR5 and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase 8 and anti-cytochrome *c* antibodies were purchased form Stressgen (Stressgen, British Columbia, Canada). Soluble recombinant TRAIL and DR5-specific blocking chimera antibody were purchased from R&D Systems (Minneapolis, MN). β-D-glucose and glucose

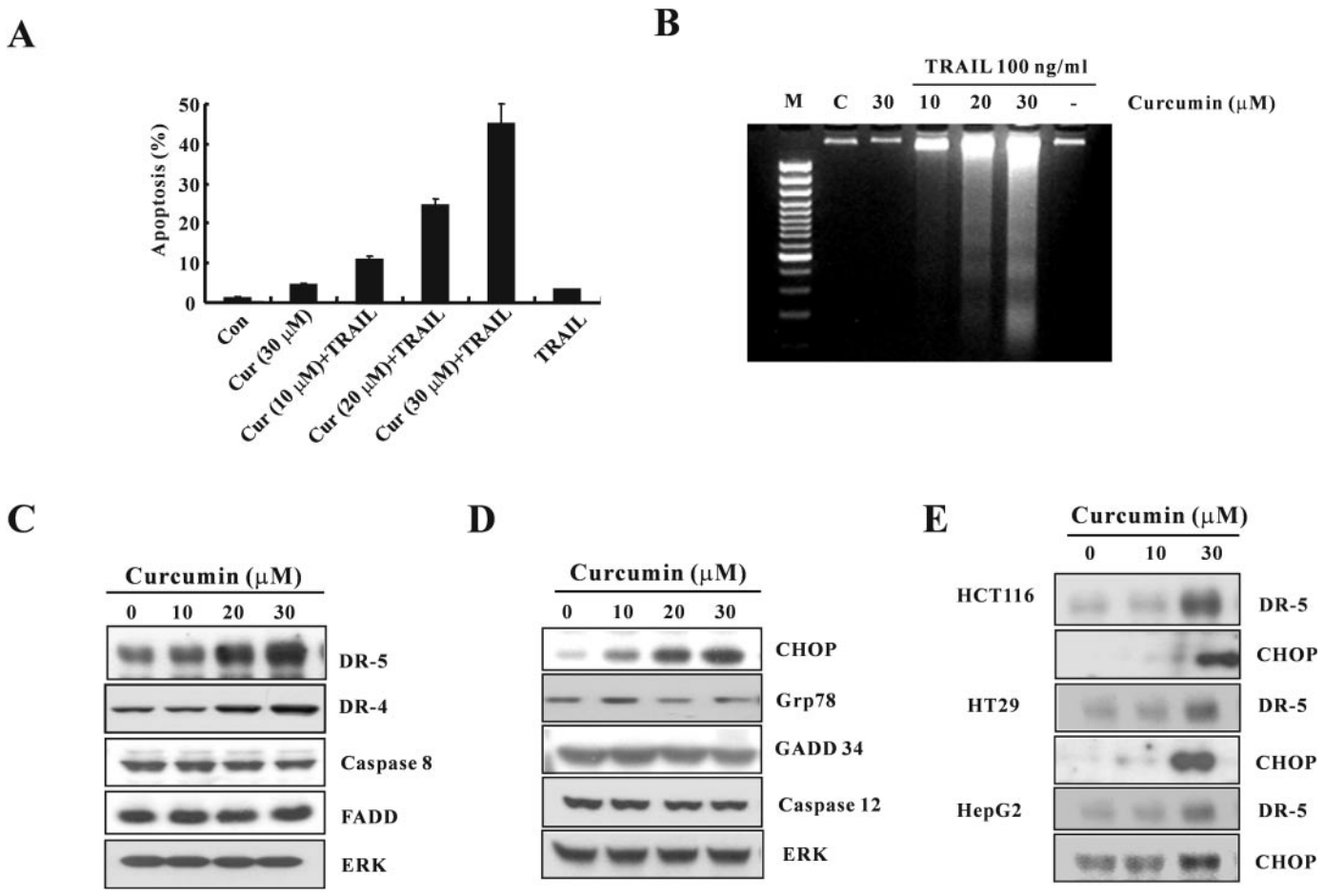

Fig. 1. Significant induction of apoptosis by curcumin plus TRAIL and the expression of **a**poptosis regulatory proteins by curcumin treatment. (**A**) Flow cytometric analysis of apoptotic cells. Caki cells were treated with 100 ng/ml TRAIL in the absence or presence of curcumin at the indicated concentrations for 24 h. Apoptosis was analyzed as a sub-G_1 fraction by FACS. (**B**) Fragmentation of genomic DNAs in Caki cells treated for 24 h with the indicated concentrations of curcumin and TRAIL. Fragmented DNA was extracted and analyzed on 2% agarose gel. (**C**) Effect of curcumin on the expression of the proteins associated with TRAIL-mediated apoptosis. Expression levels of DR5, DR4, caspase 8 and FADD were determined by blot analysis. (**D**) Effect of curcumin on the expressions of the proteins associated with ER stress-mediated apoptosis. Expression levels of CHOP, Grp78, GADD34 and caspase 12 were determined by western blot analysis. (**E**) HCT116, HT29 and HepG2 cells were treated with the indicated concentrations of curcumin. Expression of DR5 and CHOP were determined by western blot analysis.

oxidase were obtained from Sigma Chemical. Cucumin (Sigma Chemical) was prepared as a 20 mM stock solution in ethanol and final concentration of ethanol in culture medium is <0.2% volume.

Western blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The whole cell extracts (40 μg) were subjected to 10% SDS–polyacrylamide gel electrophoresis, and electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA). Detection of specific proteins was carried out with an ECL blotting kit according to the manufacturer's instructions.

Cell count and flow cytometry analysis

Cell counts were performed using a hemocytometer. Approximately 1×10^6 Caki cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

Caspase 3 activity assay

To evaluate caspase 3 activity, cell lysates were prepared after their respective treatment with TRAIL or curcumin. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl and 10% glycerol) containing the caspases substrate [Asp-Glu-Val-Asp-chromophore *p*-nitroanilide (DVAD-*p*NA)] at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Plasmids, transfections and luciferase gene assays

The human CHOP promoter-containing plasmids have been described previously (23). The pDR5/SacI plasmid [containing DR5 promoter sequence (−2500/+3)] and pDR5/−605 [containing DR5 promoter sequence (−605/+3)] were gifts from Dr T Sakai (Kyoto Prefectural University of Medicine, Japan). Point mutations of the CHOP binding sites to the DR5/−605 promoter were generated by a two-step PCR method using the following primers: mCHOP (5-CTTGCGGAGGAGGTAGTTGACGA to 5-TCGTCAACTACC-TCCTCCGAAAG). Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure. For transfection, cells were plated onto 6-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were co-transfected with 2 μg of various plasmid constructs and 1 μg of the pCMV-β-galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase

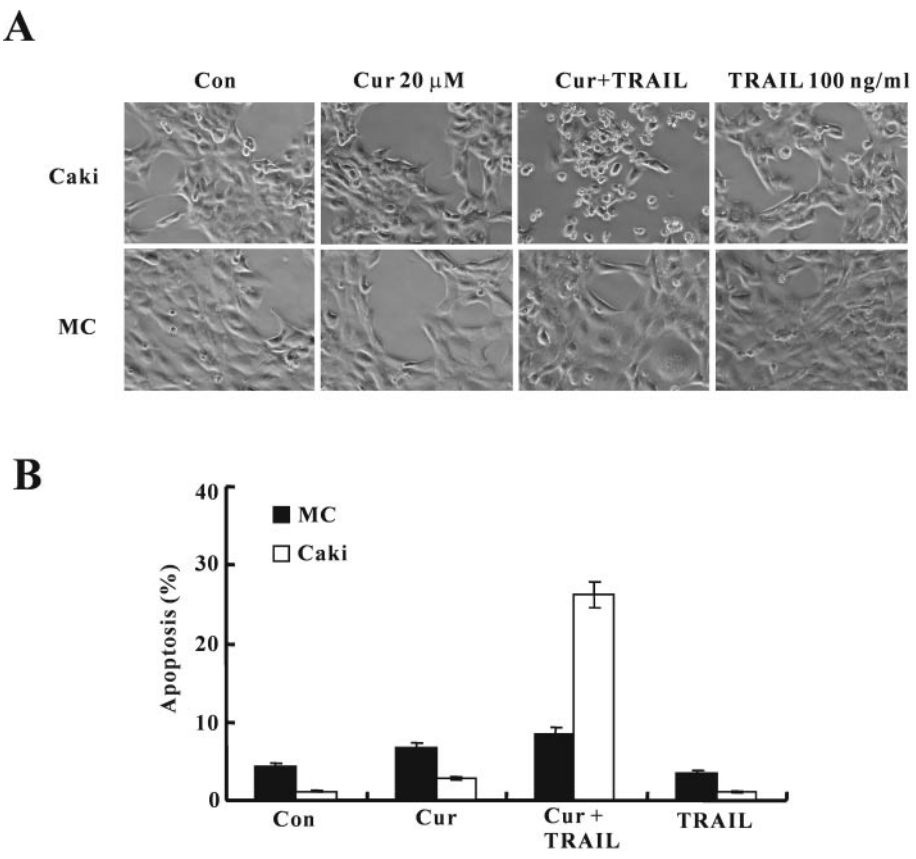

Fig. 2. Effect of curcumin and/or TRAIL on the morphological changes of Caki and normal mesangial cells. (**A**) Caki and normal mesangial cells (MC) were treated with vehicle, 20 μM curcumin alone, 100 ng/ml TRAIL or curcumin plus TRAIL for 24 h. The morphologies of cells were determined by interference light microscopy. Magnification, ×200. (**B**) Apoptosis was analyzed as a sub-G_1 fraction by FACS.

activity was normalized for β-galactosidase activity in cell lysate and expressed as an average of three independent experiments.

RNA isolation and RT–PCR

To determine whether the potential sensitizing effects of curcumin to TRAIL-mediated apoptosis were a result of increased levels of mRNA encoding CHOP, we compared the levels of CHOP in Caki cells, which were treated with or without various concentrations of curcumin. CHOP mRNA expression was determined by RT–PCR. Total cellular RNA was extracted from cells using the TRIzol reagent (Life Technologies). A cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The cDNAs for CHOP and actin were amplified by PCR with specific primers. The sequences of the sense and anti-sense primer for CHOP (accession no. S40706) were 5′-CAACTGCAGAGATGGCAGCTGA-3′ and 5′-ACTGATGCTCCCAATTGTTCAT-3′, respectively. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Small interfering RNA (siRNA)

The 25-nt siRNA duplexes used in this study were purchased from Invitrogen (Calsbad, CA) and had the following sequences: DR5, UUU AGC CAC CUU UAU CUC AUU GUC C; and green fluorescent protein (GFP), AAG ACC CGC GCC GAG GUG AAG. Cells were transfected with siRNA oligonucleotides using LipifectAMINE 2000 (Invitrogen, Calsbad, CA) according to the manufacturer's recommendations.

DNA fragmentation assay

After treatment with curcumin, TRAIL, and combination of TRAIL and curcumin for 24 h, Caki cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10 000 *g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml of ethidium bromide.

Results

Curcumin upregulates DR5 and CHOP expressions in various cancer cells

Caki cells, the human renal cancer cells, are resistant to the cytotoxic effect of TRAIL. However, we found that treatment of Caki cells with the subtoxic doses (<30 μM) of curcumin and TRAIL can effectively induce cell death in a dose-dependent manner of curcumin (Figure 1A). Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA fragmentation in Caki cells cotreated with 20 or 30 μM curcumin plus 100 ng/ml TRAIL but not in cells treated with curcumin alone, TRAIL alone and 10 μM curcumin plus 100 ng/ml TRAIL for 24 h (Figure 1B). To assess the molecular mechanisms underlying this synergistic induction of apoptosis by curcumin and TRAIL in Caki cells, we examined the effect of curcumin on the expression of apoptosis regulatory proteins by western blotting. Treatment with subtoxic concentrations of curcumin (10–30 μM) did not induce any significant changes in the protein levels of Bcl-2, Bax, XIAP (X-linked inhibitor of apoptosis) and cIAP2 (cellular inhibitor of apoptosis 2) (data not shown). We further analyzed the expressions of various proteins related with DR-mediated apoptotic signaling pathway by western blotting, following treatment of Caki cells with various concentrations of curcumin. Interestingly, DR5, a TRAIL-interacting DR, was significantly and dose-dependently induced in curcumin-treated cells. As shown in Figure 1B, treatment with curcumin slightly induced the

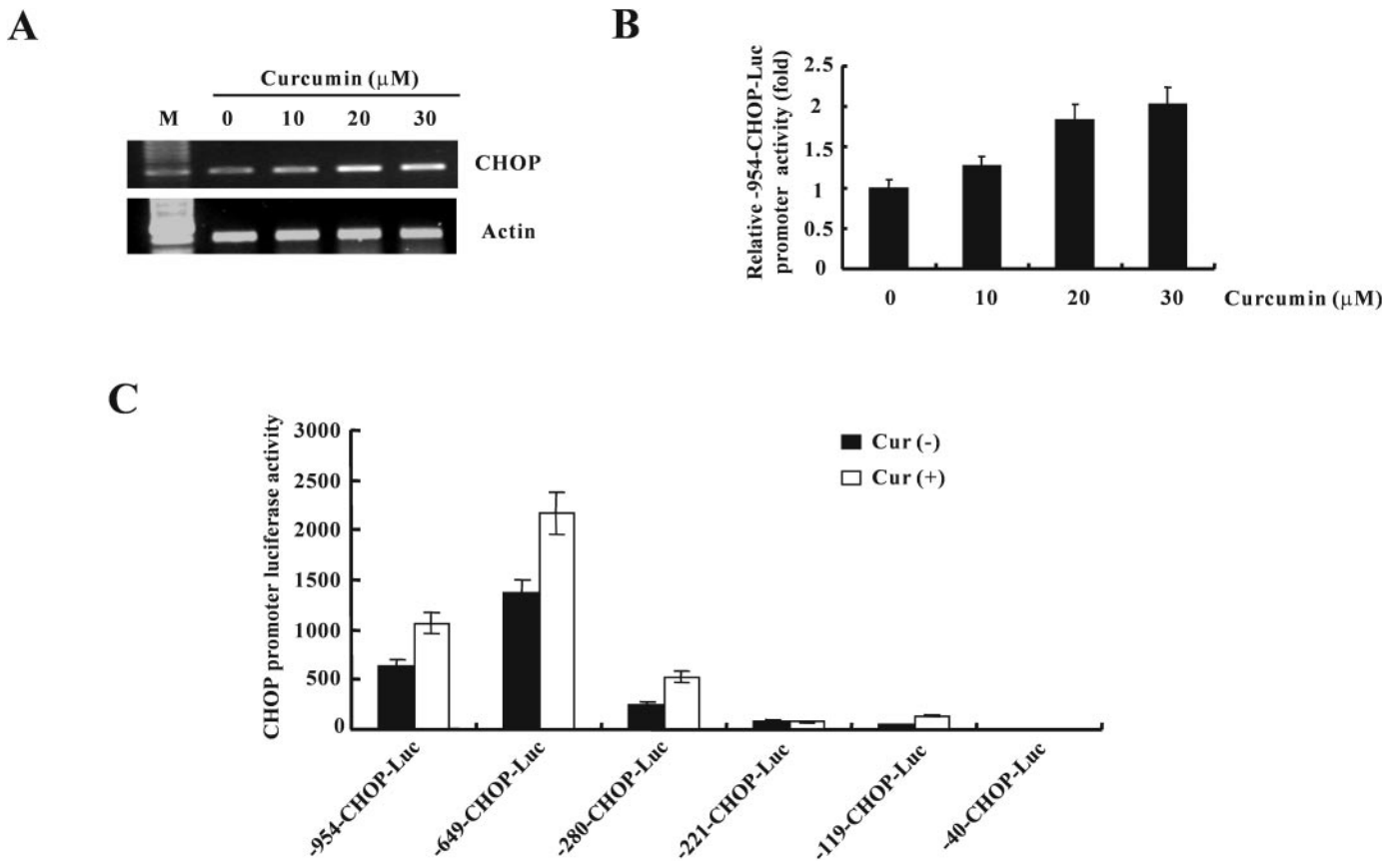

Fig. 3. The expression levels of the CHOP mRNA and CHOP protein by treatment with curcumin in Caki cells. (**A**) Caki cells were treated with the indicated concentrations of curcumin. Total RNA was isolated and RT–PCR analysis was performed as described in Materials and methods. A representative study is shown; two additional experiments yielded similar results. (**B**) CHOP-954 promoter plasmid was transfected, and treated with varying concentrations of curcumin. The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least three independent experiments. (**C**) Caki cells were transiently transfected with LUC constructs containing progressive 5′ deletions of the CHOP promoter and further cultured with curcumin (30 μM). The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least three independent experiments.

expression of DR4 protein in a dose-dependent manner. However, there were no major changes in the protein levels of caspase 8 and Fas associated death domain (FADD) (Figure 1C).

Recently, it has been shown that endoplasmic reticulum (ER) stress causes apoptosis (24) and CHOP is a key transcription factor in this process (22). The ER stress-generated unfolded protein response (UPR) is characterized by increased transcription of ER chaperones, such as Bip/Grp78, which are meant to increase the ER capacity to cope (25). To investigate whether the sensitizing effect of curcumin on TRAIL-induced apoptosis is associated with ER stress, we assessed the expression levels of CHOP, GADD34, caspase 12 and Grp78 after curcumin treatment. We found that treatment of Caki cells with curcumin induced a dose-dependent increase in the protein levels of CHOP, but did not affect the levels of GADD34, caspase 12 and Grp78 (Figure 1D). Treatment with curcumin significantly increased DR5 and CHOP protein levels also in a variety of tumor cell types (colon cancer cells; HCT116 and HT29 and hepatocellular carcinoma HepG2) (Figure 1E). These results suggest that upregulation of DR5 and CHOP is a common response of cancer cell lines to curcumin treatment.

Next, we investigated whether the combined treatment with curcumin and TRAIL affects the induction of apoptosis in normal human mesangial cells. The mesangial cells were resistant to 20 μM curcumin or 100 ng/ml TRAIL alone and their morphological change was not significantly affected by the combined treatment with 20 μM curcumin and 100 ng/ml TRAIL. However, the apoptotic characteristics, such as cell shrinkage, apoptotic bodies and detachment from the plate, were frequently observed in Caki cells treated with curcumin plus TRAIL (Figure 2A). Furthermore, curcumin did not enhance TRAIL-induced apoptosis in normal mesangial cells (Figure 2B).

Curcumin activates transcription from the CHOP promoter

To examine whether curcumin-induced CHOP upregulation is controlled at the transcription level, we performed RT–PCR analysis of CHOP in curcumin-treated Caki cells. RT–PCR analysis demonstrated that curcumin enhanced CHOP mRNA levels in a dose-dependent manner (Figure 3A). We further attempted to identity the curcumin responsive element in the CHOP promoter. We first examined the effects of curcumin on the promoter activities of reporter constructs containing −954 to +91 region of the *CHOP* gene promoter region (23). This construct was transiently transfected into Caki cells, and the response to curcumin was determined by luciferase assay, in the presence or absence of curcumin. As shown in Figure 3B, curcumin increased the promoter activities of −954-CHOP-Luc in a dose-dependent manner. Luciferase assay using a series of deletions in this region demonstrates that deletion

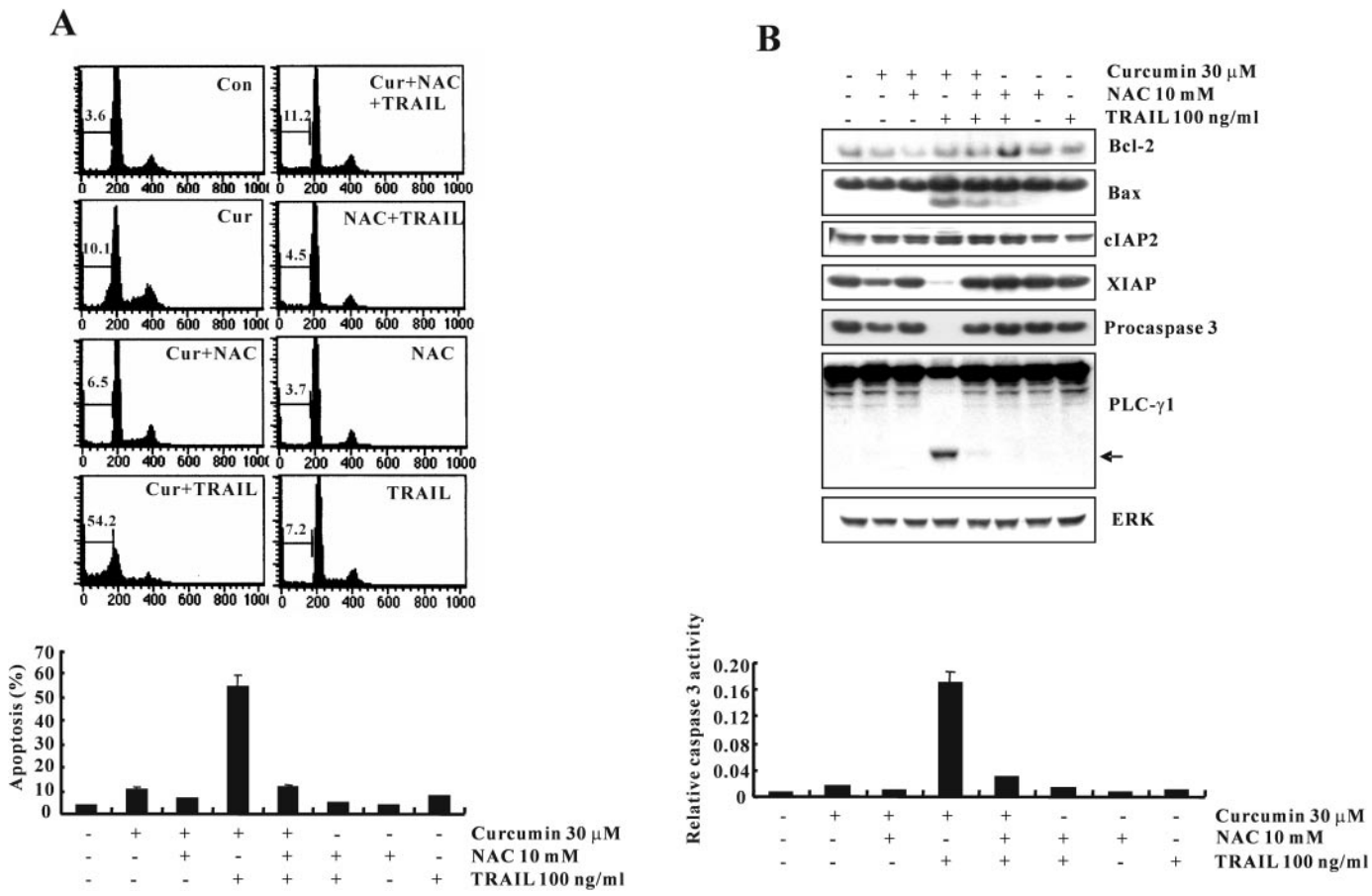

Fig. 4. Effect of NAC on curcumin plus TRAIL-induced apoptosis. (**A**) Caki cells were treated with curcumin plus TRAIL in the presence or absence of NAC (10 mM). Apoptosis was analyzed as a sub-G_1 fraction by FACS. (**B**) Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane and probed with specific antibodies, anti-caspase 3, anti-PLC-γ1, anti-Bcl2, anti-Bax, anti-cIAP2, anti-XIAP or with anti-ERK antibody to serve as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results. Enzymatic activities of caspase 3 were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD-*p*NA) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (*p*NA) was monitored spectrophotometrically (405 nm). Data shown are mean SD ($n = 3$).

to −649 had significant effect on the activation of the CHOP promoter by curcumin treatment (Figure 3C). In contrast, deletion from −280 decreased the curcumin inducibility and basal activity, suggesting that the region between −649 and −280 contains positive regulatory elements involved in the transcriptional activation of CHOP by curcumin or basal condition.

Pretreatment with N-acetyl-cysteine prevents apoptosis by curcumin plus TRAIL

We and other groups have reported that curcumin promotes formation of reactive oxygen species (ROS) (19,26). Therefore, we next examined the role of ROS in curcumin plus TRAIL-induced apoptosis using the thiol antioxidant *N*-acetylcysteine (NAC) (27). Pretreatment of Caki cells with NAC markedly blocked curcumin plus TRAIL-induced apoptosis (Figure 4A). As shown in Figure 4B, cotreatment with curcumin and TRIAL induced downregulation of XIAP proteins. Since XIAP has been previously reported to be a substrate of caspases during apoptosis (28) and pretreatment with z-VAD, a pan-caspase inhibitor, blocked downregulation of XIAP following treatment with curcumin and TRAIL (data not shown), this reduction of XIAP protein levels might be the result of caspase activation in response to the combined treatment, rather than the cause of curcumin-stimulated TRAIL-induced apoptosis. NAC pretreatment significantly inhibited caspase 3 activation, attenuated the cleavage of PLC-γ1 and prevented downregulation of XIAP (Figure 4B). These data clearly indicate that prevention of curcumin plus TRAIL-induced apoptosis by NAC is associated with the blocking of ROS generation and the subsequent activation of caspases.

Upregulation of CHOP by curcumin appears to be dependent on the formation of reactive oxygen metabolites

To investigate whether ROS generation is directly associated with curcumin-induced CHOP upregulation, we assessed the CHOP expression levels in cells treated with an antioxidant NAC or glutathione (GSH) in addition to 30 μM curcumin. As shown in Figure 5A and B, treatment with curcumin significantly increased both the protein and mRNA levels of CHOP, but pretreatment with NAC or GSH markedly inhibited this curcumin-induced CHOP upregulation. Curcumin-mediated increase in CHOP promoter activity, assessed by transient transfection and luciferase assay, was also blocked by pretreatment with NAC (Figure 5B). Involvement of ROS in CHOP upregulation was further examined by western blotting of CHOP in cells treated with H_2O_2 or 10 mM glucose plus glucose oxidase. As shown in Figure 5C, treatment with

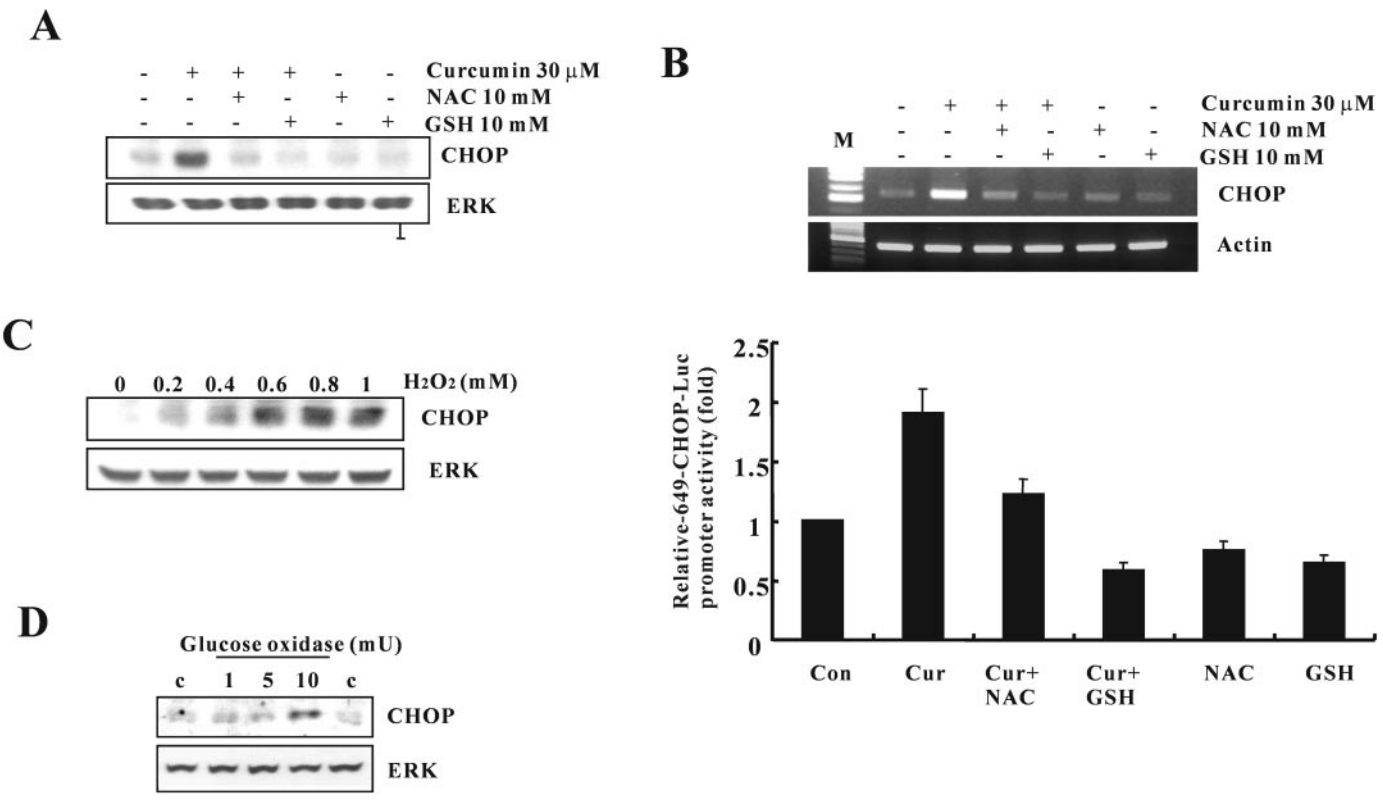

Fig. 5. Effect of NAC and GSH on curcumin-induced CHOP protein and mRNA expression. (**A**) Caki cells were incubated with indicated concentration of NAC or GSH for 1 h before challenge with curcumin (30 μM) for 24 h. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with anti-CHOP antibody. (**B**) CHOP mRNA level was measured by RT–PCR analysis. A representative study is shown; two additional experiments yielded similar results. Caki cells were transfected with −649-CHOP-Luc promoter plasmid and further cultured with curcumin (30 μM) in the presence or absence of NAC or GSH. The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least three independent experiments. (**C**) Caki cells were treated with the indicated concentrations of H_2O_2 for 24 h. CHOP protein level was determined by immunoblot analysis with anti-CHOP. Equal loading of the protein samples was confirmed by western blotting of ERK. A representative study is shown; two additional experiments yielded similar results. (**D**) Caki cells were treated with β-D-glucose (10 mM) and the indicated concentrations of glucose oxidase for 20 h. CHOP protein level was determined by immunoblot analysis with anti-CHOP. ERK protein levels served as the control for the loading of protein levels.

H_2O_2 induced CHOP protein levels in a dose-dependent manner. Western blotting analysis showed a significantly induction of CHOP in the cells treated with β-D-glucose (10 mM) and glucose oxidase (10 mU/ml) for 20 h (Figure 5D). These data clearly indicate that ROS generation is critical for curcumin-induced CHOP upregulation. Previously, we have shown that ROS generation mediates curcumin-induced DR5 upregulation (29). Therefore, these results suggest the possibility that the blocking effect of NAC on the cell death induced by curcumin plus TRAIL may result from the suppression of curcumin-induced upregulation of CHOP and/or DR5.

CHOP is not involved in the curcumin-induced DR5 upregulation and the cell death induced by curcumin plus TRAIL

As Yamaguchi *et al.* (30) and other groups (31,32) recently reported that ER stress-mediated CHOP is a potential transcription factor for DR5. To determine whether CHOP plays a role in DR5 upregulation by curcumin, we employed CHOP siRNA. CHOP siRNA markedly reduced the CHOP protein levels but not DR5 protein levels by curcumin treatment (Figure 6A). To further clearly determine whether the CHOP is associated with curcumin-mediated transcriptional activation of DR5, we mutated the potential CHOP site (−281 to −261) of the *DR5* gene (32). As shown in Figure 6B, this mutation did not alter the activation of the DR5 promoter by curcumin, demonstrating that CHOP is not involved in the curcumin-mediated upregulation of DR5. Moreover, suppression of CHOP expression by CHOP siRNA did not prevent the sensitization to TRAIL-induced apoptosis by curcumin (Figure 6C). These results show that curcumin-induced CHOP upregulation is dispensable for the recovery of TRAIL-sensitivity in Caki cells.

Upregulation of DR5 by curcumin contributes to the enhancement of TRAIL-induced apoptosis in Caki cells

We next examined whether curcumin-induced DR5 upregulation is critical for enhancement of TRAIL-induced apoptosis by curcumin. We first used a recombinant human DR5/Fc chimeric protein, which has a dominant-negative effect by competing with endogenous DR5. As shown in Figure 7A, the curcumin-mediated enhancement of TRAIL-induced apoptosis was significantly blocked by the DR5/Fc chimeric protein. In addition, treatment with DR5/Fc chimeric protein significantly inhibited caspase 3 activation, and attenuated the cleavage of PLC-γ1 (Figure 7B). These data suggest that interaction of TRAIL with DR5 receptor is important for the sensitizing effect of curcumin on TRAIL-mediated apoptosis. To further determine the protective effect of the

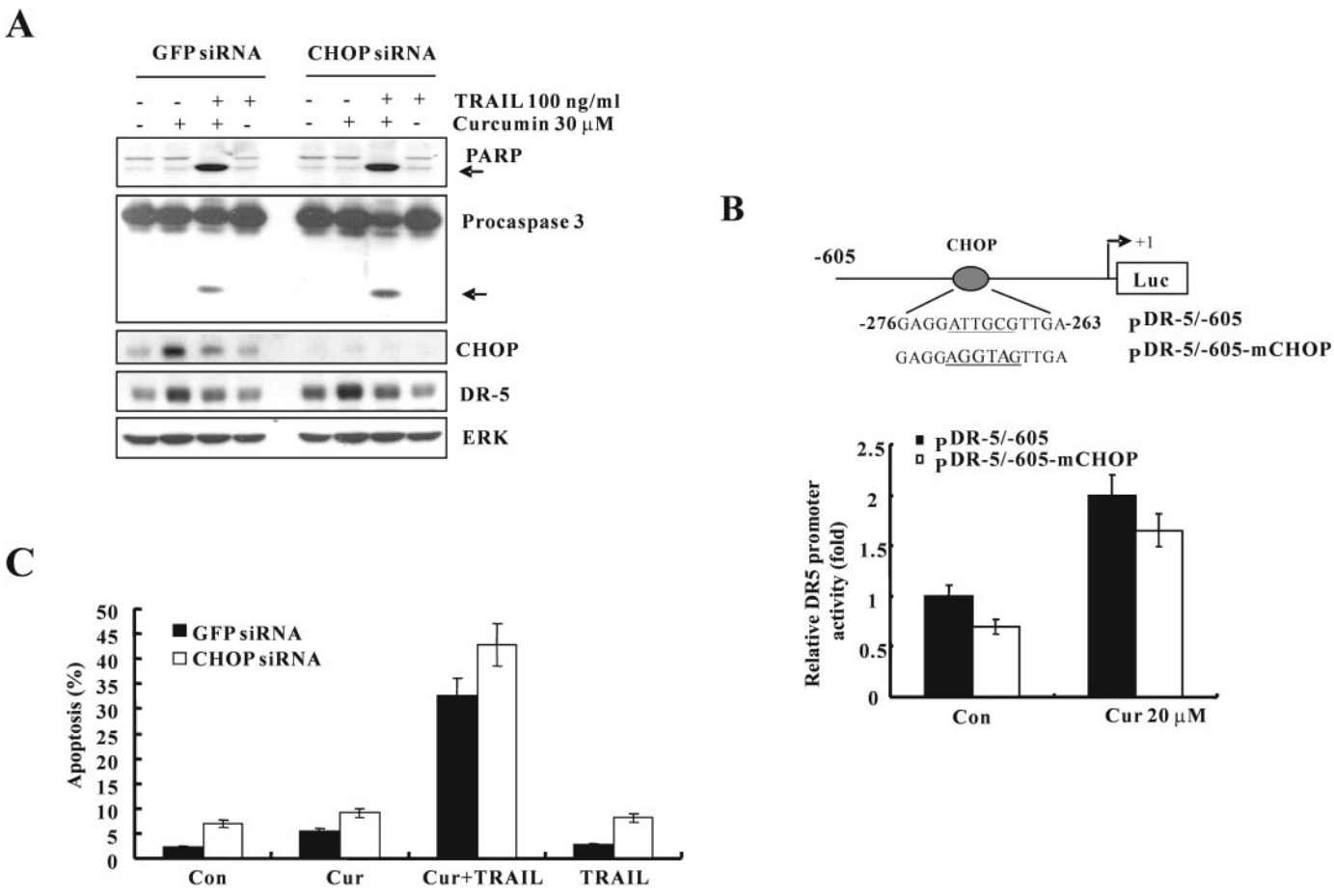

Fig. 6. CHOP is not associated with curcumin-mediated upregulation of DR5. (**A**) Caki cells were transfected with CHOP siRNA or GFP siRNA. Post-transfection, after 24 h, the cells were treated with 30 μM curcumin and/or 100 ng/ml TRAIL for 24 h. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane and probed with specific antibodies, anti-caspase 3, anti-PARP, anti-DR5, anti-CHOP or with anti-ERK antibody to serve as control for the loading of protein level. (**B**) pDR5/605 promoter and CHOP-mutated pDR5/605 plasmids were transfected, and treated with or without curcumin. The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least three independent experiments. (**C**) Apoptosis was determined by FACS analysis of the DNA fragmentation of propidium iodide-stained nuclei as described in Materials and methods.

DR5/Fc chimeric protein, we performed DNA fragmentation analysis for apoptosis. As shown in Figure 7C, treatment with DR5/Fc chimeric protein effectively inhibited DNA fragmentation induced by curcumin plus TRAIL.

To further confirm the functional significance of curcumin-induced DR5 upregulation, we employed the siRNA duplex against DR5 mRNA. Caki cells transfected with the control GFP or DR5 siRNA were treated with curcumin alone (30 μM), TRAIL alone (100 ng/ml), or combination of curcumin and TRAIL for 24 h and subjected to immunoblot analysis. As shown in Figure 8B, transfection of siRNA against DR5 resulted in a suppression of DR5 expression induced by curcumin in Caki cells, compared with cells transfected with control GFP siRNA. When we evaluated apoptosis by FACS analysis, GFP siRNA-transfected cells showed ~54% apoptosis following treatment with curcumin plus TRAIL, however, DR5 siRNA-transfected cells decreased this to 15% (Figure 8A). Suppression of DR5 expression in cells transfected with DR5 siRNA significantly inhibited not only caspase 3 activity (Figure 8B) but also DNA fragmentation (Figure 8C) induced by the combined treatment with curcumin and TRAIL. Taken together, these results suggest that curcumin-induced of DR5 is indispensable for the enhancement of TRAIL-sensitivity in Caki cells.

Discussion

TRAIL is proposed as a based treatment of cancer since it appears to target cancer cells to induce apoptosis while normal cells are resistant to it (5,7). The relative resistance of normal cells to TRAIL has been explained by the presence of large numbers of DcR on normal cells (5,7). However, recent studies have been shown that an increasing number of cancers are resistant to TRAIL. One proposed mechanism for this resistance is the presence of intrinsic proteins that can act as inhibitors of apoptosis. FLIP structurally resembles caspase 8 but lacks proteolytic activity, thus functioning as a dominantnegative inhibitor of caspase 8 (33–35). Further downstream in the TRAIL-induced apoptotic pathway, Bax mutations (13) or increased expression of IAP family members such as XIAP and survivin, also causes TRAIL resistance (36,37). Therefore, new strategies are necessary to overcome TRAIL resistance in cancer cells.

Recently, Deeb *et al*. (38,39) reported that a cancer chemopreventive agent curcumin sensitizes prostate cancer cells to

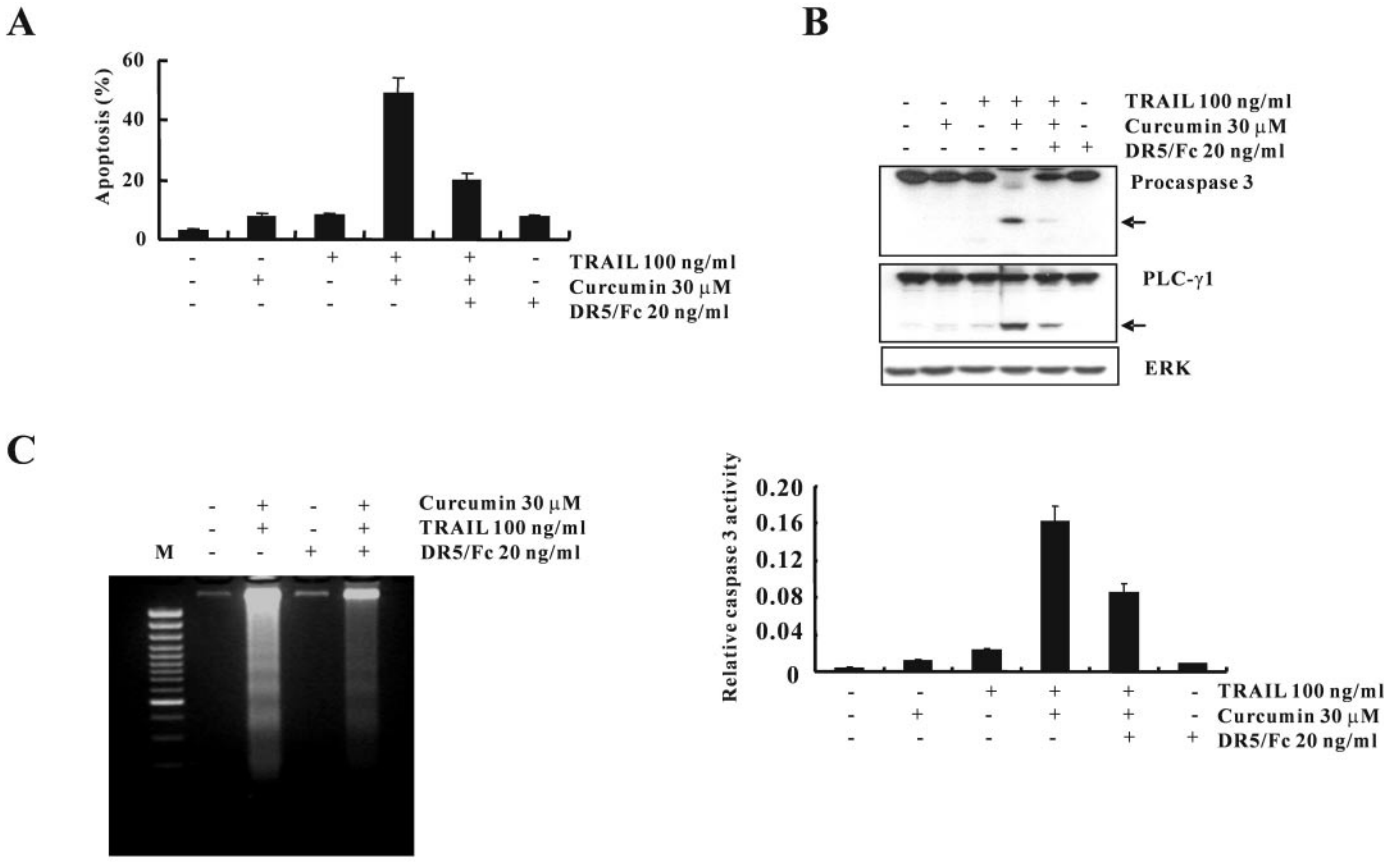

Fig. 7. Effect of DR5/Fc chimeric protein on curcumin plus TRAIL-induced apoptosis. (**A**) Caki cells were treated with curcumin plus TRAIL in the presence or absence of DR5/Fc chimeric protein (20 ng/ml). Apoptosis was analyzed as a sub-G_1 fraction by FACS. (**B**) Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane and probed with specific antibodies, anti-caspase 3, anti-PLC-γ1 or with anti-ERK antibody to serve as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 and caspase 3 are indicated by an arrow. A representative result is shown; two additional experiments yielded similar results. Caspase 3 activity was determined as described in Figure 3B. Data shown are mean ± SD. ($n = 3$). (**C**) Fragmentation of genomic DNAs in Caki cells treated for 24 h with the indicated concentrations of curcumin, TRAIL and DR5/Fc chimeric protein. Fragmented DNA was extracted and analyzed on 2% agarose gel.

TRAIL by inhibiting NF-κB through suppression of IκBα phosphorylation. In this study, we further investigated the ability of curcumin to sensitize TRAIL-induced apoptosis in human renal cancer cells. Recently, we have shown that curcumin induces DR4 and DR5 upregulation (29), suggesting that curcumin-induced upregulation of these TRAIL receptors may be associated with the significant apoptosis in cells treated with curcumin and TRAIL. Recently studies using affinity assay and phage display of DR-selective TRIAL variants have revealed that DR5 may play a more prominent role than DR4 in TRAIL-mediated apoptotic signals (40,41). Following experimental evidences in the present study demonstrate that curcumin-induced DR5 upregulation is essential for curcumin plus TRAIL-induced apoptosis. First, curcumin induces DR5 expression levels more than DR4. Second, both a DR5-specific blocking chimeric antibody and suppression of DR5 expression by siRNA duplexes inhibited the cell death induced by the combination of curcumin and TRAIL in Caki cells. In an attempt to explore the underlying mechanisms involved in curcumin-induced DR5 expression, we tested the possibility that CHOP, a member of the C/EBP family of transcription factors, might be associated with curcumin-induced DR5 upregulation. Several papers provide evidence that DR5 upregulation is critical for ER stress (induced by thapsigargin, tunicamycin and brefeldin A)-mediated apoptosis in human cancer cells and CHOP is involved in this process (30,32,42). Additionally, *CHOP* gene expression is also upregulated when cells were deprived of essential nutrients, and exposed to DNA-damaging agents and anticancer drugs (22,23). Our results obtained by luciferase assay using the *CHOP*-deletion mutants showed that CHOP promoter region from −649 to −280 contain *cis*-positive elements that are essential for curcumin-induced CHOP regulation. The region from −649 to −280 includes the binding sites for many transcriptional factors such as C/EBP, ATF-2 and SP-1 (23). C/EBPβ-deficient cells did not affect the induction of CHOP mRNA by leucine starvation, whereas lack of ATF-2 resulted in a complete loss of the CHOP mRNA induction (23). In contrast, the induction of CHOP by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 has been demonstrated to require C/EBP and ER stress element (43). Therefore, further studies will be necessary to identify the precise *cis*-regulatory elements in curcumin-induced CHOP induction.

Recently, Scott *et al*. (44) have reported that treatment of colon cancer cells with curcumin significantly induced apoptosis accompanying the upregulation of CHOP through the generation of ROS. Similar to their results, subtoxic doses of curcumin induced CHOP upregulation in Caki cells and pretreatment with NAC significantly decreased curcumin-induced CHOP upregulation. However, curcumin treatment did not affect the levels of Grp78 (an ER stress response gene product), GADD34 and caspase 12, suggesting that

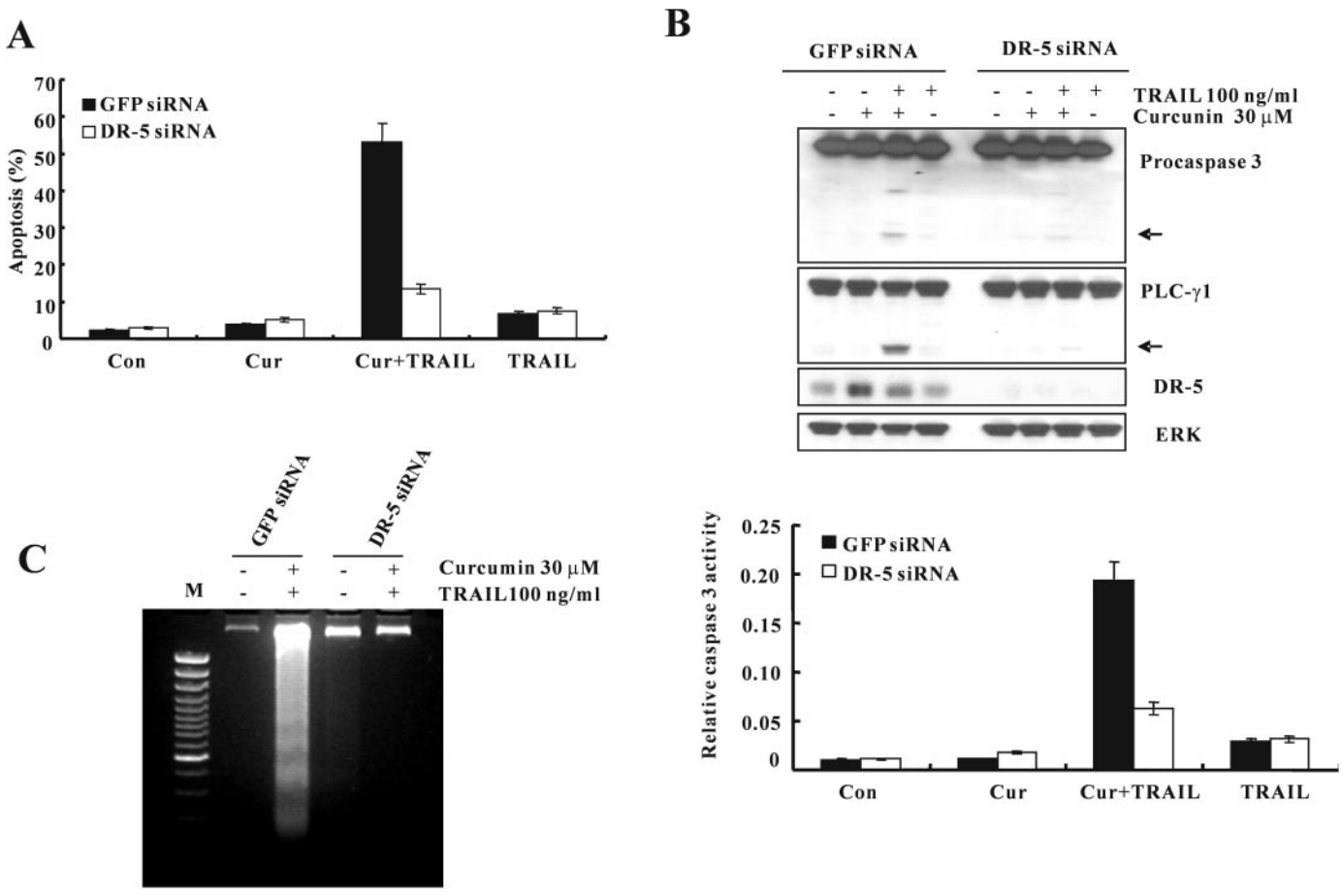

Fig. 8. Downregulation of DR5 attenuates curcumin-mediated TRAIL-induced apoptosis in Caki cells. (**A**) Caki cells were transfected with DR5 siRNA or GFP siRNA. Post-transfection, after 24 h, the cells were treated with 30 μM curcumin and/or 100 ng/ml TRAIL for 24 h. Apoptosis was analyzed as a sub-G1 fraction by FACS. (**B**) Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-caspase 3, anti-PLC-γ1, anti-DR5 or with anti-ERK antibody to serve as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results. Caspase 3 activity was determined as described in Figure 3B. Data shown are mean ±SD ($n = 3$). (**C**) Caki cells were transfected with DR5 siRNA or GFP siRNA. Post-transfection, after 24 h, the cells were treated with 30 μM curcumin and/or 100 ng/ml TRAIL for 24 h. Fragmented DNA was extracted and analyzed on 2% agarose gel.

curcumin induces upregulation of CHOP expression though ER stress-independent manner. When we treated Caki cells with curcumin in CHOP transfected cells, neither curcumin-induced DR5 upregulation nor the cell death induced by curcumin plus TRAIL was affected. Furthermore, results of the luciferase assay showed that the potential CHOP-binding site is not essential for curcumin-mediated transcriptional activation of the DR5 luciferase plasmid (Figure 5B). Previously, we have shown that pretreatment with NAC completely blocks curcumin-induced DR5 upregulation (29). Taken together, these results suggest that ROS are commonly involved in curcumin-induced DR5 and CHOP upregulation. However, suppression of CHOP expression by its siRNA cannot attenuate the sensitizing effect of curcumin on TRAIL-induced apoptosis, since CHOP is not associated with curcumin-induced DR5 upregulation in Caki cells.

In conclusion, we provide the clear evidences here that curcumin enhances TRAIL-induced apoptosis through the induction of DR5 expression in renal cancer cells. These data raise the possibility that the combination of curcumin and TRAIL may provide an effective therapeutic strategy for the treatment of cancer.

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