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RU486 Induces Pro-Apoptotic Endoplasmic Reticulum Stress Through the Induction of CHOP Expression by Enhancing C/EBPδ Expression in Human Renal Carcinoma Caki Cells

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ABSTRACT

RU486 (Mifepristone) is known as an antagonist of the progesterone receptor and glucocorticoid receptor. Here, we investigated the mechanism underlying anti-tumor activity of RU486 in renal carcinoma Caki cells. Treatment of Caki cells with RU486 was found to induce several signature ER stress markers; including ER stress-specific XBP1 splicing, and the up-regulation of glucose-regulated protein (GRP)-78 and CCAAT/enhancer-binding protein homologous protein (CHOP) expression. RU486-induced expression of CHOP involves the putative C/EBPδ site within the CHOP promoter region. Using a combination of C/EBPδ cDNA transfection, the luciferase assay with a mutated C/EBPδ binding site and siRNA-mediated C/EBPδ knockdown, we found that the C/EBPδ site is required for RU486-mediated activation of the CHOP promoter. In addition, RU486-induced CHOP expression is down-regulated by inhibition of the p38 MAPK and JNK signaling pathways at the post-translational levels. RU486 dose-dependently induced apoptotic cell death in renal carcinoma cells. Suppression of CHOP expression by CHOP siRNA attenuated RU486-induced apoptosis. Taken together, RU486 induces pro-apoptotic ER stress through the induction of CHOP expression. J. Cell. Biochem. 117: 361–369, 2016.

KEY WORDS: RU486; CHOP; ER STRESS; C/EBPδ; APOPTOSIS

RU486, a glucocorticoid receptor (GR) antagonist, has high affinity for both GR and progesterone receptor [Baulieu, 1989]. In addition, RU486 has demonstrated promising results in interference with cancer cell growth. Numerous potential therapeutic mechanisms have revealed that RU486 inhibits proliferation and induces apoptosis in endometrial cancer, breast cancer, and prostate cancer [El Etreby et al., 2000; Gaddy et al., 2004; Navo et al., 2008]. These mechanisms include estrogen and progesterone receptor down-regulation, and modulation of the expression levels of Bcl-2, Bax, and p53 [Jiang et al., 2002; Navo et al., 2008; Jang et al., 2013]. In our previous study, RU486 increased death receptor 5 (DR5) expression and decreased the protein expression levels of Bcl-2 and c-FLIP(L) in human cancer cells, resulting in the induction of TRAIL sensitivity [Min et al., 2012]. RU486 may induce TRAIL sensitization in various cancer cells via a common mechanism.

Many disturbances, including those of cellular redox regulation, cause accumulation of unfolded proteins in the endoplasmic reticulum (ER), triggering an evolutionarily conserved series of signal-transduction events, termed the unfolded protein response (UPR) [Xu et al., 2005]. When the ER dysfunction is severe or prolonged, the organelle elicits apoptotic signals [Oyadomari and Mori, 2004]. One of the components of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP). CHOP is a member of the C/EBP family of bZIP transcription factors, and it is highly expressed by ER stress [Oyadomari and Mori, 2004]. It has been shown that CHOP induces death by promoting protein synthesis by dephosphorylation of α subunit of eukaryotic translation initiation factor 2 (eIF2α) in the ER stressed cells [Marciniak et al., 2004].

To gain a better understanding of the molecular effects of RU486 on renal carcinoma cells, we focused on two major findings. One is the molecular mechanisms of RU486-mediated CHOP expression involved in the ER stress signaling pathway. The other is the potential cell death inducing effect of RU486 via up-regulation of CHOP expression.

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MATERIALS AND METHODS

CELLS AND MATERIALS

Caki, SK-Hep1, HT29, and Huh-7 cells were obtained from the American Type Culture Collection (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. CHOP+/+ MEF and CHOP-/- MEF were a gift from Dr. K.M. Park (Kyungpook University, Korea). Mouse kidney cells (TMCK-1) were supplied as a gift from Dr. T.J. Lee (Yeungnam University, Korea). A primary culture of human mesangial cells (Cryo NHMC) was purchased from Clonetics (San Diego, CA). We used less than 0.1% dimethyl sulfoxide (DMSO) concentration in all cell culture experiments. Anti-CHOP, anti-PARP, anti- GRP78, anti-XBP-1 and anti-C/EBPδ antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phospho-ERK, anti- phospho-JNK, anti-phospho-p38 MAPK, anti-p38 MAPK, and anti-JNK were purchased from Cell Signaling Technology (Beverly, MA). Anti-ERK antibody was obtained from Transduction Laboratories, (Lexington, KY). The anti-actin antibody was obtained from Sigma (St. Louis, MO). PD98059, SP600125, SB203580 and z- VAD-fmk were obtained from Calbiochem (San Diego, CA). PCR primers were purchased from Bioneer (Daejeon, Korea), and other chemicals were from Sigma (St. Louis, MO).

CELL VIABILITY ASSAY

XTT assay was employed to measure the cell viability using WelCount^{TM} Cell Viability Assay Kit (WelGENE, Gyeongsan, Korea). Briefly, 24 h after drug treatment, add reagent to each well and then measure with multi-well plate reader (at 450 nm/690 nm).

WESTERN BLOT ANALYSIS

For the Western blotting experiments, the cells were washed with cold PBS and lysed on ice in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 mM EDTA). The lysates were centrifuged at 10,000*g* for 10 min at 4°C, and the supernatant fractions were collected. The proteins were separated by SDS–PAGE electrophoresis and transferred to Immobilon-P membranes. The specific proteins were detected using an enhanced chemiluminescence (ECL) western blotting kit (Millipore Corporation, Bedford, MA) according to the manufacturer's instructions.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated using the TRIzol reagent (Life Technologies; Gaithersburg, MD), and the cDNA was prepared using M-MLV reverse transcriptase (Gibco-BRL; Gaithersburg, MD) according to the manufacturer's instructions. The following primers were used for the amplification of human CHOP, GRP78, XBP-1 and actin: CHOP (sense) 5′-CAA CTG CAG AGA ATT CAG CTG A-3′ and (antisense) 5′-ACT GAT GCT CTA GAT TGT TCA T-3′, GRP78 (sense) 5′- ACT GCT GTT TTC AGA TGG AGG T-3′ and (antisense) 5′- CTA GGA GCC AGC TCA GAT GC-3′, XBP-1 (sense) 5′-CCT TGT AGT TGA GAA CCA GG-3′, and (antisense) 5′-GGG GCT TGG TAT ATA TGT GG-3′, and actin (sense) 5′-GGC ATC GTC ACC AAC TGG GAC- 3′ and (antisense) 5′-CGA TTT CCC GCT CGG CCG TGG-3′. The PCR amplifications were carried out using the following cycling conditions: 94°C for 3 min followed by 17 (actin) or 25 cycles (CHOP, GRP78, and XBP-1) of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified products were separated by electrophoresis on a 1.5% agarose gel and detected under UV light.

PREPARATION OF NUCLEAR EXTRACTS

Following the required treatments, Caki cells were trypsinized and suspended in buffer A (10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After incubation on ice for 30 min, cells were centrifuged at 2,500 rpm for 3 min to obtain a nuclear pellet. Buffer C (20 mM HEPES at pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) was added, followed by rotation for 30 min at 4°C. The resulting lysates were centrifuged at 12,000 rpm at 4°C for 5 min. Supernatant fractions were collected as the nuclear extract.

PLASMIDS, TRANSFECTIONS, AND LUCIFERASE GENE ASSAYS

CHOP promoter constructs were generously provided by Dr. P. Fafournoux (U.R. 238 de Nutrition Cellulaire et Moléculaire, France). The human CHOP promoter-containing plasmids have been described previously [Bruhat et al., 2000]. Point mutation of the c/EBP binding sites in the CHOP (-649) promoter was generated by a two-step PCR method using the following primers: mC/EBP (5′- TGC CAA ACC ATG TAG CAT CCC CGC -3′ and 5′-GCG GGG ATG CTA CAT GGT TTG GCA -3′). The clone representing this point mutation was sequenced to ensure the accuracy of the PCR amplification procedure. For transfection, in brief, cells were plated onto 6-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were transfected with 2 μg of various plasmid constructs using Lipofectamine. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase activities were assayed according to the manufacturer's protocol (Promega).

SMALL INTERFERING RNA (siRNA)

The siRNA duplexes used in this study were purchased from Invitrogen (Carlsbad, CA) and had the following sequences: CHOP, AAG ACC CGC GCC GAG GUG AAG; and green fluorescent protein (GFP [control]), AAG ACC CGC GCC GAG GUG AAG. C/EBPδ siRNA was purchased from Santa Cruz Biotechnology. Cells were transfected with siRNA oligonucleotides using Oligofectamine reagent (Invitrogen) according to the manufacturer's recommendations.

FLOW CYTOMETRY ANALYSIS

For flow cytometry, the cells were resuspended in 100 μl of phosphate-buffered saline (PBS), and 200 μl of 95% ethanol was added while the cells were being vortexed. Next, the cells were incubated at 4°C for 1 h, washed with PBS, resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) with 12.5 μg of RNase and incubated for an additional 30 min at 37°C. The cellular DNA was then stained by adding 250 μl of a propidium iodide solution (50 μg/ml) to the cells for 30 min at room temperature. The stained cells were analyzed by fluorescent-activated cell sorting on a FACScan flow cytometer to determine the relative DNA content, which was based on the red fluorescence intensity.

4′,6′-DIAMIDINO-2-PHENYLINDOLE (DAPI) STAINING

To examine cellular nuclei, the cells were fixed with 1% paraformaldehyde on glass slides for 30 min at room temperature. After fixation, the cells were washed with PBS and a 300 nM 4′,6′-diamidino-2-phenylindole solution (Roche, Mannheim, Germany) was added to the fixed cells for 5 min. After the nuclei were stained, the cells were examined by fluorescence microscopy.

DNA FRAGMENTATION ASSAY

The cell death detection ELISA plus kit (Boehringer Mannheim; Indianapolis, IN) was used to determine the level of apoptosis by detecting fragmented DNA within the nuclei of RU486-treated cells. Briefly, each culture plate was centrifuged for 10 min at 200*g*, the supernatant was removed, and the cell pellet was lysed for 30 min. Next, the plate was centrifuged again at 200*g* for 10 min, and the supernatant that contained the cytoplasmic histone-associated DNA fragments was collected and incubated with an immobilized anti-histone antibody. The reaction products were incubated with a peroxidase substrate for 5 min, and the absorbance was measured by spectrophotometry at 405 and 490 nm (reference wavelength) using a microplate reader. The signals in the wells containing the substrate alone were subtracted as the background.

ASP-GLU-VAL-ASP-ASE (DEVDASE) ACTIVITY ASSAY

To evaluate DEVDase activity, cell lysates were prepared after their respective treatments with RU486. 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, at pH 7.5, 137 mM NaCl, 10% glycerol) containing a caspase substrate (Asp-Glu-Val-Asp-chromophore-p-nitroanilide [DVAD-pNA]) at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured using a spectrophotometer.

DENSITOMETRY

The band intensities were scanned and quantified using the gel analysis plugin for the open source software ImageJ 1.46 (Imaging Processing and Analysis in Java; ttp://rsb.info.nih.gov/ij).

STATISTICAL ANALYSIS

The data were analyzed using one-way ANOVA and post-hoc comparisons (Student–Newman–Keuls) using the Statistical Package for Social Sciences 22.0 software (SPSS Inc.; Chicago, IL).

RESULTS

RU486 INDUCES CHOP EXPRESSION AND SPLICING OF XBP-1 mRNA

To examine whether induction of ER stress-related proteins was caused by RU486, we analyzed the expression of CHOP and GRP78 in response to RU486 treatment. As shown in Figure 1A, treatment with RU486 (10–50 μM) increased the expression of ER stress-related proteins in a dose-dependent manner. Next, we investigated whether RU486-induced CHOP and GRP78 induction is controlled at the transcriptional level. RT-PCR analysis demonstrated that RU486-induced CHOP and

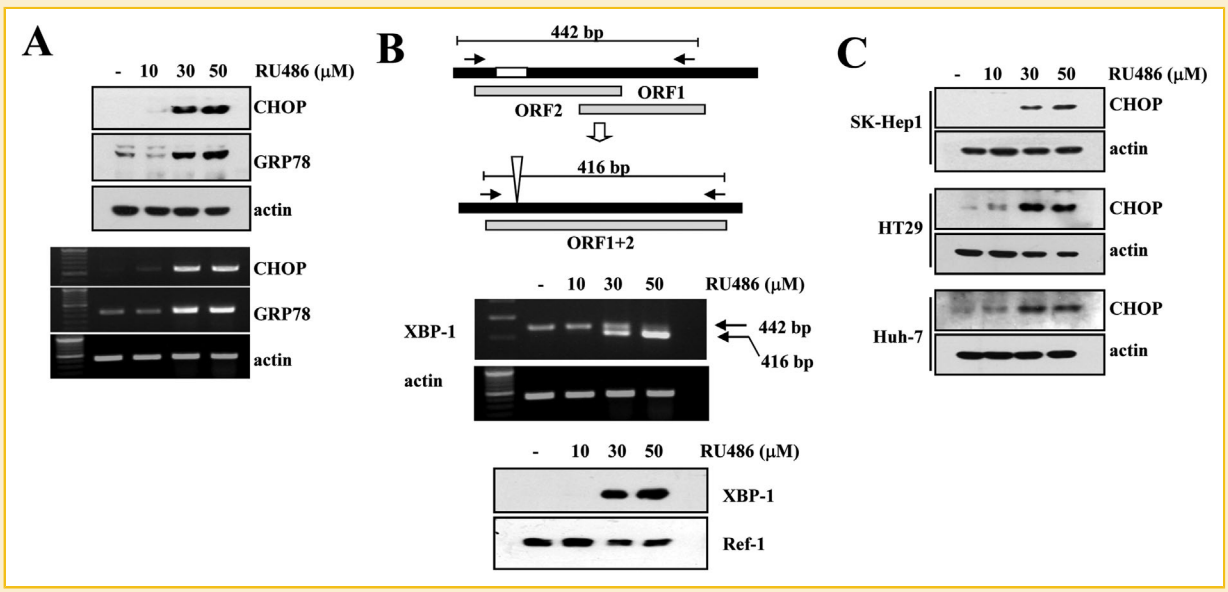

Fig. 1. Expression levels of ER stress-related proteins in Caki cells following RU486 treatment. (A) Caki cells were treated with the indicated concentrations of RU486 (10, 30, 50 μM) for 6 h. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to membranes, and probed with anti-CHOP and anti-GRP78 antibodies. Total RNA was isolated, and RT-PCR analysis was performed as described in the Materials and Methods section. Actin was used as an internal control. A representative study is shown, and two additional experiments yielded similar results. (B) Caki cells were treated with the indicated concentrations of RU486 for 6 h. Total RNA was isolated, and RT-PCR analysis was performed as described in Materials and Methods section. The 416-bp form appearing in treated cells represents the spliced form of the mRNA. Nuclear extracts were analyzed for XBP1 and Ref-1 by western blotting as described in the Materials and Methods section. (C) SK-Hep1, HT29 and Huh-7 cells were treated with the indicated concentrations of RU486 for 6 h. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to membranes, and probed with anti-CHOP antibodies.

GRP78 mRNA levels in a dose-dependent manner. The ER stress pathway is involved in IRE1α activation and subsequent XBP-1 mRNA splicing [Yoshida et al., 2001]. The spliced XBP-1 protein functions as a transcription factor that is involved in activating CHOP [Oyadomari and Mori, 2004]. To evaluate whether the potential effects of ER are related to alternative splicing of XBP-1 in RU486-treated cells, alternative splicing of XBP-1 mRNA was determined by RT-PCR. As shown in Figure 1B, RU486 induced the splicing of XBP-1 mRNA and expression of spliced XBP-1 protein. RU486-induced up-regulation of CHOP was also observed in other cancer cell lines such as SK-Hep1, HT29 and Huh-7 (Fig. 1C). These results suggest that up-regulation of CHOP is a common response of cancer cell lines to RU486 treatment.

RU486 INCREASES CHOP EXPRESSION THROUGH ACTIVATION OF THE C/EBPδ TRANSCRIPTION FACTOR

To further confirm the transcriptional regulation of CHOP, constructs containing serial deletions of the 5′ end of the CHOP promoter were transiently transfected into Caki cells, and promoter activities were measured. As shown in Figure 2A, deletion from −649 to −280 markedly reduced the RU486-mediated activation, suggesting that the region between −649 and −280 contains *cis*-positive elements involved in the transcriptional activation of CHOP by RU486. To identify potential transcription factor binding sites in the region between −649 and −280 of the CHOP promoter, the genomic nucleotide sequence was analyzed for a known transcription factor binding consensus with a computer based program (MacVector sequence analysis software). As shown in Figure 2B, the region spanning −318 to −286 contains a C/EBP-like sequence and a putative Sp1 binding site. To determine the role of each site in the RU486 responsiveness of the CHOP promoter, we transfected Caki cells with each transcription factor cDNA (Sp1 and C/EBPδ) and then treated them with RU486. Ectopic expression of Sp1 markedly increased the basal promoter activity, but did not significantly increase the RU486 responsive promoter activity in −649/CHOP-Luc (Fig. 2B). However, ectopic expression of C/EBPδ significantly increased the RU486 responsive promoter activity in −649/CHOP-Luc in a dose-dependent manner (Fig. 2C).

To confirm that C/EBPδ plays a role in RU486-induced transcriptional activation of the CHOP promoter, we performed a luciferase assay with the mutated C/EBPδ binding site (−649/CHOP-mC/EBPδ)

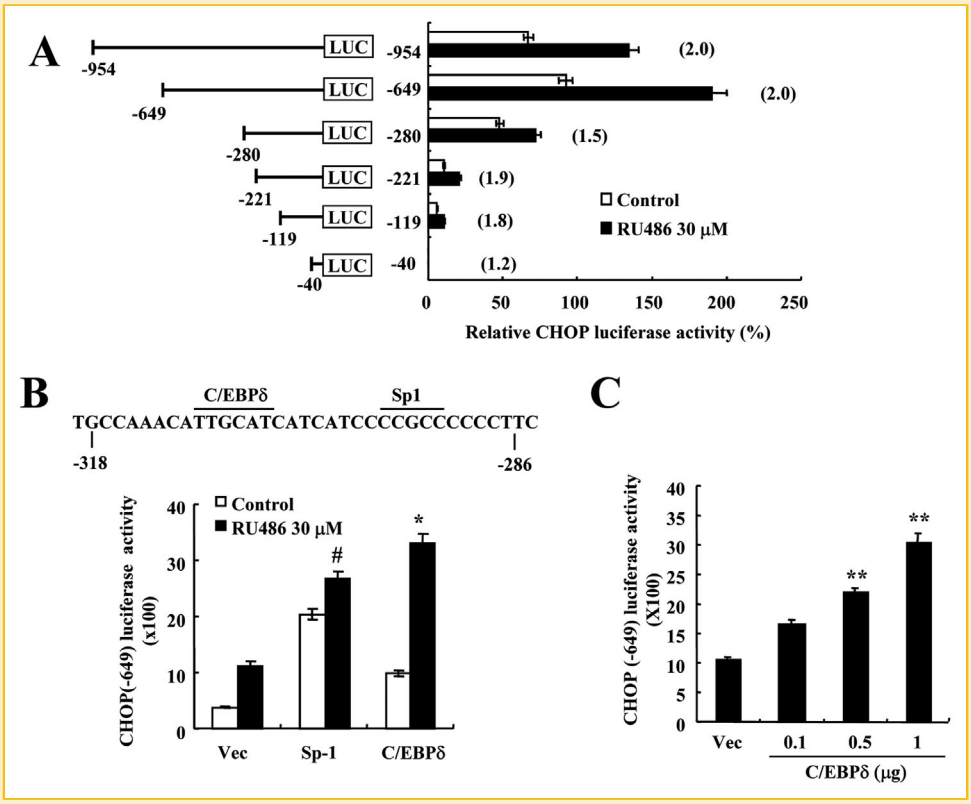

Fig. 2. Effect of RU486 on CHOP promoter activity. (A) Caki cells were transiently transfected with progressive 5′-deletion mutants of the CHOP promoter. Twenty-four hours after transfection, cells were treated with RU486 (30 μM) for 16 h. The cells were lysed, and then the luciferase activity measured. Data represent the mean ± SD of at least three independent experiments. The relative fold induction, defined as the ratio of the relative luciferase activity of RU486-treated cells to untreated cells, is indicated in parentheses to the right of the bars. (B) Nucleotide sequence from −318 to −286 relative to the start site of CHOP promoter. The positions of the C/EBPδ and Sp1 sites are indicated. Caki cells were cotransfected with Sp1 and C/EBPδ cDNA and −649/CHOP-Luc promoter plasmids. The cells were treated with or without RU486, and lysed and then the luciferase activity was measured. (C) Caki cells were cotransfected with the indicated concentrations of C/EBPδ cDNA and -649/CHOP-Luc promoter plasmids. The cells were lysed, and then the luciferase activity was measured. The values in (A, B, and C) represent the mean ± SD from three independent experiments. * $P < 0.01$ compared with the RU-486-treated vector. # $P < 0.05$ compared with the control-Sp1. ** $P < 0.01$ compared with the vector.

reporter construct. We found that RU486 treatment increased the transcriptional activity of the CHOP promoter. However, the C/EBPδ-mutated CHOP promoter was not activated by treatment with RU486 (Fig. 3A). Furthermore, siRNA-mediated suppression of C/EBPδ expression inhibited the RU486-induced increase in CHOP protein levels (Fig. 3B). In addition, we assessed the time kinetics of the expression levels of C/EBPδ in cells treated with RU486. We choose 30 μM concentration of RU486 that gave consistent results. Caki cells were treated with 30 μM RU486 at the indicated times, and the expression levels of C/EBPδ were determined by western blot analysis. RU486 increased C/EBPδ expression within 3 h, and peaked at 6 h. The CHOP expression pattern showed similar kinetics to that of C/EBPδ expression (Fig. 3C). Taken together, these results suggest that the expression of C/EBPδ is critical for RU486-induced CHOP expression.

THE p38 MAPK AND JNK SIGNALING PATHWAYS REGULATE RU486-INDUCED CHOP PROTEIN EXPRESSION AT THE POST-TRANSLATIONAL LEVEL

To investigate whether the ERK, JNK, or p38 MAPK pathways are involved in RU486-induced CHOP expression, we examined the activation of the three MAPKs by detecting their dually phosphorylated forms in western blots probed with specific anti-phosphokinase antibodies (Fig. 4A). RU486 treatment induced a transient increase in the phosphorylated p38 MAPK and JNK levels, but not ERK. Next, we examined whether selective MAPK inhibitors could affect RU486-induced CHOP expression. Pretreatment of cells with SB203580 (SB; a p38 MAPK inhibitor) or SP600125 (SP; a JNK inhibitor) profoundly inhibited RU486-induced CHOP protein expression but not CHOP mRNA expression (Fig. 4B). Furthermore, JNK and p38 MAPK inhibitors did not decrease RU486-induced CHOP promoter activity in Caki cells transfected with the −954/CHOP-Luc plasmid (Fig. 4C). We next investigated the effect of p38 MAPK and JNK on the protein stabilities of CHOP. Caki cells were treated with 30 μM RU486 for 6 h induce CHOP expression, and then the cells were washed with fresh media. Washed cells were treated with cycloheximide (CHX; an inhibitor of de novo protein synthesis) alone, CHX plus SB or CHX plus SP for the indicated time points. Although treatment with CHX alone down-regulated CHOP expression, combined treatment with CHX plus SB or CHX plus SP more markedly reduced CHOP expression (Fig. 4D and E). These data suggested that RU486-induced CHOP expression is down-regulated by inhibition of the p38 MAPK and JNK signaling pathways at the post-translational level.

SUPPRESSION OF CHOP EXPRESSION ATTENUATES RU486-INDUCED APOPTOSIS

To investigate whether RU486 induces apoptosis, Caki cells were treated with the indicated concentrations of RU486 for 48 h. We first determined PARP cleavage and apoptosis in Caki cells using flow cytometric analysis to detect hypodiploid cell populations. As shown in Figure 5A, treatment with RU486 for 48 h in Caki cells resulted in a markedly increased PARP cleavage and accumulation of sub-G1 phase cells in a dose-dependent manner. However, RU486 treatment for 24 h did not induce PARP cleavage (data not shown). Treatment with RU486 altered the morphology of Caki cells to a typical apoptotic pattern, including cellular shrinkage and blebbing, and blocked chromatin in the nuclei (Fig. 5B and C). Next, we analyzed DNA fragmentation, which is another hallmark of apoptosis. Treatment with RU486 for 48 h increased cytoplasmic histone-associated DNA

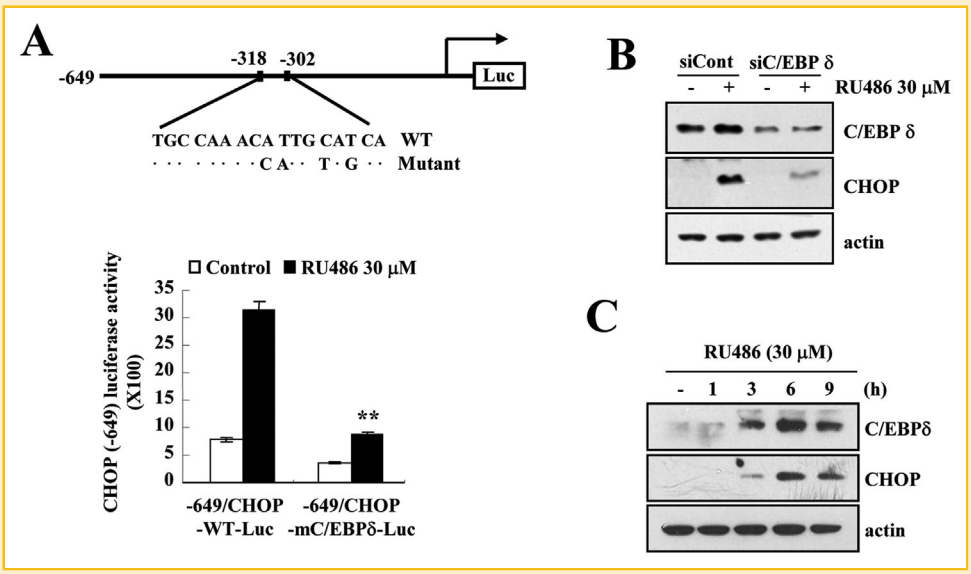

Fig. 3. C/EBPδ mediates RU486-induced CHOP up-regulation. (A) Caki cells were transfected with −649/CHOP or C/EBPδ- mutated −649/CHOP and then were treated with the indicated concentrations of RU486. After 24 h, cell lysates were lysed and assayed for luciferase activity. Data represent the mean ± SD of at least three independent experiments. (B) Caki cells were transfected with C/EBPδ siRNA or control siRNA. Twenty-four hours after transfection, cells were treated with or without RU486 for 12 h. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to membranes, and probed with specific antibodies C/EBPδ and anti-CHOP or with anti-actin antibody to serve as control for the loading of protein level. (C) Caki cells were treated with 30 μM RU486 for the indicated time periods. The expression levels of C/EBPδ, CHOP and actin were determined using western blotting. The values in A represent the mean ± SD from three independent experiments. ** $P < 0.01$ compared with RU-486-treated −649/CHOP.

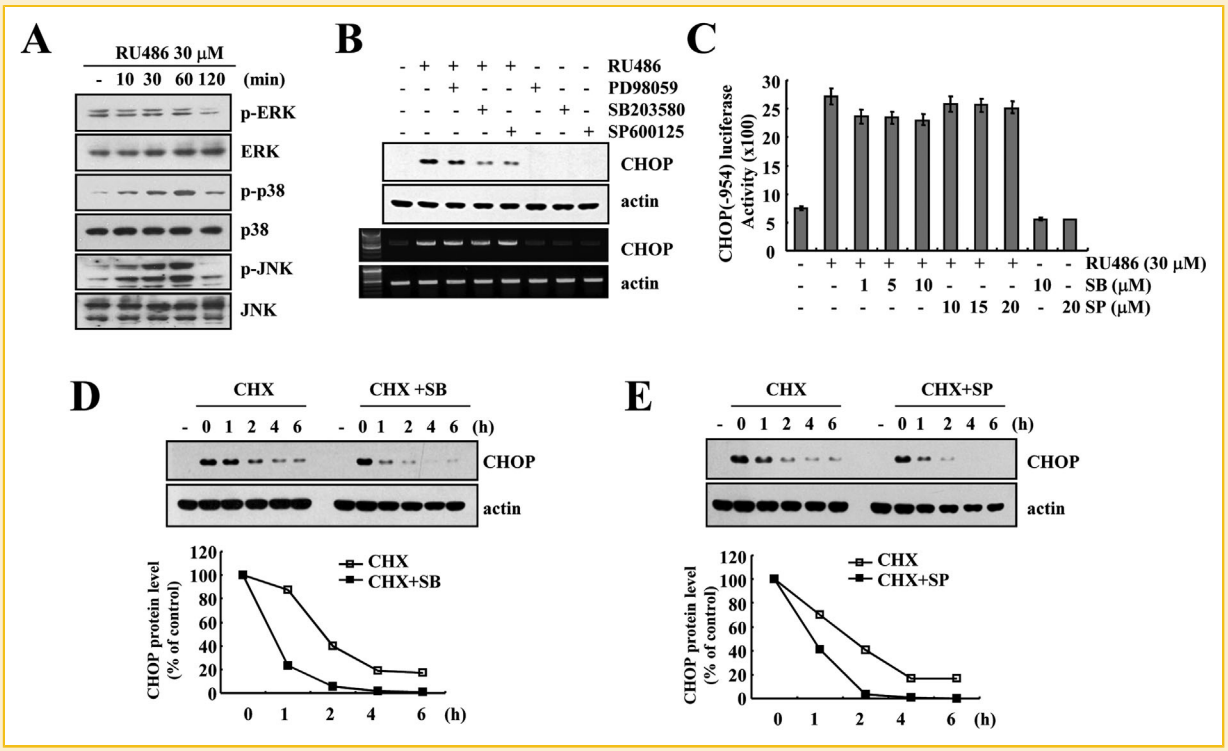

Fig. 4. Effect of the MAPK signaling pathway on RU486-induced up-regulation of CHOP protein expression in Caki cells. (A) Caki cells were treated with 30 μM RU486 for the indicated time periods. The expression levels of p-ERK/ERK, p-p38MAPK/p38MAPK and p-JNK/JNK were determined using western blotting. (B) Caki cells were pretreated with various inhibitors (50 μM PD98059, 10 μM SB203580, 20 μM SP600125) for 30 min followed by treatment with RU486 (30 μM) for 12 h. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against CHOP. The equal loading in each lane was demonstrated by the similar intensities of actin. Total RNA was isolated and RT-PCR analysis was performed. (C) Caki cells were transfected with CHOP promoter plasmid (−954/CHOP-Luc) and further cultured with RU486 (30 μM) in the presence or absence of the indicated concentrations of SB203580 or SP600125. The cells were lysed, and the luciferase activity was measured. Data represent the mean ± SD of at least three independent experiments. (D and E) Caki cells were treated with 30 μM RU486 for 6 h and washed with serum-free media, and then were further cultured with or without SB203580 (10 μM) or SP600125 (20 μM) in the presence of cycloheximide (CHX) (20 μg/ml) for the indicated time periods. The protein expression levels of CHOP and actin were determined by western blotting. The level of actin was used as a loading control. The band intensities of CHOP protein were measured using the public domain JAVA image-processing program ImageJ. The values in C represent the mean ± SD from three independent experiments.

fragments and DEVDase (caspase-3) activity in a dose-dependent manner (Fig. 5D and 5E). However, RU486 did not increase caspase-2, -8 and -9 activation (Supplementary Figure 1). To address the significance of caspase-3 activation in RU486-induced apoptosis, we used a general inhibitor of caspases, z-VAD-fmk. RU486-induced apoptosis and PARP cleavage were significantly prevented by treatment with z-VAD-fmk (Fig. 5F). In addition, normal cells showed less sensitive than cancer cells to RU486-induced cell death (Fig. 5G).

To determine the functional significance of RU486-induced CHOP up-regulation, we used a siRNA duplex against the CHOP mRNA. Caki cells were transfected with the indicated siRNA and treated with or without RU486 for 48 h. Under these conditions, the RU486-induced accumulation of the sub-G1 cell population was attenuated in cells transfected with the CHOP siRNA relative to the accumulation observed in control siRNA-transfected cells (Fig. 6A). Immunoblot analyses demonstrated that transfection of the CHOP siRNA suppressed the RU486-induced CHOP expression in Caki cells relative to the control GFP siRNA (Fig. 6B). CHOP siRNA significantly attenuated PARP cleavage (Fig. 6B). In addition, to further examine whether RU486-induced cell death is mediated through CHOP, we investigated the effects of RU486 on cell death in wild-type MEF cells and CHOP-/- MEF cells. RU486 reduced cell viability in wild type MEF cells, whereas RU486 had less effect on cell viability and PARP cleavage in CHOP-/- MEF cells (Fig. 6C and D). These data indicate that CHOP up-regulation appears to be involved in RU486-induced apoptosis.

DISCUSSION

CHOP gene expression is induced in response to stresses such as ER stress, deprivation of essential nutrients, DNA-damaging agents, and anticancer drugs [Luethy and Holbrook, 1992; Gately et al., 1994; Bruhat et al., 1999; Oyadomari and Mori, 2004]. Transcriptional induction of the CHOP gene in response to ER stress is mediated by the ER stress responsive element (ERSE), which is located between −104 and −75 of the CHOP promoter region [Ubeda and Habener, 2000]. Numerous *cis*-acting elements such as AP1, Sp1, C/EBP and ATF in the CHOP promoter contribute to the rapid and dramatic increase in CHOP transcription induced by stress [Guyton et al., 1996; Bruhat et al.,

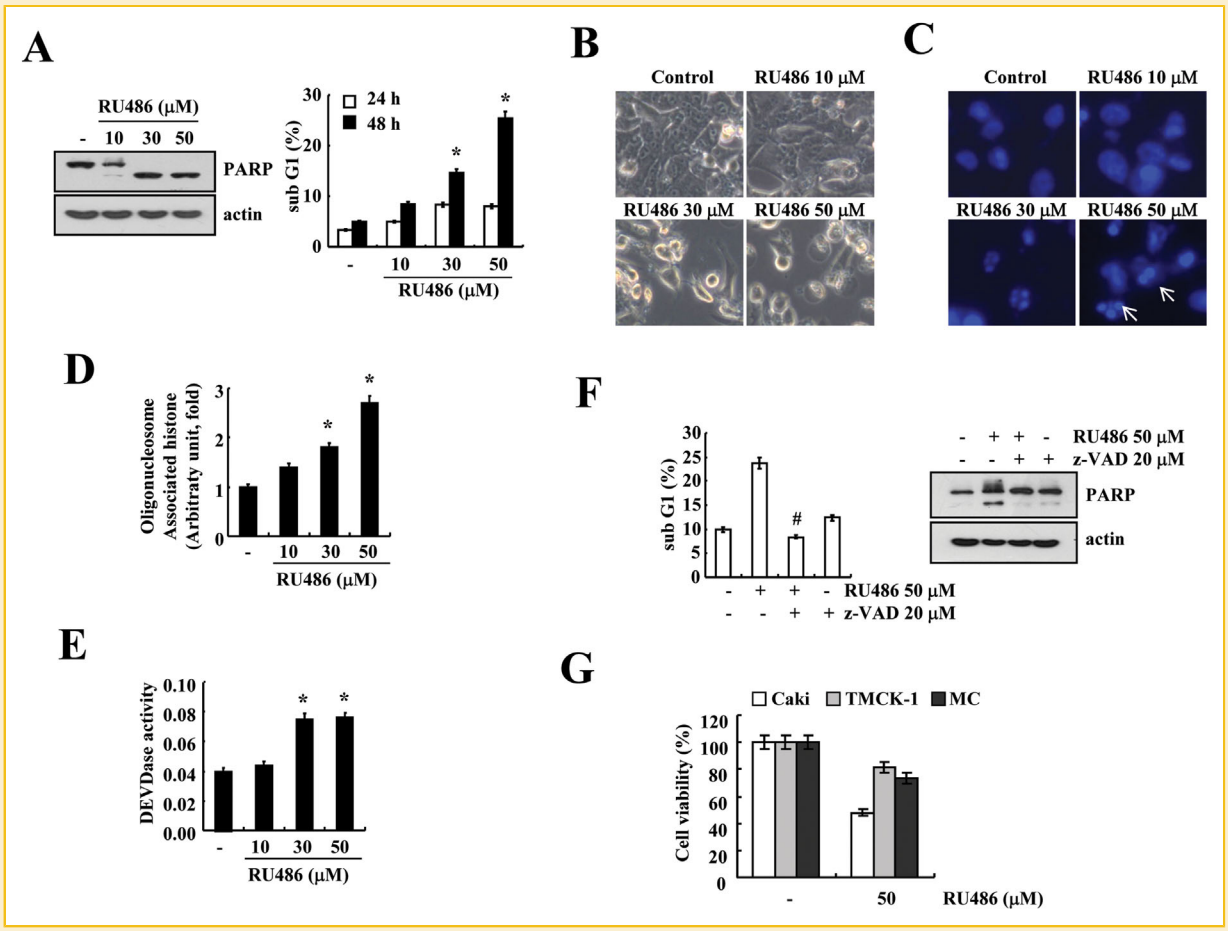

Fig. 5. RU486 induces apoptosis in Caki cells. (A) Caki cells were treated with the indicated concentrations of RU486 for 48 h. The sub-G1 fraction was measured by flow cytometry as an indicator of the level of apoptosis. The protein expression levels of PARP and actin were determined by western blotting. The level of actin was used as a loading control. (B) Cell morphology was detected by interference light microscopy. (C) The condensation and fragmentation of the nuclei were detected by 4′,6′-diamidino-2-phenylindole staining. (D) The cytoplasmic histone- associated DNA fragments were determined by a DNA fragmentation detection kit. (E) Caspase activities were determined with colorimetric assays using caspase-3 (DEVDase) assay kits. (F) Caki cells were treated with 50 μM RU486 for 48 h in the presence or absence of 20 μM z-VAD- fmk (z-VAD). The sub-G1 fraction was measured by flow cytometry. The protein expression levels of PARP and actin were determined by western blotting. The level of actin was used as a loading control. (G) Caki, TMCK-1 and human mesangial cells (MC) were treated with 50 μM RU486 for 48 h. Cell viability was analyzed using XTT assay. The values in (A, D, E, F, and G) represent the mean ± SD from three independent samples. * $P < 0.01$ compared with the control. # $P < 0.01$ compared with the RU486.

1997; Fawcett et al., 1999; Jousse et al., 1999]. CHOP expression is itself regulated by other basic leucine zipper (bZIP) class transcription factors, such as C/EBP proteins and activating transcriptional factor-3 (ATF3) [Fawcett et al., 1999]. In our study, serial deletion assays of the CHOP promoter suggest that the region between −649 and −280 is involved in the transcriptional activation of CHOP by RU486 (Fig. 2A). RU486-mediated CHOP up-regulation is critically regulated by the transcription factor C/EBPδ in cancer cells (Fig. 3A and B). The dependency of the transcriptional activation of the CHOP promoter on the C/EBPδ binding site was confirmed by the following evidence: (a) ectopic expression of C/EBPδ increased RU486-induced CHOP promoter activity; (b) RU486-induced CHOP promoter activation is abrogated by a mutation in the C/EBPδ binding site of the CHOP promoter; and (c) siRNA-mediated C/EBPδ knockdown significantly inhibited RU486-induced up-regulation of CHOP.

CHOP expression can be regulated through MAP kinase signaling pathways. JNK and p38 MAPK were reported to play a role in the transcriptional and post-translational regulation of CHOP expression [Guyton et al., 1996; Wang and Ron, 1996; Brenner et al., 1997]. The induction of CHOP by anisomycin has been demonstrated to require activation of the p38 MAPK signaling pathway [Chen et al., 2010]. The down-regulation of RU486-induced CHOP expression by JNK- and p38 MAPK-specific inhibitors, suggested that RU486 enhances CHOP protein stability via activation of JNK and p38 MAPK. However, the MEK/ERK inhibitor did not prevent or noticeably attenuate the increased CHOP mRNA and CHOP protein expression caused by RU486. Interestingly, ATF4, an ER stress-related transcription factor, was markedly increased by treatment with RU486 (Supplementary Figure 2). To examine whether RU486-induced CHOP expression is dependent on ATF4 activity, we employed the siRNA duplex against AFT4 mRNA. Suppression of ATF4 expression by transfection with its siRNA induced inhibition of RU486-induced CHOP up-regulation in these cells (Supplementary Figure 2). In addition, to identify relationship between reactive

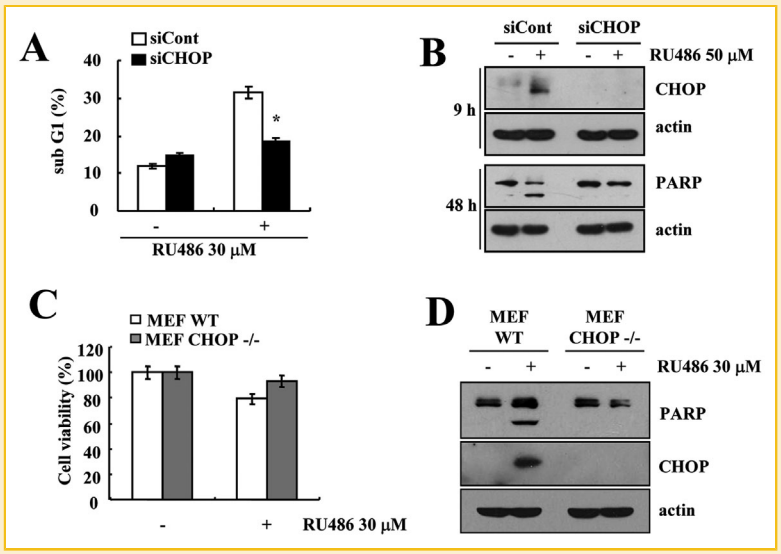

Fig. 6. Down-regulation of CHOP attenuates RU486-induced apoptosis in Caki cells. (A and B) Caki cells were transfected with CHOP siRNA or control siRNA. Twenty-four hours after transfection, cells were treated with 30 μM RU486 for 48 h. The sub-G1 fraction was measured by flow cytometry as an indicator of the level of apoptosis (A). Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to Immobilon-P membranes, and probed with anti-CHOP (9 h) or anti-PARP (48 h) antibodies. An anti-actin antibody was used to verify equal protein loading (B). (C and D) MEF WT and MEF CHOP -/- cells were treated with 30 μM RU486 for 48 h. Cell viability was analyzed using XTT assay (C). Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to Immobilon-P membranes, and probed with anti-CHOP or anti-PARP antibodies. An anti-actin antibody was used to verify equal protein loading (D). A representative study is shown, and two additional experiments yielded similar results. The values in A represent the mean ± SD from three independent experiments. * $P < 0.05$ compared with RU486-treated control siRNA.

oxygen species (ROS) production and CHOP expression in RU486-treated cells, we tested the effects of RU486 on ROS production. RU486 markedly increase intracellular ROS levels, however, RU486-induced CHOP expression was not inhibited by pre-treatment with antioxidants (Supplementary Figure 3).

Although the full significance of induction of CHOP gene expression in RU486-treated cells is not known, the effect of RU486 on CHOP in particular might contribute to the capacity of RU486 to induce apoptosis. There is substantial recognition that CHOP plays a role in triggering apoptosis [Woo et al., 2007; Sanchez-Lopez et al., 2013]. In this study, we showed that CHOP is a critical regulator of RU486-induced apoptosis. CHOP was induced by RU486 in a dose-dependent manner (Fig. 1A), and down-regulation of CHOP by siRNA attenuated RU486-induced apoptosis (Fig. 6A and B). The involvement of CHOP expression in RU486-induced apoptosis in Caki cells indicates that induction of ER stress may play an important role for the cytotoxic activity of RU486.

Several papers reported the anti-cancer effect of RU486 in xenograft models. For example, RU486 (15 mg/kg/d) markedly potentiated paclitaxel induced apoptosis in triple negative breast cancer (MDA-MB-435) cells- injected xenograft models [Skor et al., 2013], and RU486 (40 mg/kg/d) also markedly reduced ovarian cancer cell growth in xenograft models [Goyeneche et al., 2007]. In addition, RU486 (50 mg/kg/d) inhibit invasive and metastatic potential in human gastric adenocarcinoma cells-injected xenograft models [Li et al., 2004]. Although anti-cancer effect of RU486 in vivo has been well-known, the dosage of RU486 might be dependent on cell types and contexts. When RU486 is orally administered (100–800 mg), concentration of RU486 reaches to micromolar in serum for 48 h [Heikinheimo et al., 1987]. Nevertheless, patients with nonresectable meningioma received long-term treatment with RU486 (200 mg/d) (4-month ~13 years), but severe adverse effect did not detect [Spitz et al., 2005]. Furthermore, combined treatment with anti-cancer drug (cisplatin, Adriamycin, and TRAIL) also demonstrated promising results in cancer therapy [Jurado et al., 2009; Huang et al., 2010; Min et al., 2012]. Although RU486 engages certain components of the apoptotic machinery, additional in vivo studies are needed to establish the role of RU486 as a therapeutic agent for cancer.

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