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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 induces apoptosis through activation of the CHOP gene in HeLa cells☆,☆☆

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Abstract

Cyclopentenone prostaglandins (PGs) of the J series, which are produced by dehydration of PGD_2, have been reported to induce apoptosis in various cell lines. One of these cyclopentenone PGs, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ_2), is the most potent inducer of apoptosis in the series, but the signaling pathways by which it induces apoptosis are poorly understood. We recently reported that cyclopentenone PGs accumulate in the endoplasmic reticulum (ER) and it has been shown that the transcription factor CHOP is induced by ER-stresses and elicits apoptosis. In the present study we demonstrated that 15-d-PGJ_2 induces CHOP mRNA/protein in HeLa cells via activation of the conserved regions in the CHOP promoter. Using several mutants of the CHOP promoter fragments, we found that two regions, CCAAT/enhancer-binding protein (C/EBP) site at −313 and ER-stress element (ERSE) at −93, are involved in activation of the CHOP gene by 15-d-PGJ_2. These results suggest that 15-d-PGJ_2 activates the CHOP promoter in two distinct pathways that could induce apoptosis of HeLa cells.

Keywords: 15-d-PGJ_2; CHOP; Apoptosis; ERSE; C/EBP

Cyclopentenone prostaglandins (PGs) of the J series are produced in response to various stress stimuli and are transported actively into cells, and they exert a variety of biological actions [1,2]. The terminal derivative of PGJ_2 metabolism, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ_2), performs several functions, including growth arrest, apoptosis, differentiation, induction of stress-related gene expression, and suppression of macrophage activation and inflammation. However, despite many researchers' efforts little is known about the mechanisms of 15-d-PGJ_2-induced apoptosis.

The C/EBP homologous protein (CHOP), also referred to as growth arrest and DNA damage gene 153 (GADD153), is induced in response to cellular stresses, especially by endoplasmic reticulum (ER) stress [3]. CHOP is involved in the regulation of apoptosis associated with ER-stress [4], although the mechanism is still unclear.

A previous study showed that one of the cyclopentenone PGs, PGA_2, increases the accumulation of CHOP mRNA [5], although the increased accumulation of CHOP mRNA was not demonstrated to have been caused by transcriptional induction of the CHOP gene. We recently reported that cyclopentenone PGs specifically accumulate in the ER [6], and we also showed that cyclopentenone PGs transcriptionally induce the BiP gene, a member of the heat shock protein family and a

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☆☆ *Abbreviations:* PG, prostaglandin; 15-d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; ER, endoplasmic reticulum; C/EBP, CCAAT/enhancer-binding protein; ERSE, ER-stress element; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.

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resident of the ER, via the ER-stress response region [7]. Based on all of this evidence taken together, we suspected that 15-d-PGJ_2 may transcriptionally induce the CHOP gene as well as the BiP gene and that the expression of CHOP protein elicits apoptosis.

In the present study we investigated the mechanism of the induction of apoptosis by 15-d-PGJ_2. We showed that 15-d-PGJ_2 transcriptionally induces the CHOP gene and that the induction is mediated by two distinct elements in its promoter. We also found that one of the elements is composed of ERSE (ER-stress element), which is constitutively regulated by transcription factor NF-Y.

Materials and methods

Materials, cell culture, and cell proliferation study. 15-d-PGJ_2, annexin V-FITC, and propidium iodide were purchased from Cayman Chemical (Ann Arbor, MI), Trevigen (Gaithersburg, MD), and Roche Diagnostic (Indianapolis, IN), respectively. Human cervical cancer cell line, HeLa, a kind gift from Dr. Y. Shiio of the University of Tokyo (Japan), was grown in high-glucose (22 mM) DMEM supplemented with 10% fetal bovine serum. Twenty-four hours after 2×10^5 HeLa cells had been seeded per 35-mm-diameter tissue culture dish, the cells were exposed to 15-d-PGJ_2. The number of viable cells was counted by a trypan blue dye-exclusion test.

Flow cytometric analysis of cell death. To assay for apoptosis cells were stained with 10 μl of annexin V and 5 μl of propidium iodide according to the manufacturer's instructions. Briefly, after the exposure to 15-d-PGJ_2, both floating and adherent cells were collected and washed twice with phosphate-buffered saline (PBS). The cells were then resuspended in 85 μl of binding buffer containing 10 mM Hepes–KOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mg/ml annexin V-FITC. After incubation for 15 min at room temperature in the dark, the cells were analyzed by flow cytometry. Data acquisition and analysis were performed in a flow cytometer (FACScan using CellQuest software: Becton–Dickinson, Mountain View, CA).

Preparation of ^{32}P-labeled CHOP probe. Total RNA from HeLa cells was isolated as described previously [6]. Single-stranded cDNA was synthesized from the total RNA by using the SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA was amplified by the polymerase chain reaction with the following primers: 5′-AGATGAGCGGGTGGCAGCGAC-3′ and 5′-TGGGAAAGGT GGGTAGTGTGGC-3′, and the resulting product was cloned into pGEM-T Easy (Promega, Madison, WI). A CHOP fragment was isolated from 1% agarose gel by digestion with *Eco*RI and it was labeled with [α-^{32}P]dCTP by using a BcaBest labeling kit (Takara; Japan).

Northern blots. The RNA-transferred membranes were hybridized with a ^{32}P-labeled CHOP probe at 65 °C in 6× SSC and washed at 65 °C in 2× SSC.

Western blots. The cells were washed in cold PBS twice and resuspended in 50 μl of lysis buffer [50 mM Tris–HCl (pH 7.9), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin]. The cells were then subjected to mild sonication and used as a whole cell extract. The extract was subjected to 12% SDS–polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with the primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibody. The immune complexes were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Anti-Gadd153 (sc-793), anti-C/EBP (sc-150), and anti-NF-YB (sc-10779) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-α-tubulin was purchased from Oncogene Research Product (San Diego, CA).

Construction of plasmid DNA. We obtained a 3-kilobase (3K) promoter fragment from a human genomic λPS library (MoBiTec; Germany) with a ^{32}P-labeled CHOP probe and inserted it into the upstream of the luciferase reporter gene of pGVB2 vector (Toyo ink; Japan). Several deletion mutants were generated with the deletion kit (Takara; Japan) and point mutations were generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).

DNA transfection and luciferase assay. For expression of CHOP protein, 1.5×10^5 HeLa cells in 35-mm-diameter dishes were transfected with 2 μg/dish of CHOP plasmid DNA in Lipofectamine Plus (Invitrogen, Carlsbad, CA) for 3 h and the medium was changed. At 12, 24, and 36 h after transfection, collected cells or cell lysates were subjected to flow cytometry analysis and Western blotting, respectively. A luciferase assay was carried out as described previously [8].

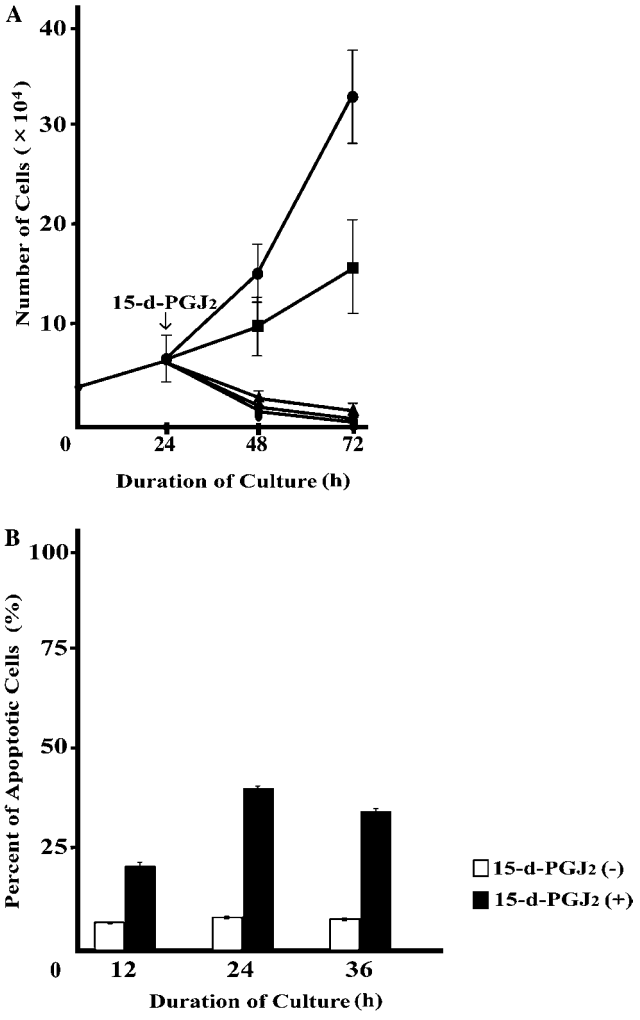

Fig. 1. 15-d-PGJ_2 induces apoptosis in HeLa cells. (A) Cell proliferation. At 24 h after plating, HeLa cells were exposed to the vehicle or the concentration of 15-d-PGJ_2 indicated and the number of cells was counted at the times indicated. (B) Flow cytometry analysis. HeLa cells were exposed to or not exposed to 15-d-PGJ_2 for 12, 24, and 36 h. Apoptotic cells were detected labeled annexin V and measured by flow cytometry.

Electrophoretic gel mobility shift assay. After HeLa cells (2×10^6 cells) had been exposed to 15-d-PGJ_2 and cultured for 24 h, nuclear extracts were prepared and an electrophoretic mobility shift assay (EMSA) was carried out as described previously [6].

Results and discussion

Several studies have demonstrated that 15-d-PGJ_2 induces apoptosis in a variety of cell lines [9–13], however, the pathway and the factor(s) involved in its action remain largely unknown. Recent reports have implicated CHOP in apoptosis induced by ER-stress in various cell lines [4,14,15], while in our previous study we showed that a cyclopentenone PG is specifically accumulated in the ER after being transported into cells [6]. We have also shown that cyclopentenone PGs induce the BiP gene transcriptionally via the ER-stress response region in the promoter [7]. Based on all of these findings taken together, we hypothesized that 15-d-PGJ_2 transcriptionally induces the CHOP gene that elicits apoptosis.

15-d-PGJ_2 induces apoptosis in HeLa cells

We first invested the effect of 15-d-PGJ_2 on HeLa cell proliferation by performing a trypan blue dye-exclusion test. Fig. 1A shows the proliferation of HeLa cells in the presence of various concentrations of 15-d-PGJ_2. Exposure of the cells to 5 μM or higher concentrations of 15-d-PGJ_2 resulted in significant inhibition of cell proliferation and 7.5 μM or higher concentrations caused marked cell death within 24 h after addition. Since the cell death was accompanied by morphological characteristics of apoptosis, such as nuclear condensation, shrinkage, rounding, and detachment of the cells, we measured the DNA content of the nuclei of the cells by flow cytometry. As shown in Fig. 1B, the ratio of apoptotic cells increased from 20% of the total cells at 12 h to 38% at 24 h and 34% at 36 h after exposure to 10 μM of 15-d-PGJ_2.

15-d-PGJ_2 induces CHOP mRNA and protein in HeLa cells

One of the cyclopentenone PGs, PGA_2, had been reported to increase the expression of CHOP mRNA, but 15-d-PGJ_2 had never been reported to induce CHOP expression [5,16]. The results of the Northern blot analysis in Fig. 2A shows the level of CHOP mRNA markedly increased (6- to 8-fold compared with 0 h value) 24 h after exposure to 7.5 μM or more of 15-d-PGJ_2. A time course study showed that CHOP mRNA induction started at 3 h (about 2.7-fold) and that accumulation of mRNA had increased until 36 h (13-fold) (Fig. 2B). These results indicate that 15-d-PGJ_2 increases the expression of CHOP mRNA in HeLa cells. We also investigated whether de novo protein synthesis is required for by 15-d-PGJ_2-induced expression of CHOP gene. Cycloheximide strongly abolished the 15-d-PGJ_2-induced accumulation of CHOP mRNA

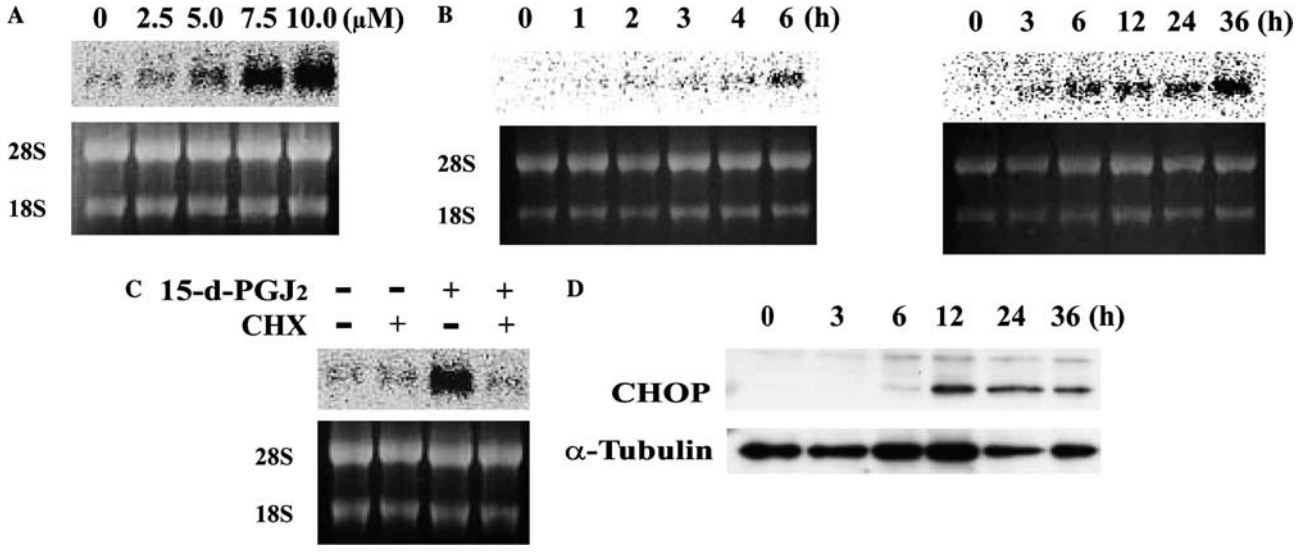

Fig. 2. 15-d-PGJ_2 induces CHOP mRNA and protein in HeLa cells. *Northern blots.* (A) Dose-dependency. At 24 h after HeLa cells had been exposed to the concentrations of 15-d-PGJ_2 indicated, expression of mRNA was examined. (B) Time course. After exposing the cells to 10 μM of 15-d-PGJ_2, total cellular RNA was extracted at the times indicated. (C) After incubating cells with either 1 μg/ml cycloheximide (CHX) (+) or vehicle (−) for 30 min, they were exposed to (+) or not exposed to (−) 15-d-PGJ_2 for 12 h and total cellular RNA was extracted. Expression of CHOP mRNA was analyzed as described under "Materials and methods." The bottom panels in (A–C) show ethidium bromide staining of the formaldehyde gel. *Western blots.* (D) HeLa cells were exposed to 10 μM 15-d-PGJ_2 at the times indicated. The cells were then collected and analyzed for expression of CHOP protein as described under "Materials and methods."

(Fig. 2C, lane 4) and this result is very consistent with a previous report that cyclopentenone PGs induced apoptosis in L1210 cells over 24–72 h and required de novo protein synthesis [9]. This finding suggests that it requires newly synthesized protein(s) (for example, transcription factors or regulatory factors against transcription factors). Western blot analysis showed that CHOP protein expression was observed at 12 h after exposure to 15-d-PGJ_2 (Fig. 2D), and as shown in Fig. 2B, this finding is consistent with the induction of CHOP mRNA.

CHOP induces apoptosis in HeLa cells

A number of previous reports have indicated that CHOP is involved in the process of apoptosis in a variety of cell lines [4,14,15], and we performed flow cytometry and Western blot analysis to determine whether ectopic expression of the CHOP induces apoptosis in HeLa cells. As shown in Figs. 3A and B, the ratio of apoptotic cells dramatically increased more than 12 h after the expression of CHOP protein, indicating that the expression of CHOP protein induces apoptosis in HeLa cells. However, since as shown in Figs. 1B and 2D it takes 12 h for 15-d-PGJ_2 to induce CHOP protein and another 12 h to induce apoptosis, the time lag suggests the existence of several steps downstream of CHOP before apoptosis and they should be elucidated.

15-d-PGJ_2 stimulates CHOP promoter activity

In 1992 Choi et al. [5,16] found that cyclopentenone PGs increased the accumulation of CHOP mRNA, but they failed to prove that the promoter fragment of the CHOP gene is responsive to cyclopentenone PGs. In this study we investigated whether 15-d-PGJ_2 is capable of stimulating the promoter activity of CHOP gene and found that a 3K-promoter fragment inserted upstream of a luciferase-reporter is responsive to 15-d-PGJ_2 in a dose-dependent manner (Fig. 4A). Marked induction of luciferase activity (7.7-fold) was observed after the exposure to 10 μM 15-d-PGJ_2, a finding consistent with our observation of the effect of 15-d-PGJ_2 on CHOP mRNA expression by Northern analysis. These results indicate that the 3K-promoter fragment is necessary and sufficient for the response of the CHOP gene to 15-d-PGJ_2.

Analysis of the 15-d-PGJ_2-responsive elements in the CHOP promoter

To identify the element responsible for the 15-d-PGJ_2-stimulated promoter activity, we generated a series of deletion constructs that included various lengths of the 5′-flanking region of the CHOP gene fused to a luciferase gene. As shown in Fig. 4B, deletion up to position −369 (relative to the transcription start site) retained ~12-fold activation after exposure to

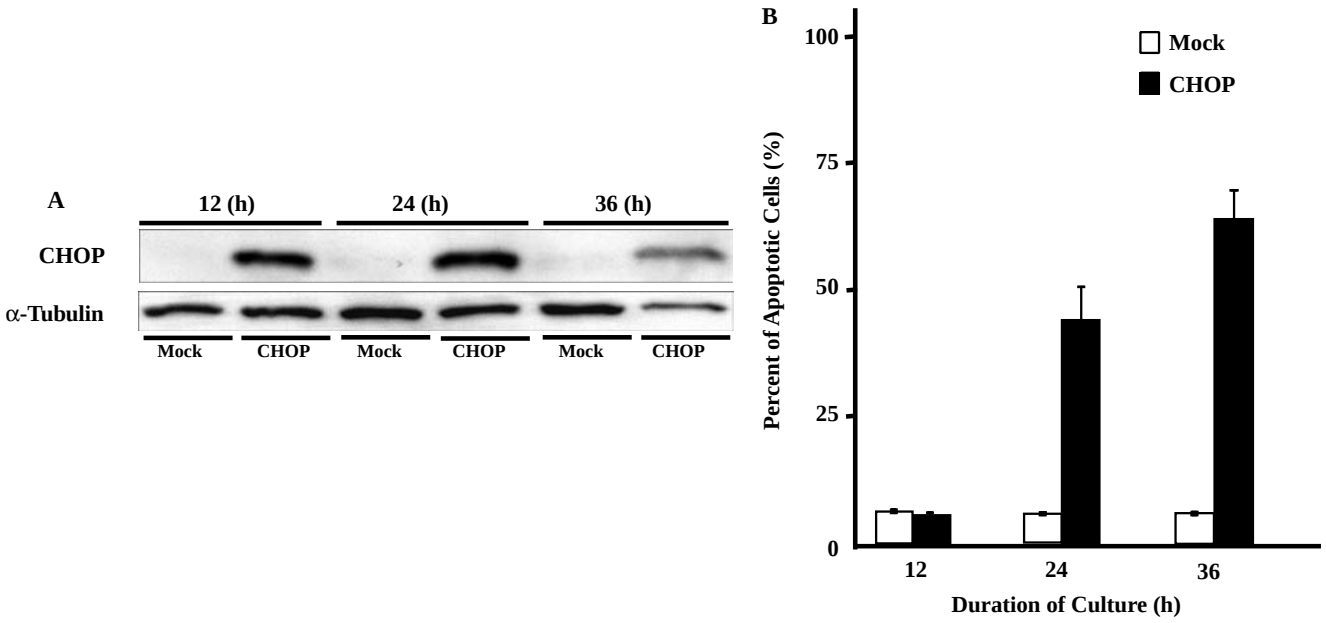

Fig. 3. Expression of CHOP protein induces apoptosis in HeLa cells. (A) HeLa cells were transiently transfected with either the CHOP expression plasmid (*CHOP*) or the control plasmid, pcDNA 3.1 (−) vector (*Mock*). At the times indicated after transfection of each plasmid, all of the cells were lysed and analyzed for expression of CHOP protein as described under "Materials and methods." (B) HeLa cells were transfected with either the CHOP expression plasmid (*CHOP*) or the control vector (*Mock*) used in (A). Detection of apoptotic cells using labeled annexin V was measured by flow cytometry.

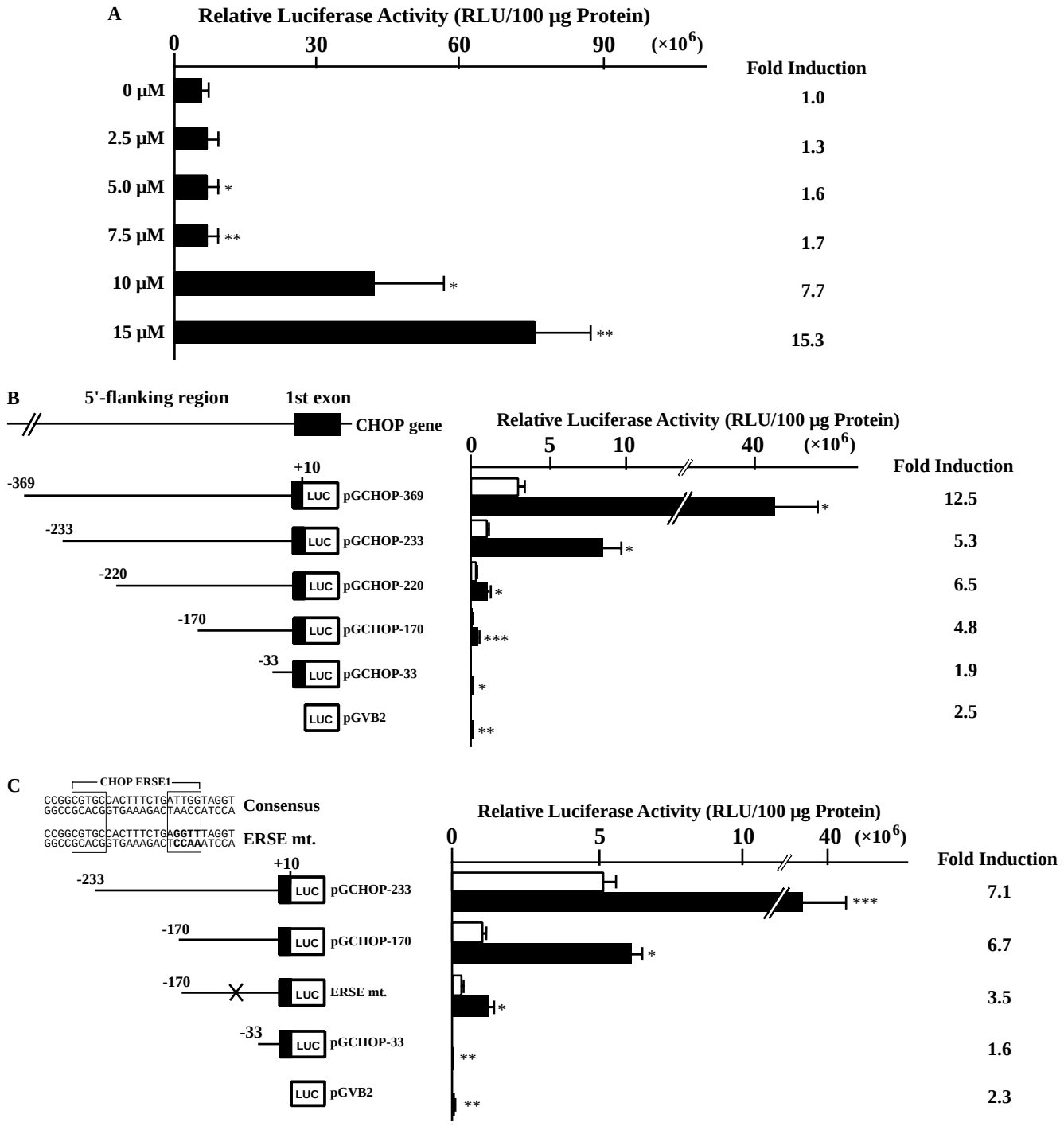

Fig. 4. Activation of the CHOP promoter by 15-d-PGJ_2. *Luciferase assay.* (A) Dose dependency. HeLa cells were exposed to the concentration indicated of 15-d-PGJ_2 for 24 h and luciferase activity was measured. (B) Deletion analysis of the CHOP promoter. After transiently transfecting HeLa cells with various deletion mutants, they were exposed to or not exposed to 10 μM of 15-d-PGJ_2 for 24 h. (C,E) Mutation analysis of the CHOP promoter. Each mutant was identical to the wild-type of the same length except for the mutation in bold letters. After transfection of these mutant plasmids, they were exposed to or not exposed to 10 μM of 15-d-PGJ_2 for 24 h and luciferase activity was measured. (D) A dominant-negative NF-YA expression plasmid suppresses 15-d-PGJ_2-responsiveness to the CHOP promoter. One microgram of pGCHOP-170 was co-transfected into HeLa cells with 1 μg of a dominant negative NF-YA expression plasmid (*pNF-YA29*) or the parental expression vector (*pSG5*), and luciferase activity was analyzed after exposure to or no exposure to 15-d-PGJ_2 for 24 h. Relative luciferase activity in the cell lysates is shown as raw light units (*RLU*) per 100 μg of protein. Fold induction by 15-d-PGJ_2 was also calculated and indicated on the *right.* Data are shown as means; *bars*, SD; $n = 3$ *p<0.05; **p<0.01; and ***p<0.001. *EMSA.* (F) EMSA was carried out with nuclear extracts prepared from 15-d-PGJ_2-exposed ($J(+)$) or vehicle-exposed ($J(-)$) HeLa cells for 24 h. Double-stranded oligonucleotides containing the C/EBP site were used as a probe.

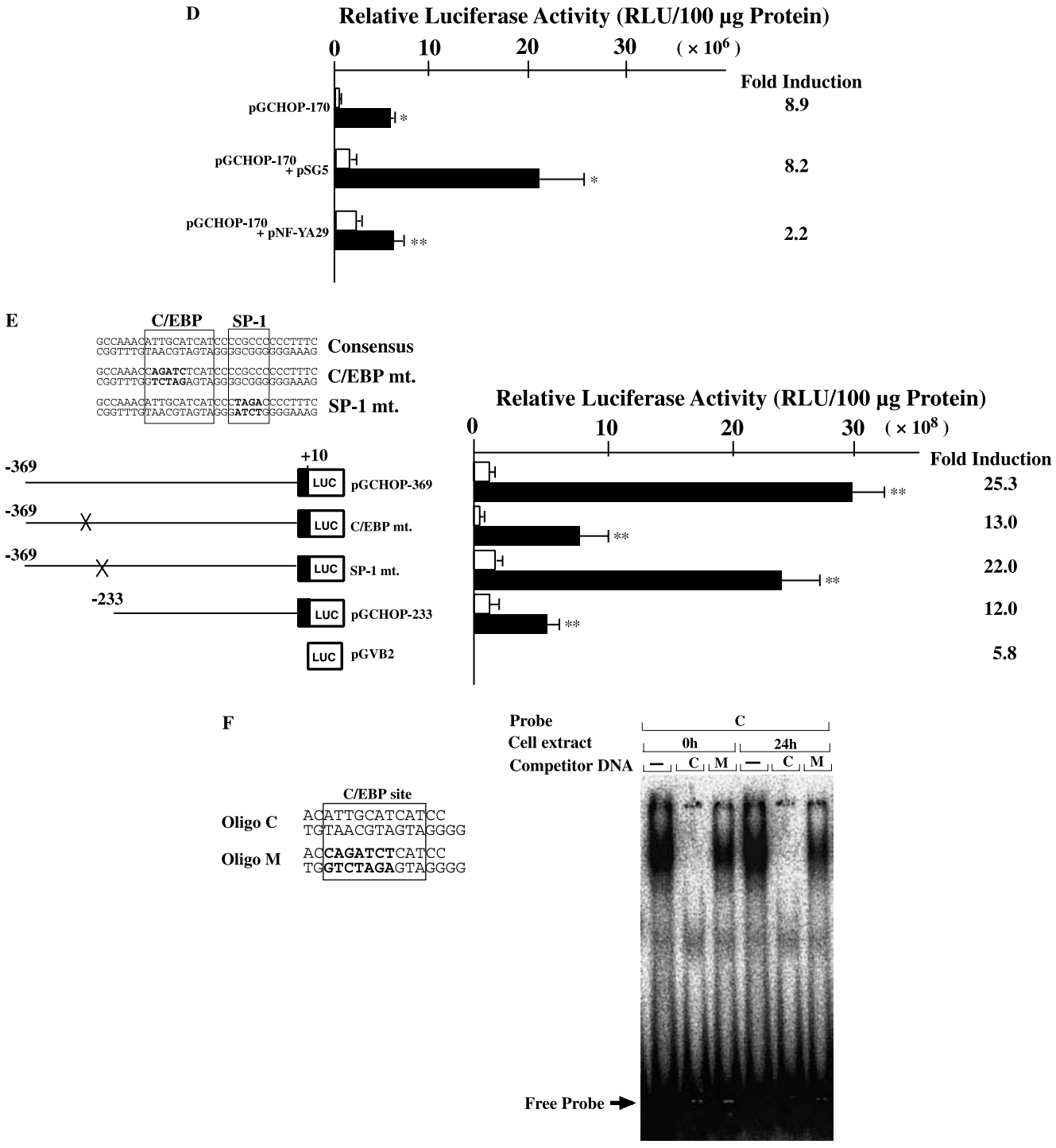

Fig. 4. (*continued*)

15-d-PGJ_2, a level comparable to that of the full-length promoter (3K), whereas deletion up to −233 reduced the activity to less than half that of pGCHOP-369, and deletion up to −33 completely abolished responsiveness to 15-d-PGJ_2. These results suggest that there are at least two regions responsive to 15-d-PGJ_2 in the CHOP promoter.

The downstream of the responsive region (between −170 and −33) harbored two ERSEs, ERSE2 (between −103 and −85), and ERSE1 (between −93 and −75). Previous reports [3,17] showed that the ERSE1 on the CHOP gene is activated by ER-stress triggered by tunicamycin or calcium ionophore, etc., and the general transcription factor NF-Y was found to be required for

constitutive activation of the CHOP gene transcription through ERSE1 [3,17]. We therefore mutated the ERSE1 site and investigated responsiveness to 15-d-PGJ_2, and as shown in Fig. 4C, a point mutant at ERSE1 (ERSE mt.) harbored about half the activity of the intact fragment (pGCHOP-170). To further confirm the involvement of ERSE1 in the regulation of CHOP transcription by 15-d-PGJ_2, we co-transfected plasmid pGCHOP-170 with a dominant negative NF-YA mutant-expression plasmid (pNF-YA29) or the parental expression vector (pSG5). As shown in Fig. 4D, the activity of pGCHOP-170 was markedly reduced by co-transfection with pNF-YA29. Taken together, these results suggest that ERSE1 is involved in the 15-d-PGJ_2-induced transcriptional activation of the CHOP gene.

On the other hand, the upstream responsive region (between −369 and −223) contained two responsible elements, a C/EBP and a SP-1 site. In order to investigate whether these elements are involved in the activation of the CHOP gene by 15-d-PGJ_2, we performed point mutation of one or the other element and examined the effect of 15-d-PGJ_2. The activity of the construct that contained the mutated SP-1 site (SP-1 mt.) was still stimulated by 15-d-PGJ_2, whereas the mutation of the C/EBP site (C/EBP mt.) reduced the 15-d-PGJ_2-induced activity to the same level as that of the deletion construct, pGCHOP-233 (Fig. 4E). These results suggest that the C/EBP site is significant and that it is involved in the response to 15-d-PGJ_2. To detect protein binding to the C/EBP consensus sequence, we performed electrophoretic mobility shift assay (EMSA) analyses using oligonucleotides spanning −312 to −299 of the CHOP promoter (Oligo C) that contained the C/EBP consensus sequence. As shown in Fig. 4F, probe Oligo C yielded a single major retarded band that was competed away by an excess of unlabeled Oligo C but not mutated Oligo M, and 15-d-PGJ_2 did not change either the intensity or the mobility pattern of the complex (Fig. 4F, lanes 1 and 4). In order to identify protein(s) that directly binds to the sequence, we performed supershift experiments using various antibodies against proteins that may bind to the C/EBP consensus sequence. However, none of the antibodies we examined, against CHOP, ATF2, ATF4, C/EBP-β, or C/EBPα, yielded any supershifted bands (data not shown), suggesting that other known or unknown protein(s) may bind to the C/EBP site on the CHOP promoter and regulates the induction, but it remains to be defined.

Cyclopentenone PGs such as 15-d-PGJ_2 are produced in response to various stress stimuli. Presumably when the stress on the cells is very severe, high concentrations of PG are generated locally and induce apoptosis of the cells so as to avoid severe damage for individuals.

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