

Anti-apoptotic hPEBP4 silencing promotes TRAIL-induced apoptosis of human ovarian cancer cells by activating ERK and JNK pathways

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in a variety of tumor cell lines but not typically in normal or nontransformed cells, which makes TRAIL a desirable therapeutic agent to fight cancer. Human phosphatidylethanolamine-binding protein 4 (hPEBP4) is a recently identified anti-apoptotic molecule and has been shown to be highly expressed in breast and ovarian cancer cells. We demonstrate that silencing of hPEBP4 in CaoV-3 ovarian cancer cells potentiates TRAIL-induced apoptosis. We found that endogenous hPEBP4 interacts with Raf-1 and MEK1 in TRAIL-treated CaoV-3 cells by co-immunoprecipitation analysis. Simultaneously, silencing of hPEBP4 in CaoV-3 cells enhances TRAIL-induced ERK and JNK activation. Moreover, the inhibitors of MEK1 or JNK can reduce hPEBP4-silence-induced TRAIL sensitivity. Therefore, silencing of hPEBP4 in CaoV-3 ovarian cancer promotes TRAIL-induced apoptosis, and the increased MAPK activation is required for the apoptosis sensitization. All these data indicate that silencing of hPEBP4, an important potential

target, may be a promising approach for the treatment of ovarian cancer.

Introduction

Ovarian cancer is the most common gynecological malignancy worldwide; yet, the 5-year survival rate for this disease has remained low at 30% for the last 20 years with relatively little recent improvement (1). Despite initial tumor response rates of 80% to front-line platinum-based chemotherapy (2), the majority of patients with advanced ovarian cancer will ultimately have cancer relapse and develop drug resistance (3,4). Drug resistance is a major problem in the clinical treatment of ovarian cancer, thus requiring new strategies to make ovarian cancer more sensitive to therapeutic agents (4).

Based on the facts that apoptosis and anti-apoptosis pathways are deeply related to drug sensitivity and resistance, treatment protocols aimed at apoptosis induction have been outlined. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is well known to trigger apoptosis in many malignant cells. Whereas cancer cells are responsive to TRAIL-induced cell death when used alone or in combination with other agents, normal cells are known to be relatively less sensitive to TRAIL, making it a desirable therapeutic agent to target a variety of cancers (5,6). The potential and safety of soluble TRAIL as an anticancer therapeutic agent has been demonstrated in mice and non-human primates (6,7). It has been reported that the combination of chemotherapy and TRAIL could induce apoptosis in some chemoresistant and TRAIL-resistant ovarian cancer cells (8).

hPEBP4 is a novel member of the phosphatidylethanolamine-binding protein (PEBP) family identified as an anti-apoptotic molecule by us (9), and selectively expressed in breast cancer tissue. Silencing of hPEBP4 potentiates TNF α -induced apoptosis and cell arrest in MCF-7 cells (10). Considering the preliminary data showing high expression of hPEBP4 in CaoV-3 ovarian cancer cells, in the present study, we investigated whether down-regulation of hPEBP4 expression in the CaoV-3 cells can sensitize the cells to TRAIL-induced apoptosis. Our results demonstrate that

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Abbreviations: ERK, extracellular signal-regulated kinase; MEK, extracellular signal-regulated kinase kinase; PI, propidium iodide; PEBP, phosphatidylethanolamine binding protein; PE, phosphatidylethanolamine; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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Key words: ovarian carcinoma, phosphatidylethanolamine binding protein, apoptosis, TRAIL, MAPK

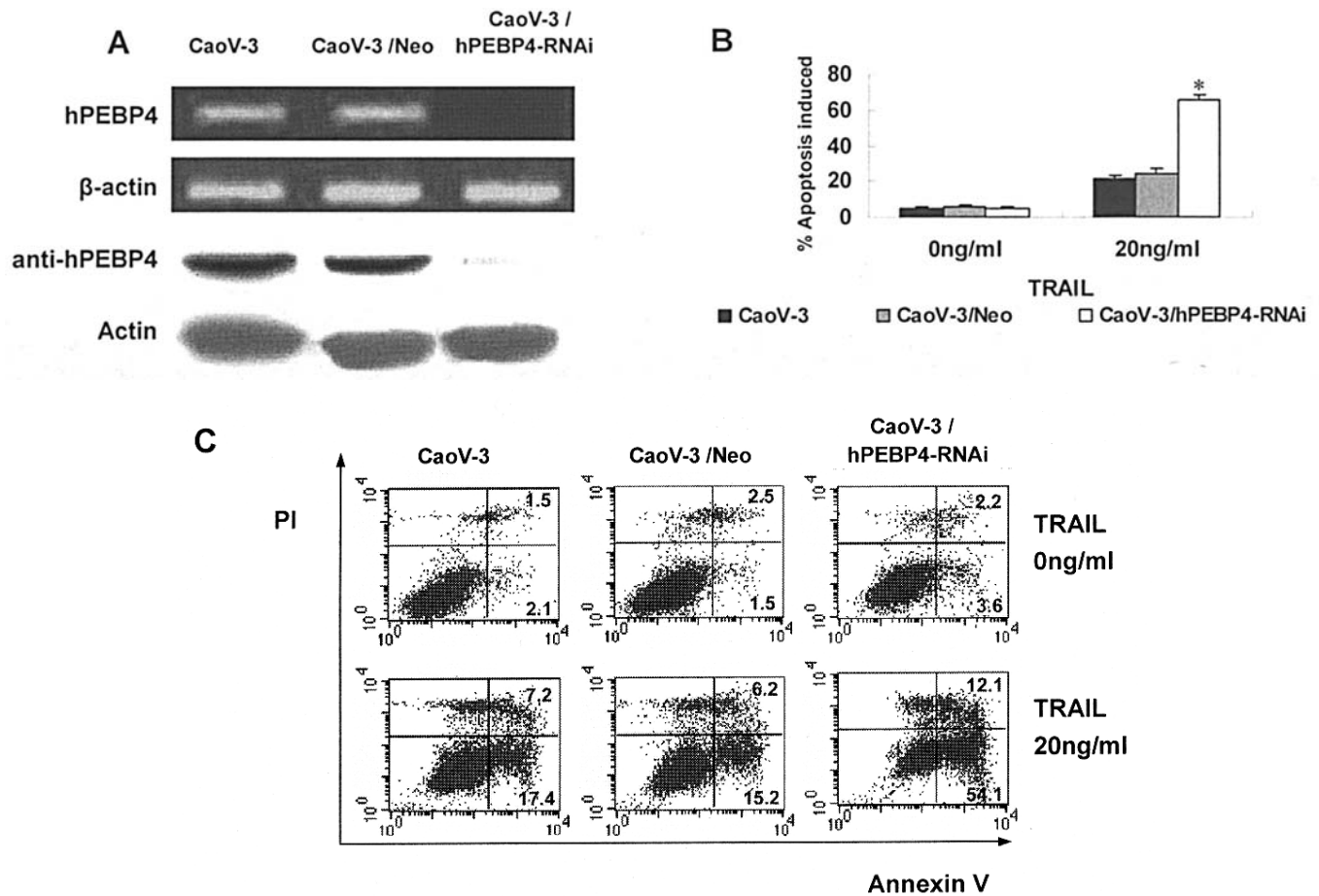


Figure 1. Silencing of hPEBP4 expression sensitizes CaoV-3 ovarian cancer cells to TRAIL-induced apoptosis. (A) Functional silencing of endogenous hPEBP4 expression by RNA interference in CaoV-3 cells. hPEBP4 was highly expressed in CaoV-3 cells by RT-PCR and Western blot analysis. CaoV-3 cells were stably transfected with hPEBP4-specific RNAi (hPEBP4-RNAi) plasmid or control RNAi plasmid (Neo), and silencing of hPEBP4 expression was confirmed by RT-PCR (upper panels) and Western blot analysis with anti-hPEBP4 antibody (lower panels). (B) The stable transfectants of CaoV-3 cells were seeded at a cell density of 2×10^6 cells/well in 6-well plates. After 20 ng/ml TRAIL simulation for 6 h, the cells were stained with Annexin V and PI, then subjected to FACS assay. * $P < 0.01$ compared with CaoV-3/Neo/TRAIL. Data are means \pm SE of at least three independent experiments. (C) The representative data of three independent experiments. The percentages of apoptotic cells in each sample are indicated.

silencing of hPEBP4 significantly promotes TRAIL-induced apoptosis in CaoV-3 cells by activating ERK and JNK pathways. Therefore, down-regulation of anti-apoptotic hPEBP4 might represent a promising approach for the treatment of ovarian cancers.

Materials and methods

Reagents and cell culture. MEK1 inhibitor, PD98059, and JNK inhibitor, SP600125, were obtained from New England Biolabs and Calbiochem, respectively. TRAIL was purchased from PeproTech EC Ltd. Ovarian carcinoma CaoV-3 cells were grown in RPMI-1640 or DMEM supplemented with 10% (v/v) fetal calf serum, 4.5 g/l D-glucose, nonessential amino acids (100 μ M each), 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO₂ atmosphere.

Generation of siRNA plasmid vector. IMG-800 (pSuppressor-Neo, Imgenex, CA) vector was used for construction of 21-bp head-to-head hairpins of hPEBP4. The single-stranded oligonucleotides specific to hPEBP4, 5'-TCGAGGGAAA AGTCATCTCTCTCCTTgagtactcAAGGAGAGAGA

TGACTTTTCCCTTTTT-3' (sense) and 5'-CTAGAAAA GGGAAAAGTCATCTCTCTCCTT cagtactcAAGGAG AGAGATGACTTTTCCC-3' (antisense), were synthesized and annealed to generate double-stranded DNAs, which were cloned into the *Sal*I and *Xba*I cloning sites of pSuppressor/Neo (Imgenex). The plasmid construct (hPEBP4-RNAi) was then confirmed by sequencing. The control plasmid, Neo, contains a scrambled sequence that does not show significant homology to rat, mouse or human gene sequences (Imgenex).

Cell transfection and stable selection. The day before transfection, ovarian carcinoma CaoV-3 cells were plated in a 24-well plate. The cells were 60–80% confluent at the time of transfection. hPEBP4-RNAi or Neo plasmid was transfected into CaoV-3 cells using LipofectAMINE reagent (Invitrogen). Forty-eight hours after transfection, the cells were screened under 0.8 mg/ml G418 (Merck) for 25 days. Clones of the stably transfected cells with Neo or hPEBP4-RNAi plasmid were obtained by limited dilution, designated as CaoV-3/Neo and CaoV-3/hPEBP4-RNAi, respectively.

Apoptosis assay. Cells were washed, resuspended in the staining buffer, and examined by ApoAlert Annexin V

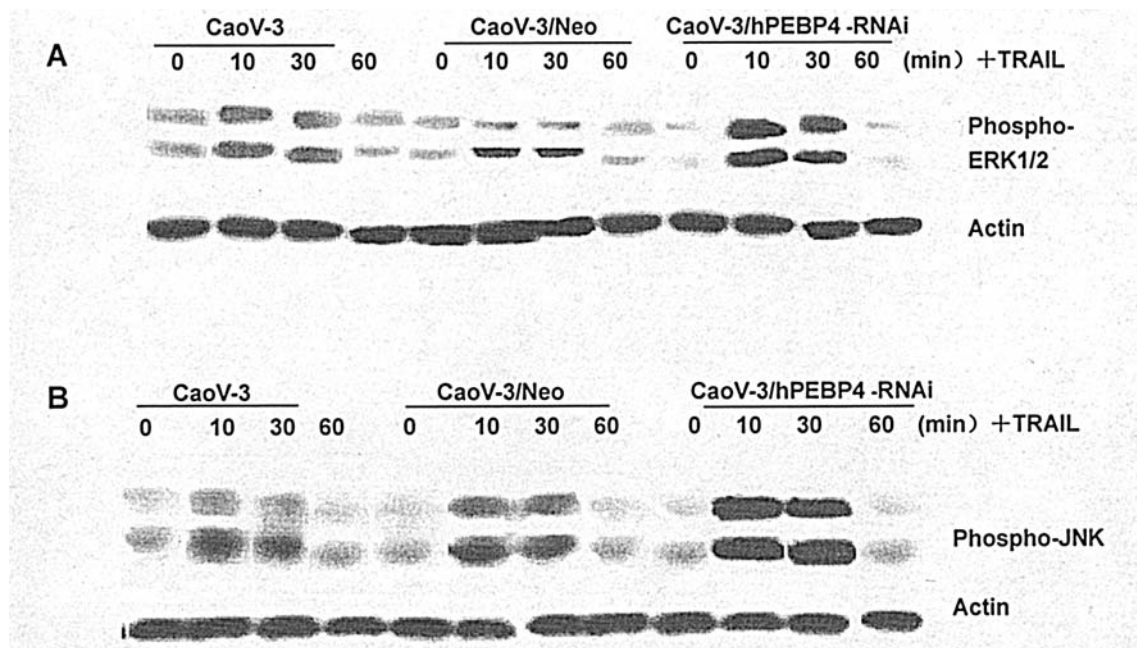


Figure 2. Silencing of hPEBP4 expression promotes TRAIL-induced MAPK activation in CaoV-3 ovarian cancer cells. (A) The effect of hPEBP4 silencing on TRAIL-induced activation of ERK1/2, MEK1 in CaoV-3 cells. The stable transfectants of CaoV-3 cells were serum-starved for 24 h and then treated with 10 ng/ml TRAIL for the indicated time. Cell lysates were subjected to immunoblotting analysis using anti-phospho-ERK1/2. (B) hPEBP4 RNA interference enhanced the activation of JNK induced by TRAIL.

apoptosis kit (Becton-Dickinson) or R123 (R-302, Molecular Probes) and PI according to the manufacturer's instructions, as described previously (10). Stained cells were analyzed by FACS (FACScalibur, Becton-Dickinson).

Co-immunoprecipitation assay. CaoV-3 lysates were pre-cleared by protein A-sepharose beads (Sigma) for 1 h at 4°C. Supernatants were collected and incubated with anti-hPEBP4 polyclonal antibody and protein A-Sepharose beads overnight at 4°C. Immune complex beads were washed thoroughly with lysis buffer. Sample buffer (50 μ l) was added to the pellets and the proteins were released from the beads by boiling for 5 min. Samples were subjected to Western blot analysis.

Western blot analysis. A BCA protein assay reagent kit (Pierce) was used to measure protein concentration. Samples containing equal amounts of protein were separated by 12% SDS-PAGE and transferred to Protran nitrocellulose membranes (Schleicher & Schuell). Blots were probed with antibodies specific for phospho-ERK1/2, phospho-MEK-1, phospho-Raf-1, phospho-JNK1/2, Raf-1, and MEK-1 (Santa Cruz), with appropriate horseradish peroxidase-conjugated antibodies as secondary antibodies (Cell Signaling). Supersignal West Femto maximum sensitivity substrate (Pierce) was used for the chemiluminescent visualization of proteins.

Statistical analysis. Statistical analysis (Fisher's exact test) was performed using the computer program SPSS Version 6.1.

Results

hPEBP4 is highly expressed in CaoV-3 ovarian cancer cells. hPEBP4 was found to promote cellular resistance to TNF α -

induced apoptosis, and down-regulation of hPEBP4 expression sensitizes MCF-7 breast cancer cells to TNF α -induced apoptosis (9). We also observed that hPEBP4 is highly expressed in CaoV-3 ovarian cancer cells detected by RT-PCR and Western blot analysis (Fig. 1A). Because down-regulation of hPEBP4 expression in MCF-7 cells could enhance apoptosis sensitization and hPEBP4 is also highly expressed in CaoV-3 cells, we investigated whether or not silencing of hPEBP4 in CaoV-3 cells will promote the cellular apoptosis of CaoV-3 cells induced by TRAIL, which has been shown to be a potential agent to treat ovarian cancer.

Silencing of hPEBP4 expression sensitizes CaoV-3 ovarian cancer cells to TRAIL-induced apoptosis. It was reported that the combination of TRAIL and chemotherapeutic agents resulted in a significant increase in apoptosis in ovarian cancer cells (8). To evaluate the effect of hPEBP4 silencing on the sensitivity of CaoV-3 ovarian cancer cells to TRAIL-induced apoptosis, CaoV-3 cells were stably transfected with hPEBP4-RNAi or Neo plasmids. Western blotting and RT-PCR confirmed the silencing of hPEBP4 expression in CaoV-3 stable transfectants (Fig. 1A). The transfectants were treated with 20 ng/ml TRAIL for 6 h and stained with Annexin V/PI. Annexin V-positive (including PI⁻ or PI⁺) present apoptotic cells. As shown in Fig. 1B and C, 25% of CaoV-3 cells and 21% of CaoV-3/Neo transfectants exhibited apoptosis respectively, when exposed to TRAIL. However, 66% of CaoV-3/hPEBP4-RNAi transfectants exhibited apoptosis. In addition, silencing of hPEBP4 in CaoV-3 cells did not affect the cell viability when compared to CaoV-3/Neo or the parental cells. This result suggested that silencing of hPEBP4 expression in CaoV-3 cells might contribute to an increased sensitivity to TRAIL-induced apoptosis.

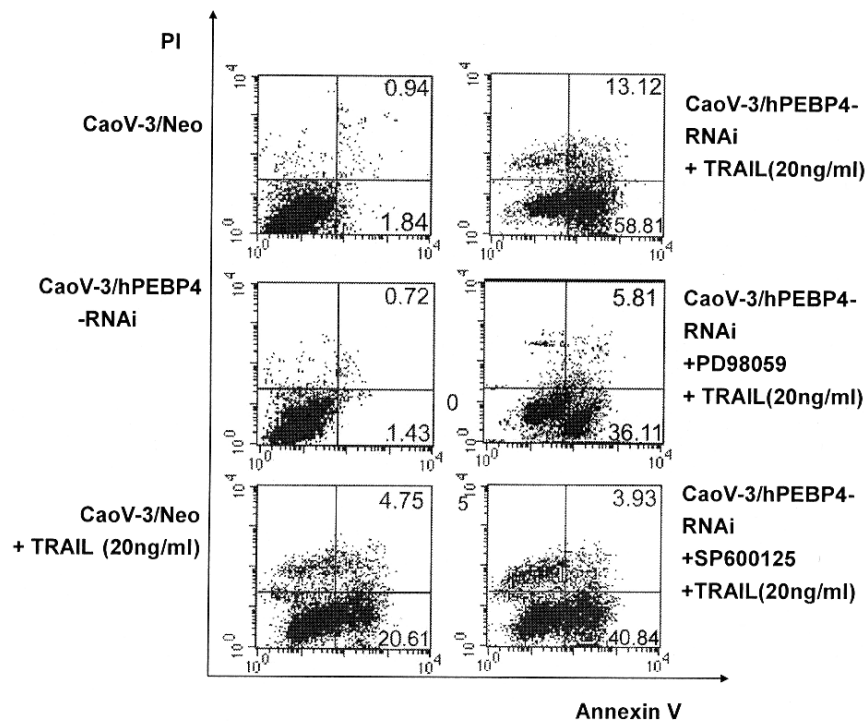


Figure 3. Enhanced ERK1/2 and JNK activation by hPEBP4 silencing in CaoV-3 cells potentiates TRAIL-induced apoptosis of CaoV-3 cells. PD98059 (MEK1 inhibitor) and SP600125 (JNK inhibitor) significantly decreased TRAIL-induced apoptosis in hPEBP4-silenced CaoV-3 cells. The stable transfectants of CaoV-3 cells were pre-incubated with 10 μ M PD98059 for 30 min or 20 μ M SP600125 for 1 h at 37°C and subsequently stimulated with 20 ng/ml TRAIL for 6 h. The cells were stained with Annexin V and PI, then subjected to FACS assay. The percentages of apoptotic cells in each sample are indicated.

Silencing of hPEBP4 expression in CaoV-3 cells potentiates TRAIL-induced MAPK activation. Next, we aimed to elucidate the mechanisms underlying the sensitization of CaoV-3 cells to TRAIL-induced apoptosis by hPEBP4 silencing. Our previous study showed that TNF α induces hPEBP4 transfer from lysosomes to the cell membrane; where hPEBP4 binds to Raf-1 and MEK1, thus inhibiting MAPK activation (9). Because TRAIL-induced apoptosis involved MAPK activation (11-15), it was necessary to observe the effect of hPEBP4 silencing on TRAIL-induced MAPK activation. The stable transfectants of CaoV-3 cells were serum-starved for 24 h and treated with 10 ng/ml TRAIL for different time periods. As determined by Western blot assays, TRAIL stimulated ERK1/2 and JNK1 activity, which occurred 10 min after treatment and returned to baseline at 1 h (Fig. 2). Interestingly, silencing of hPEBP4 expression in CaoV-3 cells significantly enhanced TRAIL-induced activation of ERK1/2 and JNK1 (Fig. 2).

Inhibition of MAPK pathway reduces the increased TRAIL-induced apoptosis in CaoV-3 cells by hPEBP4 silencing. TRAIL induces apoptosis in malignant cells by interacting with the DRs, including DR4 and DR5 (16). These receptors mediate the apoptotic signal through interaction of their intracellular death domains with adaptor proteins. It has been demonstrated that while TRAIL induces apoptosis, it also activates JNK and p44/42ERK1/2 (11-15). The roles of MAPK activation in apoptosis are highly controversial, with reports suggesting pro-apoptotic, anti-apoptotic and neutral roles (17-19). Since silencing of hPEBP4 expression in CaoV-3 cells significantly enhanced TRAIL-induced apoptosis and MAPK activation, we wondered whether the increased TRAIL-induced

apoptosis might, in part, be attributable to an increased MAPK activity by hPEBP4 silencing. Therefore, we pre-incubated hPEBP4-silenced CaoV-3 cells with MEK1 inhibitor (PD98059) for 30 min or 20 μ M JNK inhibitor (SP600125) (20) for 1 h at 37°C, then treated the cells with TRAIL. As shown in Fig. 3, pre-treatment with PD98059 or SP600125 significantly decreased the apoptosis of hPEBP4-silenced CaoV-3 cells induced by TRAIL. The apoptotic percentage decreased from 71.8% in CaoV-3/hPEBP4-RNAi cells to 41.9% after PD98059 treatment, or to 44.7% after SP600125 treatment, respectively. The data indicated that the sensitization of hPEBP4-silenced MCF-7 cells to TRAIL-induced apoptosis is due to the potentiated activation of the Raf-1/MEK/ERK pathway and JNK following TRAIL treatment.

Endogenous hPEBP4 protein associates with Raf-1 or MEK1 in CaoV-3 cells upon TRAIL treatment. Our previous study demonstrated that hPEBP4 binds to Raf-1 and MEK1 following TNF α treatment, thus inhibiting downstream MEK1/ERK activation (9). Since the above experiments proved that hPEBP4 was involved in TRAIL-induced ERK1/2 activation, we examined the interaction of endogenous hPEBP4 with Raf-1 and MEK1 in CaoV-3 cells upon TRAIL treatment. Anti-hPEBP4 polyclonal antibody was used to immunoprecipitate endogenous hPEBP4 from CaoV-3 cells stimulated with or without TRAIL. As shown in Fig. 4, the presence of Raf-1 and MEK1 was detected in the anti-hPEBP4 immunoprecipitates of CaoV-3 cells treated with TRAIL, but not in those of unstimulated cells. The results suggested that hPEBP4 binds to Raf-1 or MEK-1 upon TRAIL treatment, which might dissociate the Raf-1-MEK complex, and thus functions as a

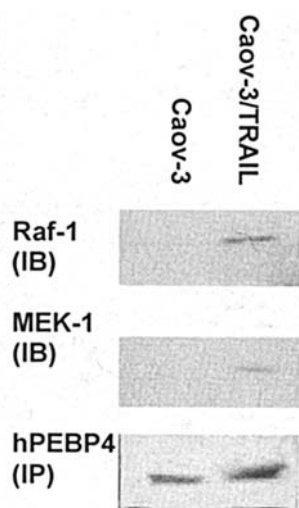


Figure 4. Endogenous hPEBP4 protein associates with Raf-1 or MEK1 in CaoV-3 cells following TRAIL treatment. Lysates of CaoV-3 cells treated with or without 10 ng/ml TRAIL for 10 min were pre-cleared by protein A-sepharose beads (Sigma) for 1 h at 4°C. Supernatants were collected and incubated with anti-hPEBP4 polyclonal antibody cross-linked to protein-A Sepharose beads overnight at 4°C. Immune complex beads were washed thoroughly with lysis buffer. Sample buffer (50 μ l) was added to the pellets and the proteins were released from the beads by boiling for 5 min. Samples were immunoblotted using Raf-1 or MEK1 or anti-hPEBP4 antibody.

competitive inhibitor of MEK phosphorylation. Therefore, silencing of hPEBP4 expression potentiates TRAIL-induced activation of the Ras/Raf-1/MEK/ERK signaling pathway, which contributes to the increased sensitivity of CaoV-3 cells to TRAIL-induced apoptosis.

Discussion

The current therapeutic approach is not very effective in treating ovarian cancer patients (21,22-24). Therefore, more efficient treatment protocols aimed at different targets need to be explored. Inducers of apoptosis have been applied in cancer treatment; however, chemotherapy or radiation is not invariably cytotoxic to all cancer cells. It has been observed that defects in the apoptotic pathway in cancer cells confer insensitivity to the cytotoxic effects of chemotherapy and may therefore represent an important mechanism for cancer cell drug resistance (25,26). Several studies have attempted to induce cancer cell apoptosis by targeting or silencing the anti-apoptotic proteins, and have shown promising results (27,28). Enhancing the chemosensitivity of cancer cells by the transfer or interference of genes that influence the death and growth of the cell is one of the most important strategies in cancer therapeutics.

The TRAIL or Apo2 ligand is a member of the TNF superfamily of cell death-inducing ligands (29). The TRAIL-mediated apoptosis signal pathway consists of activated TRAIL-R1/R2, recruited Fas-associated death domain protein (FADD). TRAIL-R1 or -R2 can also activate MAPK signaling pathways and the activation is required for the sensitization of PC3 cells to TRAIL-induced apoptosis (30,31). Our previous study demonstrated that hPEBP4 could inhibit TNF α -induced apoptosis (9). In this study we found that silencing of hPEBP4

expression in CaoV-3 cells promoted TRAIL-induced apoptosis. Simultaneously, silencing of hPEBP4 in CaoV-3 cells enhanced TRAIL-induced ERK and JNK activation. In addition, the increased TRAIL sensitivity induced by hPEBP4 silencing was partially reduced by PD98059 or SP600125, the inhibitors of ERK and JNK activation, respectively. Thus, our results suggest that ERK and JNK might act under certain circumstances in a pro-apoptotic fashion. Similar data have been reported for cisplatin-induced apoptosis and TRAIL-induced apoptosis (17-19). In this study, endogenous hPEBP4 association with both MEK1 and Raf-1 after TRAIL stimulation was observed using co-immunoprecipitation assays, confirming our assumption that hPEBP4 binds to Raf-1 or MEK1 upon TRAIL stimulation, thus dissociating the Raf-1-MEK complex, and inhibiting MEK/ERK phosphorylation. The results may explain why TRAIL-induced activation of the Ras/Raf-1/MEK1/ERK signaling pathway was further enhanced in hPEBP4-silenced cells.

Besides abundant expression in ovarian carcinoma cells, we also detected the high expression of hPEBP4 in breast and prostate cancer cells. In addition to the CaoV-3 cell model, silencing of hPEBP4 expression rendered other cancer cells, which highly express hPEBP4 such as MCF-7 cells, more sensitive to TNF α -induced cell death (9,10). hPEBP4 could be particularly important if the point at which it exerts an effect is common to apoptosis induced by other anti-cancer agents. By decreasing hPEBP4 expression in breast and ovarian cancer cells, the threshold at which chemotherapeutic agents trigger cancerous cells to undergo apoptosis may be lowered, leading to a more favorable response to chemotherapeutic agents. Therefore, its high expression in some cancers and its ability to function as an anti-apoptotic molecule outline that hPEBP4 is a promising target to be silenced for the treatment of cancers. Down-regulating hPEBP4 may prove to be an effective treatment for cancers that highly express hPEBP4, such as ovarian, prostate and breast carcinoma.

Acknowledgements

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