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Overexpression of Par-4 Sensitizes TRAIL-Induced Apoptosis Via Inactivation of NF-κB and Akt Signaling Pathways in Renal Cancer Cells

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ABSTRACT

The prostate-apoptosis-response-gene-4 (Par-4) is up-regulated in prostate cells undergoing programmed cell death. Furthermore, Par-4 protein has been shown to function as an effector of cell death in response to various apoptotic stimuli that trigger mitochondria and membrane receptor-mediated cell death pathways. In this study, we investigated how Par-4 modulates TRAIL-mediated apoptosis in TRAIL-resistant Caki cells. Par-4 overexpressing cells were strikingly sensitive to apoptosis induced by TRAIL compared with control cells. Par-4 overexpressing Caki cells treated with TRAIL showed an increased activation of the initiator caspase-8 and the effector caspase-3, together with an enforced cleavage of XIAP and c-FLIP. TRAIL-induced reduction of XIAP and c-FLIP protein levels in Par-4 overexpressing cells was prevented by z-VAD pretreatment. In addition, the surface DR5 protein level was increased in TRAIL-treated Par-4 overexpressing cells. Interestingly, even though a deletion of leucine zipper domain in Par-4 recovered Bcl-2 level to basal level induced by wild type Par-4, it partly decreased sensitivity to TRAIL in Caki cells. In addition, exposure of Caki/Par-4 cells to TRAIL led to reduction of phosphorylated Akt levels, but deletion of leucine zipper domain of Par-4 did not affect these phosphorylated Akt levels. In conclusion, we here provide evidence that ectopic expression of Par-4 sensitizes Caki cells to TRAIL via modulation of multiple targets, including DR5, Bcl-2, Akt, and NF-κB. J. Cell. Biochem. 109: 885–895, 2010.

KEY WORDS: PAR-4; TRAIL; DR5; APOPTOSIS; NF-KB; AKT

Par-4 was identified in a search for genes that are up-regulated after induction of programmed cell death in prostate cancer cells [Sells et al., 1994]. Although Par-4 is ubiquitously expressed in normal tissues and found primarily in the cytoplasm, Par-4 localizes to the cytoplasm as well as the nucleus in various cancer cells [El-Guendy and Rangnekar, 2003; El-Guendy et al., 2003]. Although endogenous Par-4 expressed in normal and cancer cells does not, by itself, cause apoptosis, blockade of endogenous expression results in the attenuation of apoptosis induced by exogenously applied apoptotic insults such as chemotherapeutic agents, serum deprivation, or ionizing radiation [Sells et al., 1997; Diaz-Meco et al., 1999; Gurumurthy et al., 2001; El-Guendy and Rangnekar, 2003]. These results suggest that Par-4 may play a role in the reduction of the apoptotic threshold. Par-4 is down-regulated in about 75% of renal cell carcinoma specimens analyzed, and reintroducing of Par-4 renders the cells susceptible to the action of TNF-α [Cook et al., 1999]. It also have been reported that Par-4 triggers apoptosis through an activation of the Fas death receptor signaling pathway by promoting the Fas/FasL translocation to the cell membrane and by protecting the Fas apoptotic pathway from the inhibitory effects of PKCζ [Chakraborty et al., 2001; de Thonel et al., 2001]. It has been shown that the Par-4 protein binds, via its leucine zipper domain, to

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the zinc finger domain of Wilms' tumor protein WT1, which resulted in inhibition of Bcl-2 expression [Johnstone et al., 1996; Sells et al., 1997; Fraizer et al., 2004]. This indicates that leucine zipper domain is required for the Par-4 protein to function in apoptosis. Conversely, it has been reported that the direct apoptosis by Par-4, entry into the nucleus is essential but the leucine zipper domain is not required [El-Guendy et al., 2003].

TRAIL therapy holds promise as a novel selective eradication of tumor cells while sparing normal cells [Pitti et al., 1996; Almasan and Ashkenazi, 2003]. Therefore, it is important to search for molecular determinants regulating the cells' sensitivity to TRAIL. It has been reported that several genes increased the sensitivity to TRAIL via activating pro-apoptotic factors or suppressing anti-apoptotic factors. For example, (i) adenoviral vector expressing CYLD increased the sensitivity to TRAIL by suppression of NF-κB survival signaling in hepatocellular carcinoma [Chu et al., 2006]; (ii) somatostatin receptor subtype 2 (sst2) up-regulated expression of DR4 and TNFRI, sensitizing the cells to death ligand-induced initiator capase-8 activation, as well as down-regulated expression of the antiapoptotic mitochondrial Bcl-2 protein [Guillermet et al., 2003]; (iii) decoy receptor 3 (DcR3) increased TRAIL-induced caspase-8 activation and Bid cleavage, enhanced Smac release from mitochondria, and augmented caspases-3 and -9 activation [Wu et al., 2004]; (iv) overexpression of reticulon 3 (RTN3) enhances TRAIL-mediated apoptosis via up-regulation of death receptor 5 (DR5) and down-regulation of c-FLIP [Lee et al., 2009]; (v) prostate apoptosis response gene-4 (par-4) enhances sensitivity towards TRAIL by an enforced cleavage of c-FLIP$_L$ together with an increased activation of the initiator caspases-8 and -10 and expression of par-4 enables cells to down-regulate the inhibitor-of-apoptosis proteins c-IAP-1, c-IAP-2, XIAP, and survivin [Boehrer et al., 2006]. However, Boehrer et al. reported that Par-4 promotes apoptosis induced by TRAIL through activation of caspase-dependent pathway, its sensitizing mechanism was not determined. Therefore, we here studied the underlying mechanism(s) that contribute to increased activation of caspases by Par-4 and whether leucine zipper domain in Par-4 is critical to the response of TRAIL-treated cancer cells.

MATERIALS AND METHODS

CELLS AND MATERIALS

Human renal carcinoma Caki cells were obtained from ATCC (Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. TRAIL was directly added to cell cultures at the indicated concentrations while untreated cells contained the solvent alone. Anti-PARP and anti-procaspase-3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-XIAP and anti-c-FLIP antibodies were obtained from R&D systems (Minneapolis, MN). Benzyloxycarbony-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) was purchased from Biomol (Biomol, Plymouth Meeting, PA). TRAIL was purchased from R&D Systems.

PAR-4 CONSTRUCTS AND STABLE CELLS

The human Myc-tagged Par-4 expression vector (pcDNA3.1-Par-4) and leucine zipper deletion construct of Par-4 (pcDNA3.1-Par-4 ΔLZ) were kindly provided by Dr. Shin J. (School of Medicine, Sunkyunkwan University, Korea). The Caki cells were transfected in a stable manner with the pcDNA3.1-Par-4, pcDNA3.1-Par-4 ΔLZ, and pcDNA 3.1 vector using LipofectAMINE as prescribed by the manufacturer (Invitrogen, Carlsbad, CA). After 48 h of incubation, transfected cells were selected in cell culture medium containing 700 μg/ml G418 (Invitrogen). After 2 or 3 weeks, single independent clones were randomly isolated, and each individual clone was plated separately. After clonal expansion, cells from each independent clone were tested for Par-4 expression by immunoblotting.

Point mutations of the putative Akt phosphorylation sites (S228A, T229A, and S230A) to the Par-4 were generated by a two-step PCR method using the following primers: 5′-AGATA-TAAAAGCACAACCAGTGTCTCT-3′ to 5′-AGATATAAAGCCACAG-CCGCTGTCT CT-3′. *Eco*RI and *Xho*I sites were included in PCR products so that after *Eco*RI and *Xho*I digestion of the PCR products they could be sub-cloned into the pcDNA3.1 site. Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure.

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 proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

FLOW CYTOMETRY ANALYSIS

Approximately 1×10^6 cells were suspended in 100 μl PBS, and 200 μl of 95% ethanol was 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 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.

ANALYSIS OF CELL SURFACE DR5

Cells were detached with 0.5 mM EDTA, and washed three times with PBS wash buffer supplemented with 0.5% BSA. Cells (5×10^5) were re-suspended in 200 μl of PBS, stained with primary antibody (1 μg/ml) and incubated for 30 min at 4°C. Unreacted antibody was removed by washing the cells twice with the same PBS buffer. Cells were stained with secondary antibody conjugated with fluorescein isothiocyanate (FITC) and incubated for 30 min at 4°C. Unbound FITC-conjugated antibody was washed twice with PBS. Cells were re-suspended in 200 μl of PBS. Cell-surface expression of DR5

receptor was determined by flow cytometry. Fluorescent intensity of the cells is directly proportional to the density of receptor.

DAPI STAINING

The TRAIL-treated cells were fixed with 1% paraformaldehyde on slide glass for 30 min at room temperature. After washing with PBS, 300 nM DAPI (40-60-diamidino-2-phenylindole, Roche, Germany) was added to the fixed cells for 5 min, after which they were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei.

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously [Baek et al., 2002]. The sequence of the double-stranded oligonucleotides used to detect the DNA-binding activities of nuclear factor-κB (NF-κB) is as follows: 5′-AGT TGAGGGGACTTTCCCAGGC-3′. The reaction mixture for EMSA contained 20 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 μg poly (dI–dC), and 5 μg nuclear proteins. Unlabeled wild type oligonucleotides were added into the reaction mixture and incubated for 10 min at room temperature. [^{32}P]-labeled probe DNA (300,000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. Mixtures were resolved on 8% polyacrylamide gels at 150 V for 4 h. Gels were dried and subjected to autoradiography.

STATISTICAL ANALYSIS

Three or more separate experiments were performed. Statistical analysis was done by Student's *t*-test. A *P*-value <0.05 was considered to have pronounced difference between experimental and control groups.

RESULTS

OVEREXPRESSION OF PAR-4 INCREASED THE SENSITIVITY TO TRAIL IN HUMAN RENAL CANCER CELLS

It has been reported that overexpression of Par-4 increased the sensitivity to TRAIL in Jurkat T cells [Boehrer et al., 2006]. To examine the role of Par-4 in TRAIL-mediated apoptosis in Caki cells, control (Caki/Vector#1 and #2) cells, and Par-4 overexpressing cell lines (Caki/Par-4 #1 and #2) were treated with 100 ng/ml TRAIL for 12 h and examined cytotoxicity using FACS analysis. As shown in Figure 1A, TRAIL treatment in Caki/Par-4 cells resulted in a markedly increased accumulation of sub-G1 phase compared with Caki/Vector cells. We next analyzed whether treatment with TRAIL gave rise to the more activation of caspases in Caki/Par-4 cells. Exposure of Caki/Par-4 #1 cells to TRAIL strongly led to a reduction of the protein levels of procaspases-8 and -3 precursor together with a concomitant cleavage of PARP, a substrate protein of caspases (Fig. 1B). To determine whether the increased sensitivity to TRAIL is associated with the expression levels of apoptosis regulating proteins, we next examined the expression levels of, c-FLIP, c-IAP family proteins, and Bcl-2 family proteins in Caki/Par-4 cells treated with TRAIL. As shown in Figure 1C, treatment of Caki/Par-4 cells with TRAIL for 12 h resulted in reduction of c-FLIP (c-FLIPs and c-$FLIP_L$), and XIAP protein levels. As previously reported, we also found that Bcl-2 protein level was low in Par-4 overexpressing cells. For an additional method to quantify apoptosis induced by TRAIL in the two cell lines, nuclear condensation and fragmentation were indentified by DAPI staining. Caki/Par-4 #1 cells treated with 100 ng/ml TRAIL showed the nuclear condensation and formation of apoptotic bodies in DAPI staining (Fig. 1D). However, the chromatin was rarely condensed and fragmented in TRAIL-treated Caki/Vector cells.

DECREASED EXPRESSION LEVELS OF XIAP AND C-FLIP PROTEINS WERE CAUSED BY ACTIVATION OF CASPASE PATHWAY IN PAR-4 OVEREXPRESSING CELLS

To investigate whether activation of caspase is directly associated with TRAIL-induced apoptosis in Caki/Par-4 cells, we determined sub-G1 population in Caki/Vector and Caki/Par-4 #1 and #2 cells after pretreatment with a pan-caspase inhibitor, z-VAD-fmk, followed by TRAIL treatment. As shown in Figure 2A, pretreatment with z-VAD-fmk completely inhibited TRAIL-induced apoptosis. This result suggests that TRAIL-induced cell death was mediated by caspase-dependent cell death pathways. To examine whether the decreases of XIAP and c-FLIP proteins were related with down-regulation of mRNA expression in TRAIL-treated Caki/Par-4 cells, we measured the mRNA expression levels of XIAP and c-FLIP by RT-PCR. XIAP and c-FLIP mRNA levels remain constant through the TRAIL treatment at different times in Caki/Par-4 cells (data not shown), suggesting that TRAIL-mediated degradation of total XIAP and c-FLIP proteins may be regulated by the post-transcriptional levels in Par-4 overexpressing cells. Since XIAP and c-FLIP have been previously reported to be a substrate of caspases during apoptosis [Deveraux et al., 1997; Lee et al., 2009], we tested whether TRAIL-mediated reduction of XIAP and c-FLIP protein levels was correlated with the enhanced caspase activities in Caki/Par-4 cells. As shown in Figure 2B, XIAP and c-FLIP degradation was prevented by pretreatment with 50 μM z-VAD-fmk in the presence of TRAIL in Caki/Par-4 cells, indicating that the decrease of XIAP and c-FLIP proteins were partly mediated by caspase-dependent pathways. Whether the decreased expression of XIAP and c-FLIP were related with sensitizing effect of Par-4 in TRAIL-treated cells, Caki/Par-4 #1 cells were transiently transfected with XIAP, c-FLIP (long and short form), and empty Vector. Treatment with TRAIL in Caki/Par-4 #1 cells that were transfected with Vector led to increase of sub-G1 populations, which were markedly blocked by ectopic expressions of c-FLIP long and short form, but not XIAP (Fig. 2C). This result indicated that the decreased c-FLIP levels were related with the induction of apoptosis in Caki/Par-4 cells and that the decreased XIAP protein was occurring in the apoptotic process.

DEATH LIGANDS/CASPASE-8 PATHWAY MIGHT BE CRUCIAL FOR PAR-4 ACTION IN CAKI CELLS

Par-4 increases CD95-mediated cell death by increasing expression of the CD95 receptor on the cell surface of malignant lymphocytes [Bergmann et al., 2004]. In addition, Par-4 drives trafficking and activation of Fas and FasL to induce prostate cancer cell apoptosis and tumor regression [Chakraborty et al., 2001]. We also checked whether the sensitivity against Fas agonistic antibody or TNF-α

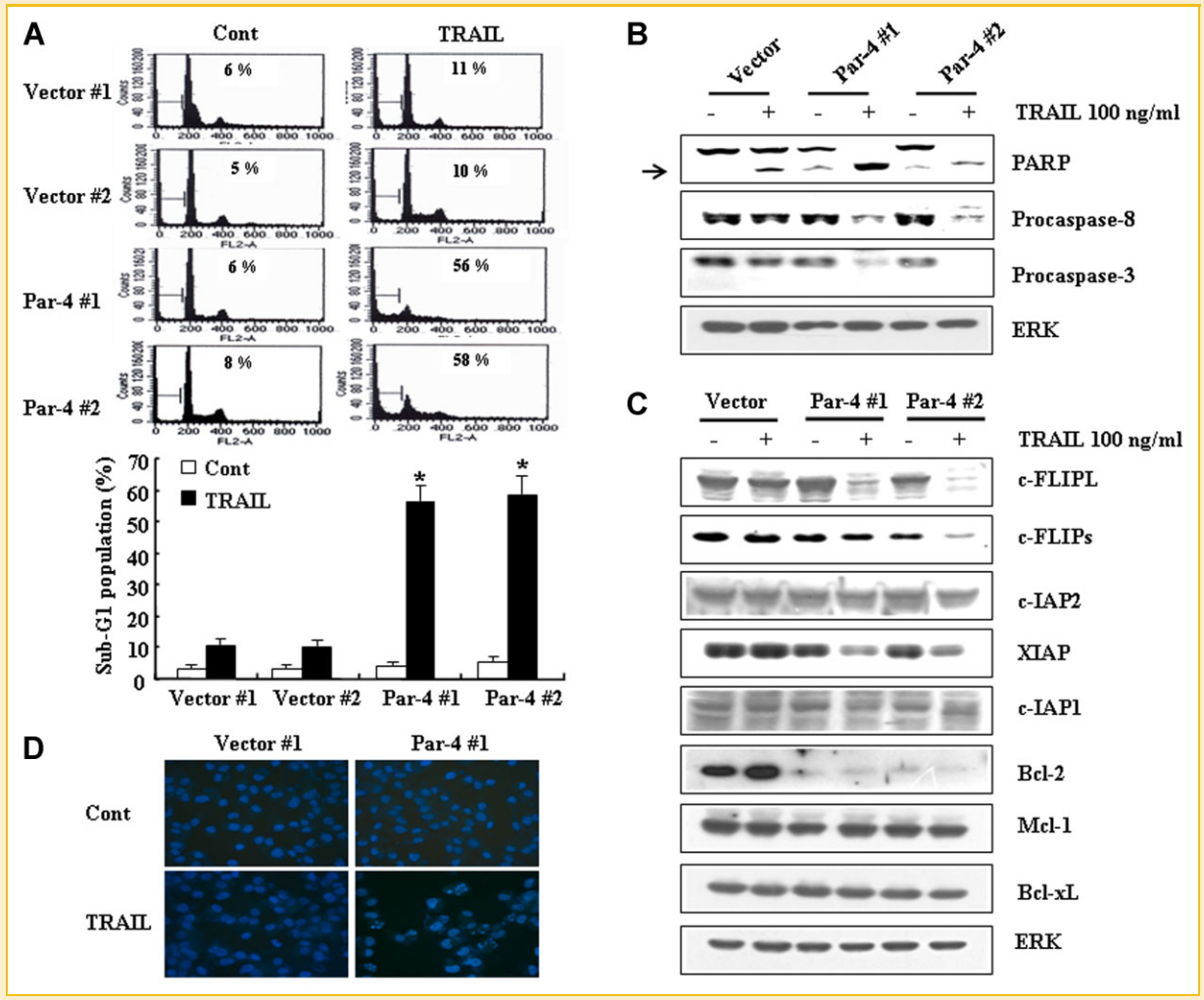

Fig. 1. Overexpression of Par-4 increased the sensitivity to TRAIL in Caki cells. A: Overexpression of Par-4 enhanced TRAIL-induced apoptosis in Caki cells. Caki/Vector (#1 and #2) and Caki /Par-4 (#1 and #2) were treated for 12 h with the indicated concentration of TRAIL and their DNA content was measured after propidium iodide staining. The FACS data were shown in upper panel. The proportion of apoptotic cells is indicated. Data shown are means SD. ($n = 3$). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated Vector cells. B: Overexpression of Par-4 enhanced cleavage of PARP, procaspase-8, and procaspase-3 proteins in TRAIL-treated cells. Cells treated as above were harvested in lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for procaspase-8, procaspase-3, and PARP. The proteolytic cleavage of PARP was indicated by arrow (Bottom panel). C: Overexpression of Par-4 enhanced cleavage of c-FLIP (c-FLIPs and c-$FLIP_L$) and XIAP proteins in TRAIL-treated cells. Cells treated as above were harvested in lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for c-FLIP, c-IAP family, Bcl-2 family, or with anti-ERK antibody to serve as control for the loading of protein level. D: Detection of TRAIL-induced apoptosis in Caki/Vector #1 and Caki/Par-4 #1 cells by DAPI staining. The cells were incubated with 100 ng/ml TRAIL for 12 h. The nuclear condensation and fragmentation were determined by fluorescence microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

could increase in Caki/Par-4 cells. As shown in Figure 3A,B, overexpression of Par-4 augmented the sensitivity to Fas agonistic antibody and TNF-α. Since overexpression of par-4 enhanced the extrinsic apoptotic pathway such as TNF-α, TRAIL- and, Fas agonistic antibody-induced apoptosis, we examined the involvement of initiator caspase-8 in Par-4 action. The cells were left untreated or treated with Fas agonist antibody, TNF-α plus cyclohexamide (CHX), and TRAIL in presence or absence of caspase-8 inhibitor, z-IETD-FMK. Par-4-inducible extrinsic apoptosis was inhibited by z-IETD-FMK (Fig. 3C), indicating that the death ligands/caspase-8 pathways were crucial for Par-4 action in Caki cells.

THE SURFACE DR5 LEVEL WAS INCREASED IN TRAIL-TREATED PAR-4 OVEREXPRESSING CELLS

Based on data from Figure 3, we postulated that the enhanced TRAIL sensitivity was occurred at upstream of the initiator caspase-8 in Caki/Par-4 cells. To elucidate whether the events for the increased sensitivity to TRAIL were present in the receptors themselves or somewhere along its apoptotic signaling pathway, we examined the expression level of TRAIL death receptor and the adaptor molecules such as FADD using Western blot analysis. No differences in expression levels of TRAIL receptors (DR4 and DR5) and FADD were observed between Caki/Vector and Caki/Par-4 cells (Fig. 4A). Next, we checked whether the enhanced TRAIL sensitivity was caused by

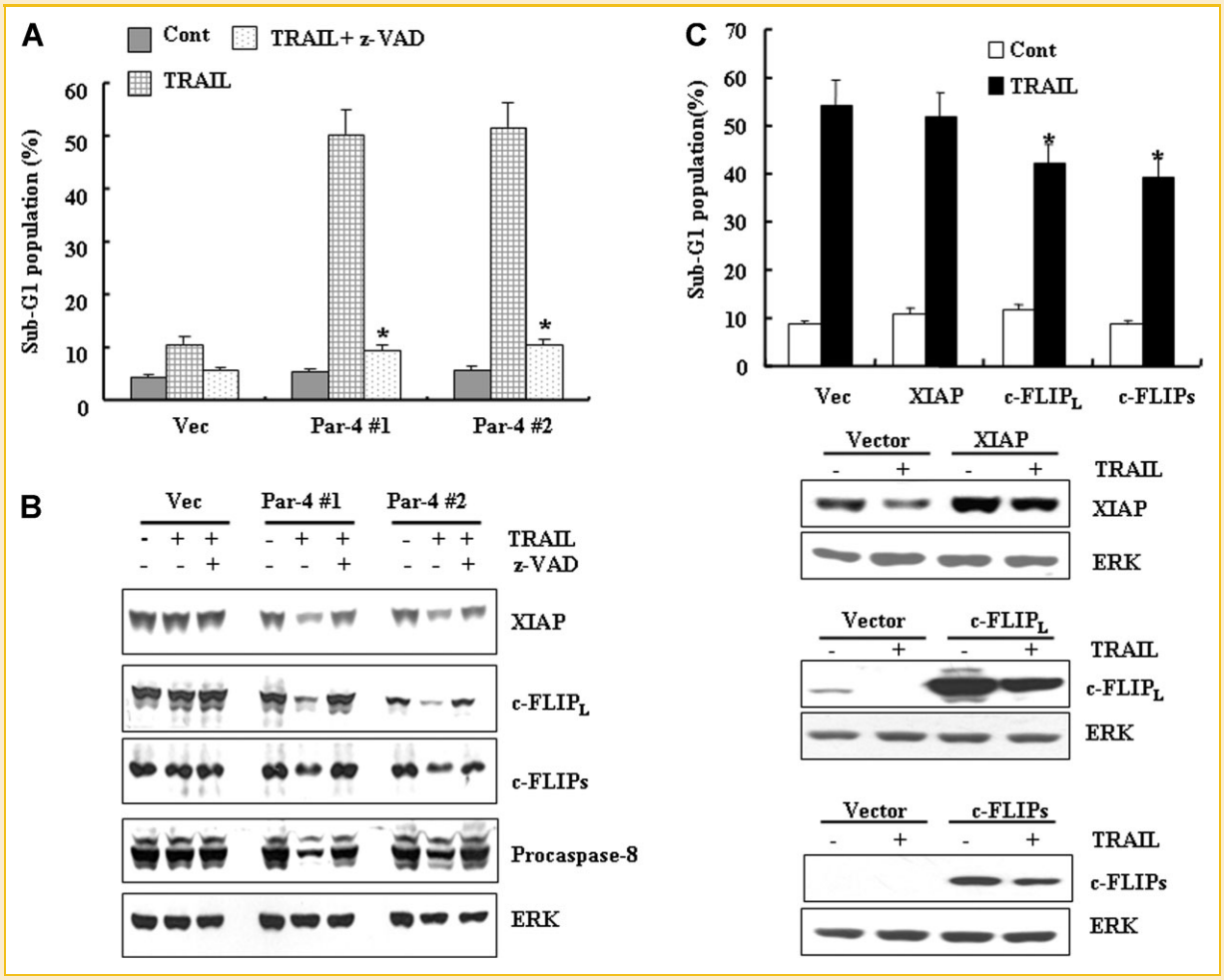

Fig. 2. TRAIL-induced apoptosis was mediated by caspase-dependent pathway in Caki/Par-4 cells and decreased expression of c-FLIP and XIAP was caused by caspase in Par-4 overexpressing cells. A: Pretreatment with z-VAD completely prevented TRAIL-induced apoptosis in Caki/Par-4 cells. Caki/Vector and Caki/Par-4 cells were pretreated with 50 μM z-VAD for 30 min, and then incubated with TRAIL for 12 h. Sub-G1 population were determined by FACS analysis. Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated Vector or TRAIL-treated Par-4 cells. B: Pretreatment with z-VAD completely prevented TRAIL-induced cleavage of XIAP, c-FLIP, and procaspase-8. Caki/Vector and Caki/Par-4 cells were pretreated with 50 μM z-VAD for 30 min, and then incubated with TRAIL for 12 h. Cells were harvested in lysis buffer and equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for XIAP, c-FLIP, and pro-caspase-8. C: Ectopic expression of c-FLIP attenuated TRAIL-induced apoptosis in Caki/Par-4 #1 cells (Top panel). Caki/Par-4 #1 cells were transiently transfected with XIAP, c-FLIP (long and short form), and empty vector (pCMV) and treated with TRAIL for 24 h. Cells were harvested and analyzed by FACS and Western blotting. Western blotting was performed using anti-XIAP, c-FLIP, and ERK antibody to confirm the transfection efficiency (Bottom panel). Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated Par-4 cells that are transfected with pCMV.

driving translocation of TRAIL receptor DR5 in Par-4 over-expressing cells. Flow cytometric analysis of the intensity of the fluorescence, which is proportional to the expression of the DR5, demonstrates that the surface expression of DR5 is augmented in TRAIL treated-Caki/Par-4 cells compared to Caki/Vector cells (Fig. 4B). However, DR5 surface expression did not increase in response to TNF/CHX in both cells (Fig. 4C). This result indicates that the increased surface DR5 expression might not be a change that occurs during apoptosis itself.

DELETION OF LEUCINE ZIPPER DOMAIN IN PAR-4 PARTIALLY DIMINISHES THE RESPONSE OF CELLS TO TRAIL

Par-4 binds to the zinc finger of WT1 via its C-terminal leucine zipper domain. This binding results in the inhibition of WT1 activated-bcl-2 transcription [El-Guendy et al., 2003]. In addition, Par-4 interacts with ZIPK, DAXX, and THAP-1 to increase apoptosis, which are mediated by the C-terminal leucine zipper domain of Par-4, suggesting that that these associations are essential for the pro-apoptotic function of this protein [Sells et al., 1997]. To determine whether the loss of leucine zipper domain of Par-4 could have an impact on the sensitivity to TRAIL in Caki cells, we established Par-4 ΔLZ overexpressing cells (Par-4 ΔLZ #1 and #2). As shown in Figure 5A, overexpression of full-length Par-4 resulted in an increased sensitivity to TRAIL, whereas deletion of leucine zipper domain in Par-4 partially attenuated TRAIL-mediated apoptosis, indicating that leucine zipper domain in Par-4 may be partly involved in the increased TRAIL sensitivity in Caki cells. Exposure of Caki/Par-4 cells to TRAIL strongly led to a reduction of

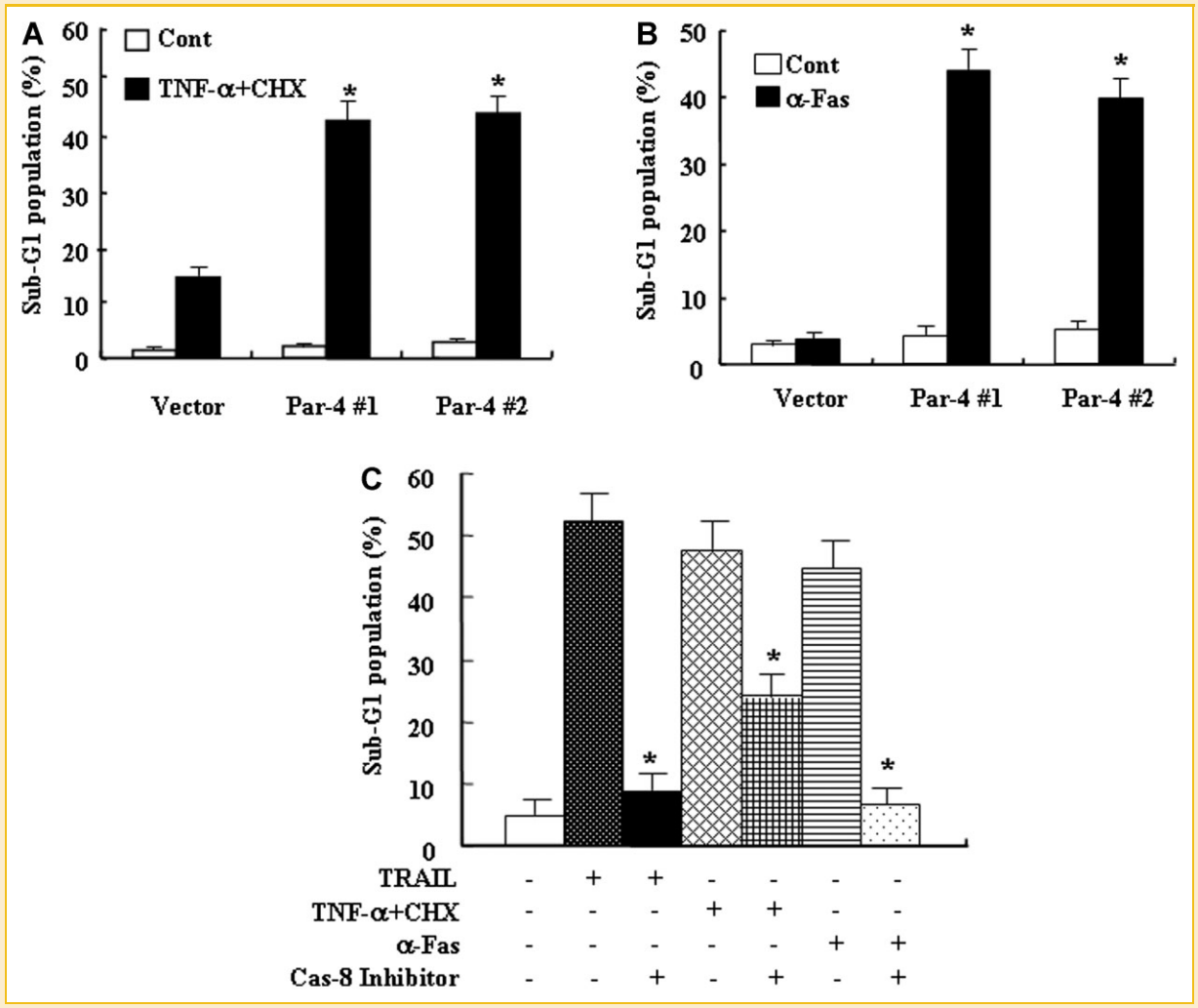

Fig. 3. Overexpression of Par-4 also enhanced TNF-α/CHX and Fas-mediated apoptosis. A: Caki/Vector and Caki /Par-4 were treated for 12 h with 100 ng/ml TNF-α plus 10 μg/ml CHX and their DNA contents were measured after propidium iodide staining. The proportion of apoptotic cells is indicated. Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus indicated TNF-α plus CHX-treated Caki/Vector cells. B: Caki/Vector and Caki /Par-4 were treated for 12 h with 300 ng/ml Fas agonistic antibody and their DNA contents were measured after propidium iodide staining. The proportion of apoptotic cells is indicated. Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus indicated Fas agonistic antibody treated Caki/Vector cells. C: Pretreatment with caspase-8 inhibitor, z-IETD-FMK, completely prevented TRAIL, TNF-α/CHX, and agonistic Fas antibody-induced apoptosis in Caki/Par-4 cells. Caki/Vector and Caki/Par-4 cells were pretreated with 50 μM z-IETD-FMK for 30 min, and then incubated with TRAIL for 12 h. Sub-G1 population were determined by FACS analysis. Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated or TNF-α/CHX-treated- or α-Fas treated-Par-4 cells.

the protein levels of procaspase-8 together with a concomitant cleavage of PARP, a substrate protein of caspases (Fig. 5B). A reduction of the protein levels of procaspase-8 and cleavage of PARP in Caki/Par-4 ΔLZ is weaker than Caki/Par-4 cells (Fig. 5B). Interestingly, the reduction of Bcl-2 protein level by Par-4 was recovered by the deletion of its leucine zipper domain (Fig. 5B), suggesting that leucine zipper domain of Par-4 is involved in Bcl-2 down-regulation in Caki cells. Even though restoration of Bcl-2 protein level by Par-4 ΔLZ, TRAIL sensitivity did not completely recovered to the level induced by wild type Par-4 (Fig. 5), indicating that reduction in Bcl-2 alone was not sufficient to explain the TRAIL sensitivity in Par-4 overexpressing cells.

INACTIVATION OF NF-*K*B PATHWAY IS ASSOCIATED WITH THE AUGMENTATION OF TRAIL SENSITIVITY IN CAKI/PAR-4 CELLS

The transcription factor NF-κB is activated in many cancer cells in response to TRAIL [Khanbolooki et al., 2006; Voortman et al., 2007; Smith et al., 2007], and numerous reports have shown that Par-4 enhanced apoptosis through the inhibition of NF-κB activation [Diaz-Meco et al., 1999; Camandola and Mattson, 2000; Chendil et al., 2002]. In this study, we examined whether abrogation of NF-κB signaling pathway was involved in Par-4-induced TRAIL-mediated apoptosis in Caki cells. We performed NF-κB gene-dependent reporter assay using a pNF-κB-Luc plasmid, using a pNF-κB-Luc plasmid containing four NF-κB binding sites. Caki/Vector, Caki/Par-4, and Caki Par-4 ΔLZ cells were transiently

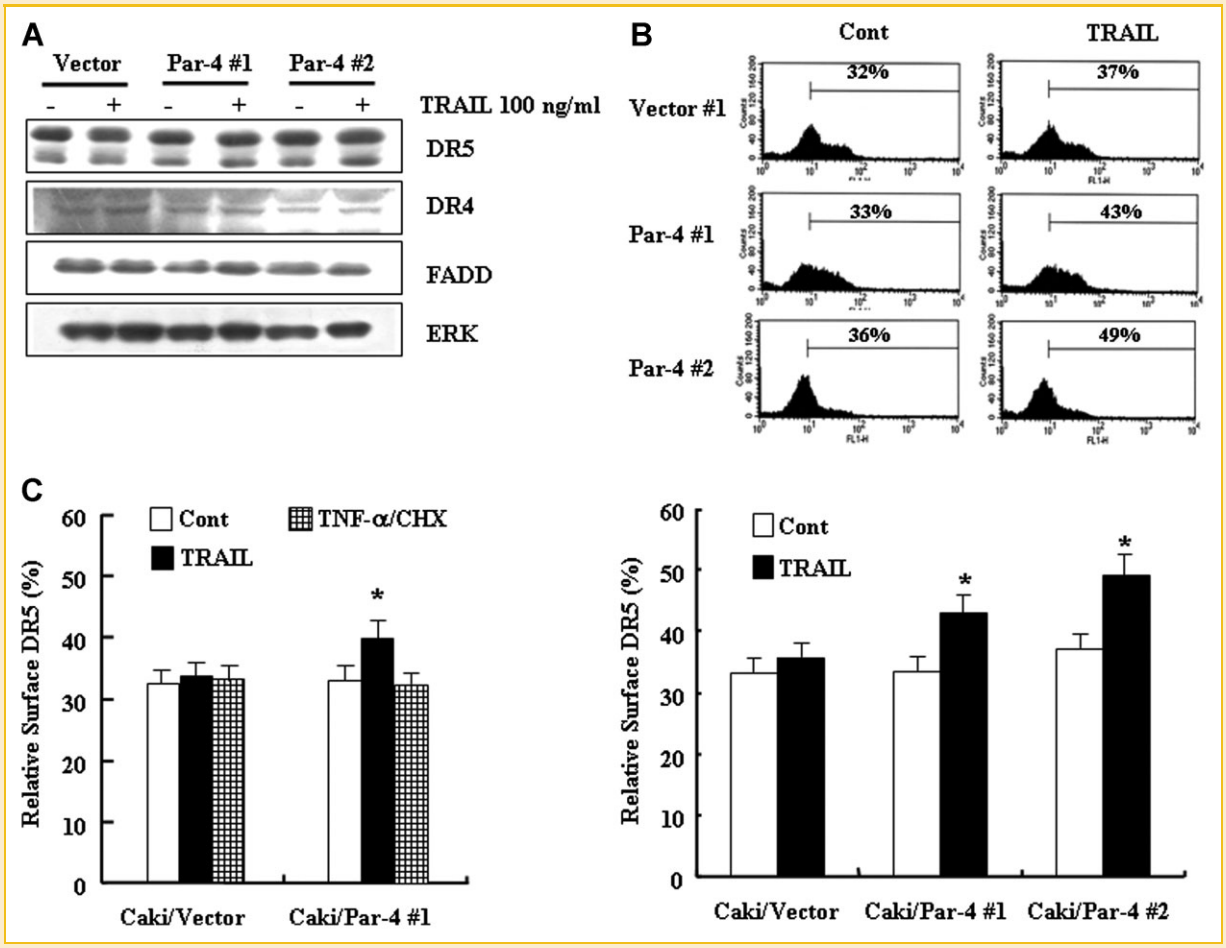

Fig. 4. The enhanced sensitivity to TRAIL was associated with the increased expression of DR5 surface proteins in Par-4 overexpressed cells. A: Caki/Vector and Caki/Par-4 cells were treated with the indicated concentrations of TRAIL for 12 h. Western blotting was performed as above using anti-FADD, -DR4, and -DR5 antibodies. Anti-ERK was served as a control to check equal loading of protein samples. B: The cell surface expression levels of DR5 in both Caki/Vector and Caki/Par-4 cells were measured by flow cytometry analysis with or without TRAIL treatment. Data shown are means SD ($n = 3$). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated-Vector cells. C: The cell surface expression levels of DR5 in both Caki/Vector and Caki/Par-4 cells were measured by flow cytometry analysis with or without TRAIL and TNF-α/CHX treatments. Data shown are means SD ($n = 3$). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated-Vector cells.

transfected with the pNF-κB-Luc plasmid and then stimulated with TRAIL for 0.5, 1.5, and 3 h. TRAIL treatment significantly induced the NF-κB-dependent luciferase enzyme expression in Caki/Vector cells. TRAIL-induced a down-regulation of NF-κB promoter activity in cells with overexpression of Par-4, and this response was diminished in cells with a deleted leucine zipper domain in Par-4 (Fig. 6A). These results indicate that TRAIL-induced down-regulation of NF-κB promoter activity was partly caused by leucine zipper domain in Par-4. To gain insight into the underlying mechanism of Par-4-induced NF-κB inhibition, we examined the effect of Par-4 on NF-κB DNA binding activity. TRAIL treatment caused a significant increase in the DNA-binding activity of NF-κB in Caki/Vector cell. In contrast, forced expression of Par-4 significantly suppressed the basal and TRAIL-induced DNA-binding activity of NF-κB (Fig. 6B). Next, we examined the functional significance of NF-κB on TRAIL-mediated apoptosis in Par-4 overexpressing cells. Ectopic expression of p50 plus p65 by transient transfection markedly lessened the sensitivity to TRAIL in Caki/Par-4 cells (Fig. 6C).

THE PHOSPHORYLATED AKT LEVEL WAS RELATED TO THE ENHANCED INDUCTION OF APOPTOSIS IN CAKI/PAR-4 CELLS

Akt physically binds to Par-4 via the Par-4 leucine zipper domain and phosphorylates Par-4 to allow 14-3-3 sequestered by the chaperone 14-3-3 in the cytosol, thereby inactivating it to ensure cell survival [Goswami et al., 2006]. We investigated whether overexpressed Par-4 induced inactivation of Akt, thereby preventing it from executing its antiapoptotic functions in TRAIL-treated cancer cells. Exposure of two Caki/Par-4 cell clones to TRAIL strongly led to reduction of the phosphorylated Akt level (Fig. 7A). These results suggested that the enhanced induction of apoptosis caused by Par-4 overexpression might be associated with a decrease in phosphorylated Akt protein level in Caki cells. However, deletion of leucine zipper domain of Par-4 did not affect the phosphorylated

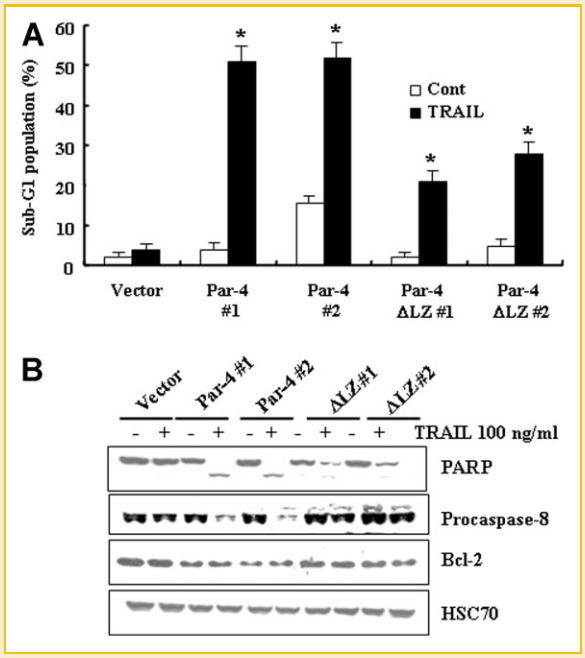

Fig. 5. Deletion of leucine zipper domain in Par-4 partially diminishes the response of cells to TRAIL. A: CakiVector, Caki/Par-4, and Caki/ΔLZ cells were treated with TRAIL for 12 h and their DNA content was measured after propidium iodide staining. The FACS data were shown in upper panel. The proportion of apoptotic cells is indicated. Data shown are means SD ($n = 3$). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated vector cells. B: Western blotting was performed as above using anti-procaspase-8 and -Bcl-2, anti-HSC was served as a control to check equal loading of protein samples.

Akt level (Fig. 7A), perhaps suggesting that not all modulation of TRAIL response by Par-4 requires activation through the leucine zipper domain. To confirm whether the decreased level of phosphorylated Akt was related with sensitizing effect of Par-4 in TRAIL-treated cells, Caki/Par-4 cells were transiently transfected with dominant active-Akt (DA-Akt) and empty vector. Treatment with TRAIL in Caki/Par-4 cells that were transfected with vector led to increase of sub-G1 populations, which was considerably blocked by ectopic expression of DA-Akt (Fig. 7B). This result indicated that restoration of Akt activities lessened TRAIL-mediated apoptosis in Caki/Par-4 cells. It has been reported that the proapoptotic function of rat Par-4 was inactivated by Akt through phosphorylation at S249 residue and cytoplasmic sequestration by 14-3-3 proteins [Goswami et al., 2005]. To identify the amino acid residues that were phosphorylated by Akt in human Par-4 protein, we used computer program (Site-Scan program, http://scansite.mit.edu). We found that that human Par-4 has putative phosphorylation sites at S228, T229, and S230 by Akt. To examine the functional role of Par-4 phosphorylation site by Akt in TRAIL-mediated apoptosis, we constructed S228A mutant construct (containing point mutations at serine 229 to alanine, threonine 229 to alanine, and serine 230 to alanine). As shown in Fig. 7C, the apoptotic populations are potentiated in S228A Par-4 trasnfected cells compared with wild type Par-4 in response to TRAIL. This result suggested that inactivation of Par-4 by Akt might be increased the sensitivity to TRAIL in Caki cells.

DISCUSSION

In the present study, we showed that overexpression of Par-4 significantly enhanced the sensitivity to TRAIL-mediated apoptosis in Caki cells. The Par-4-promoted-increase in the rate of apoptosis is associated with an increased activation of caspase pathways through processing of caspase-8, an initiator caspase, down-regulation of c-FLIP and XIAP as well as the increased expression of surface DR5 in TRAIL-treated cells. Furthermore, overexpression of Par-4 also enhanced the sensitivity to TNF-α CHX and Fas agonistic antibody in Caki cells. We also found that inactivation of NF-κB and Akt signaling pathways were related to the enhanced induction of apoptosis Caki/Par-4 cells. However, previous works demonstrated that forced expression of Par-4 caused the increased sensitivity to TRAIL in cancer cells [Boehrer et al., 2006; Lee et al., 2008], little was known about the precise mechanisms that Par-4 enhanced the sensitivity to TRAIL-mediated apoptosis. Therefore, we examined the underlining molecular mechanism by which Par-4 augments TRAIL sensitivity in human renal cancer cells.

In this study, we proved that activation of caspase-8 is critical to Par-4-prompted apoptosis by TRAIL in Caki cells, indicating that enhancement in the TRAIL-mediated apoptotic signaling pathways occur upstream of the initiator caspase-8 in Caki/Par-4 cells (Fig. 3). To elucidate the origin of the increasing TRAIL sensitivity, we examined the expression level of TRAIL death receptor and adaptor molecules such as FADD using Western blot analysis. No differences in expression levels of TRAIL receptors (DR4 and DR5) and FADD were observed between Caki/Vector and the Caki/Par-4 cells (Fig. 4A). Since recent studies using affinity assay and phage display of DR-selective TRIAL variants have revealed that DR5 has higher affinity and may contribute, more than DR4, to TRAIL-induced apoptosis in cancer cells [Truneh et al., 2000; Kelley et al., 2005], we focused on the functional role of DR5 in the enhancement of TRAIL-induced apoptosis in Par-4 overexpressing cells. Although, we failed to detect any differences in DR5 expression levels between Caki/Vector cell and Caki/Par-4 cell, the surface expression level of DR5 was higher in Caki/Par-4 cells than in Caki/Vector cells after TRAIL treatment (Fig. 4B). Par-4 has been shown to induce apoptosis of prostate cancer cells by cell membrane trafficking of Fas and FasL which causes the activation of caspase-8 pro-apoptotic pathway [Chakraborty et al., 2001]. Based on these results, it might be assumed that overexpressing Par-4 is able to enforce translocation of DR5 into surface and activation of initiator caspase-8, although it is unclear how TRAIL induces surface DR5 expression and how to increase caspase-8 activation in Par-4 overexpressing cells. Furthermore, overexpression of Par-4 enhances death receptor. TNF-α/CHX, Fas agonistic antibody and TRAIL-mediated apoptosis as well as down-regulation of c-FLIP proteins are caspase-dependent pathways, suggesting that the exogenous Par-4 possibly targeted the common component(s) of these death receptor-mediated apoptotic pathways such as c-FLIP. The observations that Par-4 increased TRAIL-mediated caspase-8

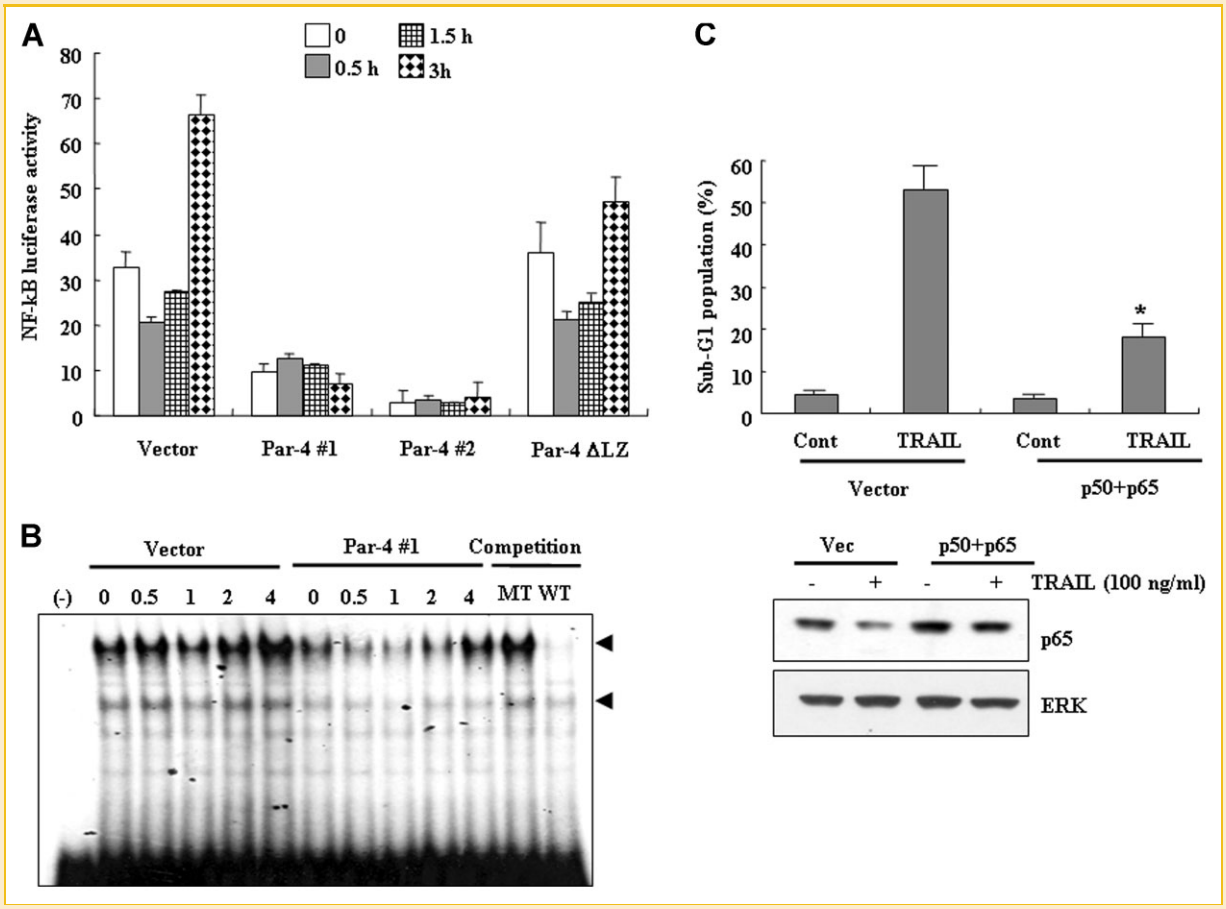

Fig. 6. Inactivation of NF-κB signaling pathways is involved in Par-4-prompted TRAIL-mediated apoptosis in Caki cells. A: Caki/Vector, Caki/Par-4, and Caki/ΔLZ cells were transfected with a NF-κB reporter plasmid, treated with TRAIL. Cells lysates were assayed for luciferase activity using a luminometer. Differences in transfection efficiency were normalized by co-transfection with a LacZ-containing plasmid. B: The cells were treated with TRAIL for the indicated times and nuclear extracts were prepared at the end of treatment. NF-κB-specific DNA-protein binding activity in nuclear extracts was determined by EMSA as described in Materials and Methods Section. Nuclear extracts were pre-incubated with 50-fold excess of NF-κB binding site consensus cold oligonucleotides (competition). C: After transient transfection with empty vector or p65 plus p50 expression vector, Cak/Par-4 cells were treated with TRAIL for 12 h and attain with propidium iodide to measure DNA content. The FACS data were shown in upper panel. The proportion of apoptotic cells is indicated. Data shown are means SD ($n = 3$). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated Vector-transfected cells.

activation and c-FLIP cleavage suggest that Par-4 promotes apoptotic events proximal to DR5 engagement. Recently, it has been reported that TRAIL causes secretion of Par-4, and that secreted Par-4 activates an extrinsic pathway involving cell surface GRP78/Bip protein [Burikhanov et al., 2009]. Based on this report, it could be assumed that Par-4 potentiated death-inducing signaling complex (DISC) formation, which is involved in the increase sensitivity to extrinsic apoptotic stimuli including TRAIL.

PI3K/Akt pathway has been reported to be one of the most important survival pathways in a variety of cell types [Song et al., 2005]. Withdrawal of growth factors or a death signal challenge decreases its activity and promotes cell death. A recent study also showed Par-4 deficiency increased phospho-Akt and overexpression of Par-4 in HEK 293 cells inhibited serum-induced phospho-Akt-S473 phosphorylation, suggesting important role of Par-4 as a negative regulator of Akt [Joshi et al., 2008]. The loss of Akt activity may thus be the result of suppression of protein phosphorylation, reduction of protein expression, or a combination of the two. In our present study, we observed that Akt was inactivated in response to TRAIL via a reduction in phospho-Akt in TRAIL-treated Caki/Par-4 cells without any change in total Akt protein level (Fig. 7). Akt physically binds to the pro-apoptotic protein Par-4 via the Par-4 leucine zipper domain and phosphorylates Par-4 to inhibit apoptosis [Goswami et al., 2005]. In our study, deletion of Par-4 leucine zipper domain blocked TRAIL-mediated inhibition of Akt activity, perhaps suggesting that not all modulation of TRAIL response by Par-4 requires activation through the leucine zipper domain. Restoration of Akt activity through transfection of a constitutively active Akt partly inhibited TRAIL-mediated apoptosis in Caki/Par-4 cells, suggesting bi-directional interplay between Par-4 and Akt. However, future experiments will have to determine bi-directional interplay between Par-4 and Akt as well as the contribution of the PI3K/Akt signaling pathway for the demonstrated enhancement of TRAIL-mediated apoptosis in Par-4 overexpressing cells.

In summary, the present study showed that overexpression of Par-4 in human renal cancer Caki cells markedly increase sensitivity

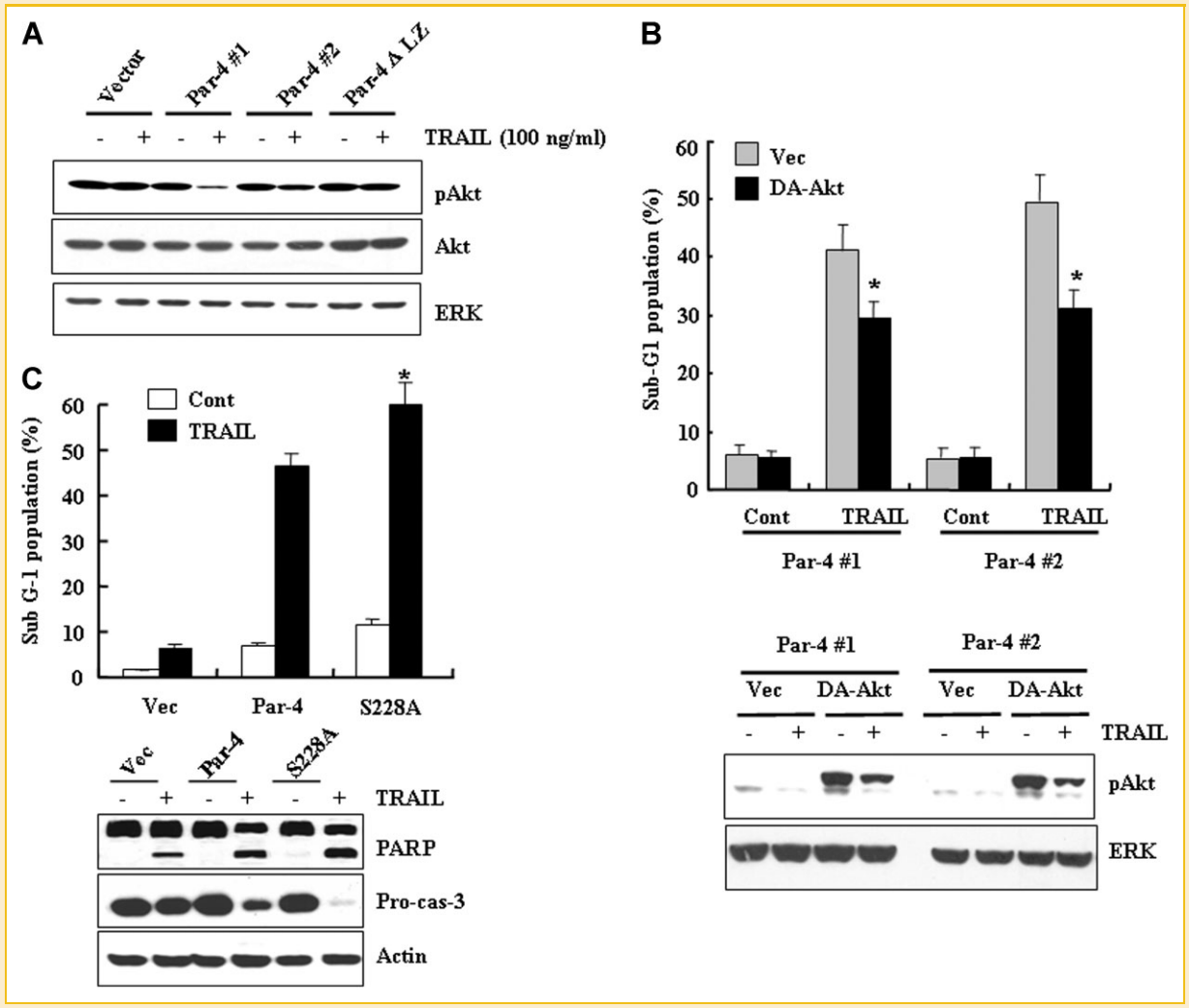

Fig. 7. Effect of Par-4 overexpression on TRAIL-induced phosphorylation of Akt in Caki/Vector and Caki/Par-4 cells. A: Overexpression of Par-4 led to reduction of phosphorylation of Akt. Caki/Vector and Caki/Par-4 cells were treated with TRAIL for 12 h. Phospho-Akt, Akt, and ERK were detected by Western blotting. B: The decreased expression of pAkt might be involved in TRAIL-induced apoptosis in Par-4 overexpression cells. Ectopic expression of dominant active Akt (DA-Akt) attenuated TRAIL-induced apoptosis in Caki/Par-4 cells (Top panel). Caki/Par-4 cells were transiently transfected with DA-Akt and empty vector and treated with TRAIL for 12 h. Cells were harvested and analyzed by FACS and Western blotting. Western blotting was performed using anti-Akt and ERK antibody to serve as control for the loading of protein level (Bottom panel). Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. $^{*}P < 0.05$ versus TRAIL-treated Par-4 cells that are transfected with Vector. C: Caki/Vector, Caki/Par-4, and Caki/S228A (mutated serine or threonine resides into alanine residues) cells were treated with TRAIL for 12 h and then their DNA content were measured after propidium iodide staining. The FACS data were shown in upper panel. The proportion of apoptotic cells is indicated (Top panel). Western blotting was performed using anti-PARP, procaspase-3, and actin antibody to serve as control for the loading of protein level (Bottom panel). Data shown are means SD (n = 3). Statistics, Student's *t*-test for unpaired values. * indicates $P < 0.05$ versus TRAIL-treated Par-4-transfected cells.

to TRAIL. We also demonstrated that overexpressing Par-4 enhanced translocation of DR5, eventually inducing more activation of caspase pathways and cleavage of XIAP and c-FLIP proteins. In addition, NF-κB and Akt signaling pathways were inactivated in Par-4 overexpressing cells in response to TRAIL treatment.

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