Biochemical and Biophysical Research Communications 436 (2013) 344–348

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

TRAF2 regulates the cytoplasmic/nuclear distribution of TRAF4 and its biological function in breast cancer cells

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ARTICLE INFO

Article history:
Received 23 May 2013
Available online 4 June 2013

Keywords:
TRAF4
TRAF2
Breast cancer
Nuclear transcription
Apoptosis

ABSTRACT

Although numerous studies have shown that tumor necrosis factor receptor-associated factor 4 (TRAF4) plays an important role in the carcinogenesis of many tumor types, its exact molecular mechanism remains elusive. In this study, we examined the regulation function of TRAF2 to the cytoplasmic/nuclear distribution of TRAF4 in the breast cancer cell line. Using cell immunofluorescent staining, we found that TRAF2 and TRAF4 were co-localized to the cytoplasm in MCF-7 cells. Co-immunoprecipitation showed that TRAF2 could interact with TRAF4 in MCF-10A, MCF-7 and MDA-MB-231 cell lines. Western blotting showed TRAF2 depletion by targeted siRNA in MDA-MB-231 cells led to reduced TRAF4 expression in the cytoplasm and augmented TRAF4 expression in the nucleus. Cytoplasmic expression of TRAF4 was augmented and nuclear expression was reduced when MCF-7 cells were transfected with hTRAF2pLPCX-HA-Flag/P874. MCF-7 cells expressing hTRAF2pLPCX-HA-Flag/P874 had enhanced cell proliferation rates. The nuclear expression of NF-κB significantly increased after TNF-α treatment. When hTRAF2pLPCX-HA-Flag/P874 and the siRNA-TRAF4 plasmid were cotransfected, the nuclear expression of NF-κB was significantly reduced compared with cells transfected with hTRAF2pLPCX-HA-Flag/P874 only. In conclusion, TRAF2 appears to interact with TRAF4 and affect the localization of TRAF4 in breast cancer cell lines. The overexpression of TRAF2 augmented the cytoplasmic expression of TRAF4 which promoted cell proliferation and inhibited cell apoptosis by activating NF-κB nuclear transcription. TRAF4 may play an important role in the activation of NF-κB via TRAF2.

1. Introduction

Tumor necrosis factor receptor associated factors (TRAFs) have emerged as the major signal transducers for the TNF receptor and interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) superfamilies. TRAFs collectively have important cellular functions that involve adaptive and innate immunity, embryonic development, stress responses and bone metabolism. These functions are mediated by TRAFs through the induction of cell survival, proliferation, differentiation and death [1–5].

TRAF4 (originally designated CART1 as it contained a C-rich domain associated with RING and TRAF) was identified by the differential screening of a cDNA library of breast cancer-derived metastatic lymph nodes [6]. TRAF4 is a unique member of the TRAF Family as it is the only protein that contains a nuclear localization signal (NLS) [6,7]. All TRAF proteins except TRAF4 have been reported to associate directly or indirectly with CD40. Only a few TNF-Rs have been reported to interact with TRAF4, including the human p75 neurotrophin receptor (p75-NGFR) and the lymphotoxin-beta receptor (LTp-R) [8,9]. Although in vivo studies have shown that TRAF4 is involved in important biological functions, its mode of action at the molecular level and the mechanism of its nuclear localization remain elusive.

All TRAF proteins share a C-terminal homology region, termed the TRAF domain which is capable of binding to the cytoplasmic domain of receptors and to other TRAF proteins [10]. As very few TNF-Rs have been verified and their interactions with TRAF4 are weak, we propose that other proteins exist that bind to TRAF4 and mediate the interaction between TRAF4 and TNF-Rs. TRAF2 is a critical member of the TRAF family as it can directly interact with many TNF-R superfamily members. Mutational analysis of TRAF2 has shown that distinct domains interact with other proteins and mediate signaling functions [11]. The two distinct TRAF-N and TRAF-C subdomains of the TRAF domain appear to mediate self-association and interactions with TRAF1 with independently. The N-terminal RING finger and two adjacent zinc fingers are required for NF-κB activation [10]. Fumihiko et al. has demonstrated that TRAF4 can interact with TRAF6 and acts as a silencer in TLR-mediated signaling through the association with TRAF6 and TRIF [12]. Therefore, we hypothesized that TRAF2 could interact with TRAF4 in breast cancer cells and regulate the nuclear translocation of TRAF4.

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http://dx.doi.org/10.1016/j.bbrc.2013.05.107

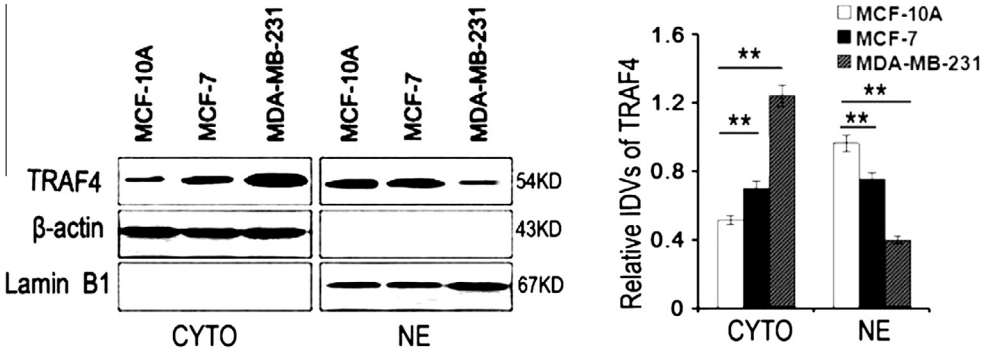

Fig. 1. The expression of TRAF4 in breast cancer cells. Western blot analysis showed that TRAF4 proteins were expressed in MCF-10A, MCF-7 and MDA-MB-231 cells. The cytoplasmic expression of TRAF4 in MCF-10A was lower than in MCF-7 and MDA-MB-231 cells ($^{**}p = 0.006$, $^{**}p = 0.001$). The nuclear expression of TRAF4 in MCF-10A was higher than in MCF-7 and MDA-MB-231 cells ($^{**}p = 0.004$, $^{**}p = 0.001$).

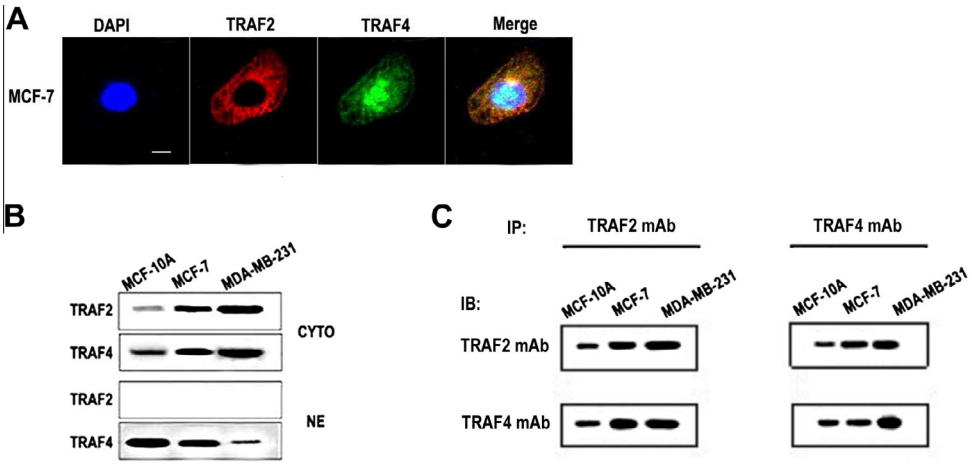

Fig. 2. TRAF2 could interact with TRAF4 in breast cancer cells. (A) The results of immunofluorescent staining showed that TRAF2 was localized to the cytoplasm in MCF-7 cells (red fluorescence), while TRAF4 (green fluorescence) was localized to both cytoplasm and nuclei. Scale bars: 10 μm. (B) Western blotting showed that TRAF2 was expressed in the cytoplasm, while TRAF4 was expressed in both the cytoplasm and nuclei. (C) Co-immunoprecipitation assays showed that TRAF2 monoclonal antibodies can effectively precipitate TRAF4 proteins (upper panel). TRAF4 monoclonal antibodies can also effectively precipitate TRAF2 proteins (lower panel) in MCF-10A, MCF-7 and MDA-MB-231 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Methods

2.1. Cell lines

The human normal breast cell line, MCF-10A, and two human breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-10A cells were cultured in DMEM/F12 (1:1) supplemented with 5% equine serum, 10 μg/ml insulin and 20 ng/ml EGF. MDA-MB-231 cells were cultured in L15 supplemented with 10% FBS and 100 units of penicillin–streptomycin. MCF-7 was routinely cultured in DMEM supplemented with 10% FBS and 100 units of penicillin–streptomycin. All of the cells were cultured at 37 °C with 5% CO_2 in a humidified incubator.

2.2. Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Scientific Inc. Meridian Rd., Rockford, USA) according to the manufacturer's instructions.

2.3. Western blot analysis

Each sample (50 μg) was separated by SDS–PAGE. After samples were transferred to a polyvinylidene fluoride (PVDF) membrane, the membrane was blocked in 5% non-fat milk and incubated overnight at 4 °C with the mouse monoclonal antibodies against TRAF2 or TRAF4 (1:1000; BD Biosciences, Inc., San Jose, CA, USA), NF-κB p65 (1:1000; Beyotime Institute of Biotechnology, China), lamin B1(1:500; Santa Cruz Biotechnology, California, USA) and β-actin(1:1000; Santa Cruz Biotechnology).

Then membranes were incubated with the secondary antibodies for 2 h at room temperature with slightly shaking. The ECL Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection. The relative protein levels were calculated by comparison to the amount of β-actin or lamin B1 proteins

2.4. Immunofluorescent staining

Cells grown on glass coverslips were fixed with ice-cold 4% paraformaldehyde for 15 min at −20 °C, followed by permeabilization with 0.2% Triton X-100. The cells were incubated with anti-TRAF4 (1:100; BD Biosciences, Inc., San Jose, CA, USA) antibodies at 4 °C overnight, followed by incubation with secondary antibody conjugated to a rhodamine label. The nuclei were counterstained with 4′ 6-diamidino-2-phenylindole. Observations were carried out with a confocal microscope (Leica SP1 and Leica SP2 UV, Leica Microsystem).

2.5. Plasmids and transfection

The hTRAF2pLPCX-HA-Flag/P874 plasmid (Addgene plasmid 20229) was purchased from Addgene (Cambridge, USA). The TRAF2

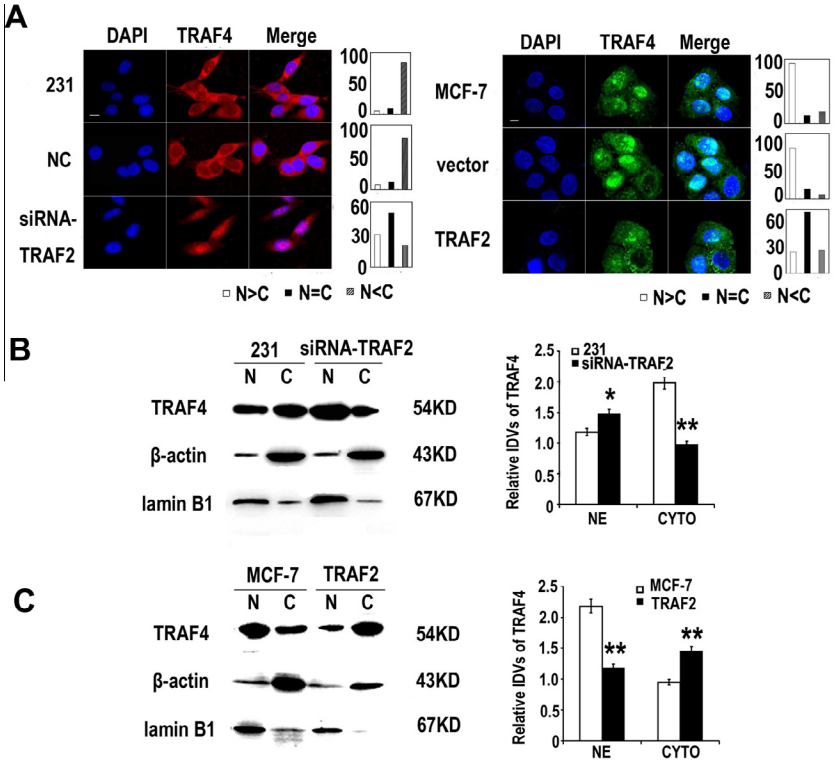

Fig. 3. TRAF2 could regulate the subcellular localization of TRAF4 in breast cancer cells. (A) MCF-7 cells were transfected with hTRAF2pLPCX-HA-Flag/P874 and MDA-MB-231 cells were transfected with siRNA-TRAF2. Their subcellular localization was analyzed by fluorescence microscopy. Left-sided panels show representative images. Right-sided panels show histograms that illustrate the percentage of cells where N exhibits the nuclear expression of TRAF4 and C exhibits the cytoplasmic expression of TRAF4. Approximately 100 cells were scored for each transfection experiment. Data represents the mean values of three independent experiments with standard deviations. Scale bars: 10 μm. (B) Western blot analyses showed that TRAF2 depletion by siRNA in MDA-MB-231 cells led to reduced TRAF4 expression in the cytoplasm (**$p = 0.001$) and augmented TRAF4 expression in the nucleus (*$p = 0.011$). (C) Augmented cytoplasmic expression (*$p = 0.001$) and reduced nuclear expression of TRAF4 (**$p = 0.001$) were detected when MCF-7 cells expressed pLPCX-HA-Flag/P874 by western blotting.

siRNA sequence was purchased from Santa Cruz Biotechnology. Cells were transiently transfected with plasmids using the Attractene Transfection Reagent or HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturers' protocols. The empty plasmid was used as a negative control.

2.6. Immunoprecipitation

Cells were washed twice with 5 ml of PBS followed by incubation on ice with lysis buffer containing 0.5% NP-40, 50 mM Tris, 150 Mm NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM EDTA for 5 min. Cells were harvested from the plates and transferred to a 1.5 ml tube. The lysate was centrifuged at 16,000 g for 5 min at 4 °C and the supernatant was transferred to a new tube. Lysates were quantified by the BCA assay and equal amounts of total protein were used for immunoprecipitation with either the anti-TRAF2 or anti-TRAF4 mAb. The immunocomplexes were then subjected to SDS–PAGE.

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation was evaluated each day for 4 days after the MTT treatment. The absorbance, which is directly correlated with the number of viable cells in the culture, was measured at 550 nm using a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA). A blank with dimethyl sulfoxide (DMSO) alone was measured and the value was subtracted from all the absorbance for cell culture specimens.

2.8. Flow cytometry

Flow cytometry was performed using the Apoptosis Detection Kit (KeyGEN BioTECH, China) according to the manufacturer's protocol on a BD FACSCalibur™ Flow Cytometer. All experiments were performed in triplicate.

2.9. Statistical analysis

All values are expressed as mean ± standard deviation (SD). The *t*-test was used to analyze all results. All analyses were performed using the statistical software package, SPSS13.0 (SPSS Inc., Chicago, IL). For all tests, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. The expression of TRAF4 in breast cancer cells

In this study, we initially examined the expression of TRAF4 in the normal breast cell line, MCF-10A, in addition to the estrogen receptor positive and negative breast cancer cell lines, MCF-7 and MDA-MB-231, by Western blotting. TRAF4 was detected in both the cytoplasmic and nuclear extract. The expression of TRAF4 in the nuclear was higher in normal breast cells than in breast cancer cells ($p < 0.01$), higher in estrogen receptor positive breast cancer cells MCF-7 than in estrogen receptor negative breast cancer cells MDA-MB-231 ($p < 0.01$). But the expression of TRAF4 in the cytoplasm was lower in normal breast cells than in breast cancer cells ($p < 0.01$), lower in estrogen receptor positive breast cancer cells

MCF-7 than in estrogen receptor negative breast cancer cells MDA-MB-231 ($p < 0.01$) (Fig. 1).

3.2. TRAF2 interacts with TRAF4 in breast cancer cells

By cell immunofluorescent staining, we found that TRAF2 and TRAF4 were co-localized in the cytoplasm of MCF-7 cells (Fig. 2A). Using Western blotting, we determined that TRAF2 and TRAF4 were both expressed in the cytoplasm of MCF-10A cells, and both the breast cancer cell lines, MCF-7 and MDA-MB-231. However, no expression of TRAF2 could be detected in the nuclear extraction (Fig. 2B). The co-immunoprecipitation results showed that TRAF2 could interact with TRAF4 in MCF-10A, MCF-7 and MDA-MB-231 cells (Fig. 2C).

3.3. TRAF2 regulates the cytoplasmic/nuclear distribution and biological function of TRAF4 in breast cancer cells

Immunofluorescent staining analyses showed that TRAF2 depletion by siRNA in MDA-MB-231 cells led to augmented TRAF4 expression in the nucleus. Reduced nuclear expression of TRAF4 was detected when MCF-7 cells expressed pLPCX-HA-Flag/P874 (Fig. 3A).

Western blot analyses confirmed that TRAF2 depletion by siRNA in MDA-MB-231 cells led to reduced TRAF4 expression in the cytoplasm ($p = 0.001$) and augmented TRAF4 expression in the nucleus ($p = 0.011$) (Fig. 3B). Augmented cytoplasmic expression ($p = 0.001$) and reduced the nuclear expression of TRAF4 ($p = 0.001$) when MCF-7 cells expressed pLPCX-HA-Flag/P874 (Fig. 3C).

After TNF-α treatment, MCF-7 cells with TRAF2 depletion by siRNA had significantly reduced nuclear expression of NF-κB ($p = 0.008$). In addition the percentage of early apoptotic cells in TNF-α-treated MCF-7 cells was increased ($p = 0.001$). MCF-7 cells that expressed hTRAF2pLPCX-HA-Flag/P874 had enhanced rates of cell proliferation ($p < 0.05$). After TNF-α treatment, the nuclear expression of NF-κB was significantly increased ($p < 0.05$). The percentage of early apoptotic cells in MCF-7 cells was reduced ($p = 0.001$).

Compared to MCF-7 cells transfected with hTRAF2pLPCX-HA-Flag/P874 alone, MCF-7 cells that expressed hTRAF2pLPCX-HA-Flag/P874 and siRNA-TRAF4 had significantly reduced nuclear expression levels of NF-κB after TNF-α treatment ($p = 0.006$). The percentage of early apoptotic cells in these MCF-7 cells was also increased ($p = 0.002$) (Fig. 4).

4. Discussion

To date, the mechanism of TRAF4 cellular localization remains unclear. The expression of TRAF4 has been detected in the cell

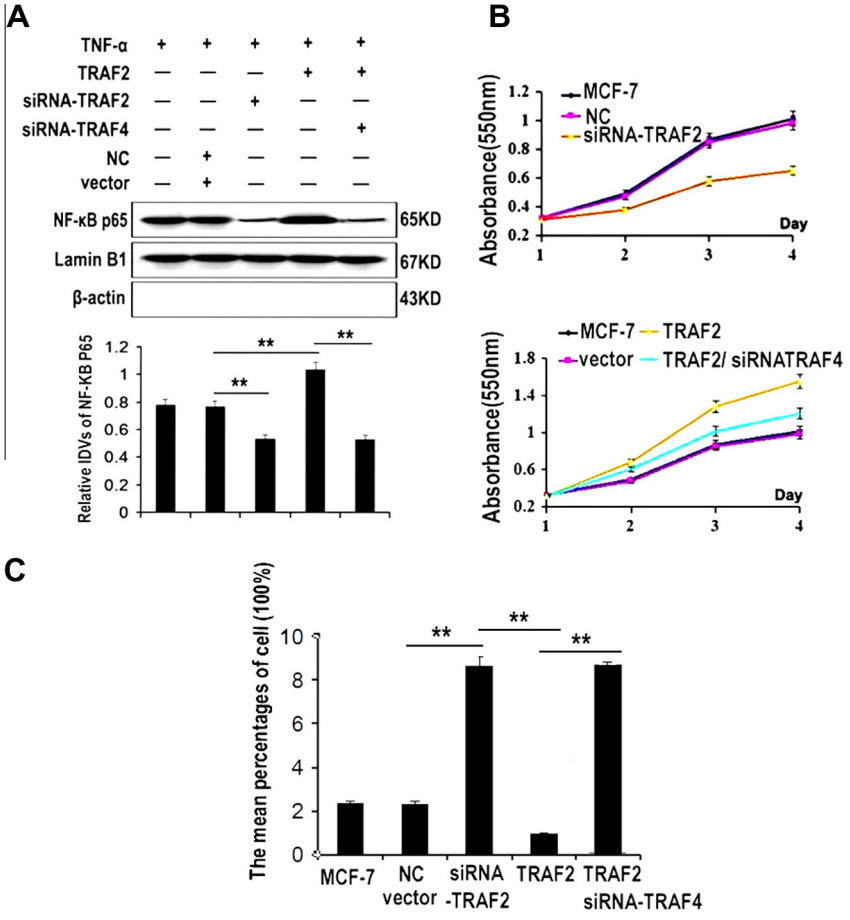

Fig. 4. The interaction of TRAF2 and TRAF4 regulates the activation of NF-κB induced by TNF-α in MCF-7 cells. MCF-7 cells were transfected with hTRAF2pLPCX-HA-Flag/P874 or siRNA-TRAF2. Control vector and negative control siRNA (NC) were used as controls. After TNF-α treatment, (A) the nuclear expression of NF-κB was detected by Western blotting ($^{*}p < 0.05$, $^{**}p < 0.01$). (B and C) MCF-7 cells expressing hTRAF2pLPCX-HA-Flag/P874 had enhanced rates of cell proliferation ($p < 0.05$) and reduced percentages of early apoptotic cells ($p = 0.001$). TRAF2 depletion by siRNA in MCF-7 cells could inhibit cell proliferation ($p < 0.05$) and the percentage of early apoptotic cells in MCF-7 cells was increased ($p = 0.001$). Compared to MCF-7 cells transfected with hTRAF2pLPCX-HA-Flag/P874 only, the percentage of early apoptotic cells was increased ($p = 0.002$), but the cell proliferation capacity was reduced when MCF-7 cells were co-transfected with hTRAF2pLPCX-HA-Flag/P874 and siRNA-TRAF4.

membrane, nucleus or cytoplasm in different cells. Some studies have concluded that TRAF4 is a dynamic tight junction-related shuttle protein involved in epithelium homeostasis [13], while Glauner, et al. thought that different structural domains could influence its cellular localization [14]. The members of the TRAF superfamily show high levels of homology. The C–TRAF domain mediates the formation of TRAFs into homo- or heterodimers or even polymers. The TRAF1, 2, 3 and 5 proteins contain R, Y and S residues that can interact with TIM motif. This motif regulates the participation of TNF-R in numerous signal transduction pathways. However these three amino acid residues have been replaced in the TRAF4 structure by S, F and F [15]. This may explain why TRAF4 rarely interacts directly with TNF-R. Some scholars have suggested that TRAF4 could indirectly participate in signal pathway regulation by TNF-R via interacting with other proteins [16].

TRAF2 can combine with various TNF-Rs and plays an important role in activating the NF-κB pathway. TNF-R1 signaling activates the transcription factor, NF-κB, and promotes cell survival. Micheau, et al. reported that TNF-R1-induced apoptosis involves two sequential signaling complexes. The initial plasma membrane bound complex (complex I) consists of TNF-R1, the adaptor TRADD, the kinase RIP1 and TRAF2, and the complex rapidly signals the activation of NF-κB [17].

In our previous studies, we showed that TRAF2 could interact with TRAF1 in breast cancer cells (data not shown). Takeshita et al. also showed that TRAF4 acts as a silencer in TLR-mediated signaling through its association with TRAF6 and TRIF [10,11]. Therefore, we hypothesized that TRAF2 could regulate the nuclear positioning of TRAF4 and may regulate the interaction between TRAF4 and TNF-R.

In this study, we firstly found that TRAF2 and TRAF4 protein colocalized in two breast cancer cell lines, MCF-7 and MDA-MB-231. TRAF2 and TRAF4 were shown to interact in normal breast cells, but this was significantly lower in breast cancer cells which may suggest that this interaction is related to the biological mechanism underlying breast carcinogenesis. To test our hypothesis, we bi-directional regulated the expression of TRAF2 in the breast cancer cell line, MCF-7 and MDA-MB-231 and detected the expression of TRAF4 in both the nucleus and cytoplasm. The results showed that lower expression level of TRAF2 significantly reduced the expression of TRAF4 in the cytoplasm, and increased its nuclear expression in MDA-MB-231 cells. Conversely, when TRAF2 was overexpressed in MCF-7 cells, the cytoplasmic expression of TRAF4 was significantly increased, but its nucleus expression was reduced. We hypothesized that the overexpression of TRAF2 in MCF-7 cells led to its increased combination with TRAF4 in the cytoplasm, which prevented TRAF4 travelling into the nucleus. And the low expression of TRAF2 in MDA-MB-231 cells led to its depressed combination with TRAF4 in the cytoplasm, which promoted TRAF4 travelling into the nucleus. This provides a reasonable explanation for the movement of TRAF4 between different subcellular structures.

Our results also showed that TRAF2 could regulate the activation of NF-κB through regulating the shuttling of TRAF4 into the nucleus. When TRAF2 was overexpressed, the cytoplasmic expression of TRAF4 was enhanced and its nuclear expression was reduced. After cells were stimulated by TNF-α, NF-κB was activated. Interestingly, when hTRAF2pLPCX-HA-Flag/P874 and siRNA-TRAF4 were co-transfected into MCF-7 cells, the nuclear expression of NF-κB did not change. Our results may suggest that TRAF4 is important in the activation of NF-κB by TRAF2. The interaction of TRAF2 and TRAF4 may promote the formation of complex I (TRADD/RIP1/TRAF2) [17], which inhibits cell apoptosis. However, the complexities of this mechanism requires further research. Interestingly, our earlier studies showed that the nuclear expression of TRAF4 in breast carcinoma is suppressed, and TRAF4 levels correlated with the invasive ability of breast cancer [18]. As the nuclear expression of TRAF4 may have important significance in the progression and invasion of breast cancer, future studies should clarify the roles of TRAF4 in apoptosis and discern whether other proteins in addition to TRAF2 affect the cellular localization of TRAF4.

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