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Ubiquitin Ligase CHIP Induces TRAF2 Proteasomal Degradation and NF-κB Inactivation to Regulate Breast Cancer Cell Invasion

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ABSTRACT

Transcriptional factor nuclear factor-kappaB (NF-κB) plays a crucial role in human breast cancer cell invasion and metastasis. The carboxyl terminus of Hsc70-interacting protein (CHIP) is a U-box-type ubiquitin ligase that induces ubiquitination and proteasomal degradation of its substrate proteins. In this study, we investigated the role of CHIP in the NF-κB pathway in the invasion of MDA-MB-231 cells, a highly aggressive breast cancer cell line. We showed that overexpression of CHIP significantly inhibits the invasion of the MDA-MB-231 cells. The overexpression of CHIP suppressed expression of urokinase plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) in MDA-MB-231 cells. Moreover, CHIP strongly inhibited the nuclear localization and the transcriptional activity of NF-κB. The activation of the IkappaB kinase complex (IKK) was also blocked by CHIP overexpression. Importantly, CHIP overexpression resulted in a significant decrease in the level of TNF receptor-associated factor 2 (TRAF2), an upstream key player in the NF-κB pathway. However, the level of TRAF2 was restored after treatment with a proteasome inhibitor, MG-132. Moreover, CHIP overexpression promoted the ubiquitination of TRAF2. We also found cell invasion significantly decreased in cells transfected with TRAF2 small interfering RNA (siRNA). In contrast, when CHIP expression was suppressed by siRNA in poorly invasive MCF-7 cells, cell invasion significantly increased in conjunction with enhanced NF-κB activation and TRAF2 levels. Taken together, these results suggest that CHIP regulates NF-κB-mediated cell invasion via the down-regulation of TRAF2. J. Cell. Biochem. 112: 3612–3620, 2011.

KEY WORDS: CHIP; INVASION; NF-KAPPAB; TRAF2

Cellular invasion is one of the fundamental steps in the metastatic process of cancer [Pantel and Brakenhoff, 2004]. The transcriptional factor nuclear factor-kappaB (NF-κB) plays a crucial role in human breast cancer progression, and its constitutive activity is associated with cancer cell invasion and metastasis [Huber et al., 2004; Park et al., 2007]. This suggests that NF-κB inhibitors could offer a therapeutic intervention to counteract the invasion and metastatic progression of cancer. The NF-κB signaling pathway is strictly controlled by its negative regulators. The inhibitor of κB (IκB), which binds to NF-κB dimers and then sequesters them in the cytoplasm, is a well-known negative regulator of NF-κB activation. In addition, some protein phosphatases (PPs), such as PP1 [Li et al., 2008], PP2A [Li et al., 2006], and PP2Cβ [Prajapati et al., 2004], may play a negative role in the NF-κB pathway. NF-κB activity can also

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be negatively regulated by several de-ubiquitinating proteins such as A20 [Wertz et al., 2004] cylindromatosis protein (CYLD) [Kovalenko et al., 2003] and ubiquitin-specific protease 31 (USP31) [Tzimas et al., 2006], although their action mechanisms need to be elucidated.

Previous studies have shown that carboxyl terminus of Hsc70-interacting protein (CHIP) is a U-box-dependent E3 ubiquitin ligase, which functions both as a cochaperone for the quality control of other proteins and as an ubiquitin ligase for proteasomal degradation of target proteins [Ballinger et al., 1999]. Increasing evidence suggests that along with its role in targeting misfolded proteins, CHIP plays an important part in the regulation of pathophysiological processes. The overexpression of CHIP controls the protein level of target molecules in various cells [Jana et al., 2005; Kumar et al., 2007; Li et al., 2009; Xie et al., 2009]. CHIP has also been implicated in the regulation of hormone receptors, such as the estrogen receptor-alpha (ERα) and the glucocorticoid receptor (GR) [Fan et al., 2005; Wang and DeFranco, 2005]. However, the way in which CHIP controls the NF-κB pathway in the invasion of breast cancer cells remains unclear. In the present study, we investigated the functional importance and the action mechanisms of CHIP in the NF-κB pathway and in cellular invasion by breast cancer cell lines.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Anti-CHIP, anti-TRAF2, anti-NF-κB p65, anti-IκBα, anti-IKKα/β, anti-β-actin, anti-histone H1, anti-PAI-1, anti-MMP-9 antibody, and protein A/G-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p65 and anti-phospho-IKKα/β antibody were obtained from Cell Signaling (Beverly, MA). Anti-uPA and anti-ubiquitin antibody were purchased from Abcam (Cambridge, UK). NF-κB inhibitor (Bay 11-7085) was purchased from Tocris (Ellisville, MO). Horseradish peroxidase-conjugated secondary antibody and MG-132 were procured from (Pierce Rockford, IL) and Sigma–Aldrich (St. Louis, MO), respectively.

CELL CULTURE

Human breast cancer cell lines MDA-MB-231 and MCF-7 cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

PLASMID CONSTRUCTION

The human CHIP DNA fragment was generated by PCR with the human cDNA library used as a template. The sense primer was 5′-GACAAGAATTCATGAAGGGCAAGGAGGAG-3′ (the underlining indicates the *Eco*RI site) and the antisense primer was 5′-GCCAAGCTCGAGTCAGTAGTCCTCCACCCA-3′ (the underlining indicates the *Xho*I site). The PCR product was digested with *Eco*RI and *Xho*I and inserted into the pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA). Cells were plated at 5×10^5 cells/ml for 24 h prior to transfection. All transfections were performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol.

CELL INVASION ASSAY

The invasion assay was performed with a cell invasion assay kit (Millipore, Bedford, MA) as per the manufacturer's protocol After CHIP transfection for 24 h, cells were added to the upper portion of a prehydrated ECMatrix-coated chamber with serum-free media. The cells were incubated at 37°C for 16 h. The non-invasion cells from the upper side of the filter were scraped and removed using a moist cotton swab. The invaded cells in the lower side of the filter were stained with staining solution and washed with phosphate-buffered saline (PBS) (pH 7.6). The invaded cells were dissolved in 10% acetic acid and quantified using a spectrophotometer at 560 nm.

SDS–PAGE AND IMMUNOBLOT ANALYSIS

Cells were solubilized in a cell lysis buffer (Cell Signaling) and centrifuged at 12,000 rpm for 20 min at 4°C. The protein samples were separated by a SDS-polyacrylamide gel and then electro-transferred to polyvinylidene difluoride membranes. The membranes were washed with Tris-buffered saline (TBS) and blocked by incubation with 5% non-fat dry milk in TBS for 1 h. The membranes were incubated with specific primary antibodies for 1 h. After washing, they were incubated with horseradish peroxidase-conjugated secondary antibody and subjected to enhanced chemiluminescence detection.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA from cells was isolated using the RNA extraction reagent Trizol (Invitrogen). The cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) according to manufacturer's protocol. Real-time PCR was performed to determine the mRNA levels of urokinase plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) in cells using LightCycler FastStart DNA Master SYBR Green I mix (Roche Applied Science, Indianapolis, IN) with a LightCycler 480 System (Roche Applied Science), in accordance with the manufacturer's instructions. The primers used were as follows: MMP-9, 5′-TGACAGCGACAA-GAAGTG-3′ (sense) and 5′-CAGTGAAGCGGTACATAGG-3′ (anti-sense); uPA, 5′-ATTTGTGAGGCCCATGGTTG-3′ (sense) and 5′-AAACCGCTGCTCCCACATT-3′ (antisense). GAPDH gene was used as an internal control using primers, 5′-GGAGTCAACGGATTTGG-TCGTA-3′ (sense) and 5′-GCAACAATATCCACTTTACCAGAGT-3′ (antisense). Relative quantification of each gene was calculated with the LightCycler 480 SW software (Roche Applied Science).

PREPARATION OF NUCLEAR FRACTION

Cells were washed, scraped with PBS, and centrifuged at 3,000 rpm at 4°C. The pellets were suspended in 10 mM Tris (pH 8.0) with 1.5 mM $MgCl_2$, 1 mM DTT, and 0.1% NP-40, and incubated on ice for 15 min. Nuclei were separated from cytosol by centrifugation at 12,000 rpm at 4°C for 15 min. The cytosolic supernatants were removed, and the precipitated pellets were suspended in 10 mM Tris (pH 8.0) containing 100 mM NaCl and stored on ice for 30 min. After

agitation for 30 min at 4°C, the lysate was centrifuged at 12,000 rpm at 4°C, and the supernatant was collected.

IMMUNOCYTOCHEMISTRY

Cells were plated in the 4-well slide chamber (Nalge Nunc, Rochester, NY) for 24 h. Cells were fixed with 4% paraformaldehyde, and then after the washing with PBS containing 0.2% NP-40, cells were blocked with 10% goat serum-containing PBS and incubated with anti-NF-κB p65. Cells were incubated with FITC-conjugated secondary antibody. Photographs of cells were taken using immunofluorescence microscopy (Olympus, Melville, NY).

NF-κB LUCIFERASE ACTIVITY ASSAY

Cells were transfected with a NF-κB/luciferase reporter vector (pNF-κB/LUC, BD Biosciences, Palo Alto, CA). The pcDNA3.1/LacZ was also co-transfected to cells as an internal control to determine the transfection efficiency. After 3 h, the cells were incubated in fresh media overnight. The luciferase activity of the cells was determined using a Luciferase Reporter Assay Kit (BD Biosciences). The activity of the NF-κB reporter luciferase was standardized to that of β-galactosidase activity.

IMMUNOPRECIPITATION

Cells were washed with PBS and lysed in a lysis buffer (Cell signaling). Cell lysates were incubated with antibody for 2 h at 4°C on a rotating plate. Precipitation was carried out with protein A/G agarose plus (Santa Cruz Biotechnology). Samples were separated by SDS–PAGE and subjected to immunoblot analysis.

IKK ACTIVITY ASSAY

Immunoprecipitated samples were incubated with 1 μg of GST-IκBα (Millipore), 1 μL of γ-[^{32}P]-ATP (>3,000 Ci/mmol) in reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 200 μM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml aprotinin, and 1 μg/ml leupeptin at 30°C for 40 min. The reaction mixture was then loaded onto 10% SDS–PAGE. The gel was vacuum-dried, and autoradiography was performed.

ZYMOGRAPHY

Cells were seeded onto 6-well plates in DMEM with 10% FBS and were cultured to 80% confluence. Cells were then transfected with a pcDNA-CHIP and maintained in the medium for 24 h. The culture media were collected and mixed with sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) and subjected to electrophoresis on a 10% SDS–PAGE gel containing 0.1% gelatin. The gel was washed in 10 mM Tris (pH 8.0) containing 2.5% Triton X-100, and then incubated in developing buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) at 37°C overnight. The gel was rinsed again in distilled water, stained with 0.5% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 2 h, and destained with 40% methanol and 10% acetic acid. Proteolytic activities appeared as clear bands and were quantified using a densitometer.

TRANSFECTION OF siRNA

TNF receptor-associated factor 2 (TRAF2) small interfering RNA (siRNA), CHIP siRNA, and scrambled control siRNA were obtained from Santa Cruz Biotechnology. Cells were seeded in 6-well plates in antibiotic-free medium for 24 h. Cells were then transfected with 10 nM of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 12 h, the medium was changed to fresh media with 10% FBS.

STATISTICAL ANALYSIS

Data are expressed as means ± SE. Statistical comparisons were performed by one-way analysis of variance and Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

CHIP OVEREXPRESSION INHIBITS INVASION OF MDA-MB-231 CELLS

To assess the involvement of CHIP in the metastatic motility of breast cancer cells, we compared basal CHIP expression in non-metastatic MCF-7 cells and highly invasive MDA-MB-231 cells. As shown in Figure 1a, CHIP expression in the MCF-7 cells was much higher than in the MDA-MB-231 cells. As the invasive capacity of the MDA-MB-231 cells was two times greater than that of MCF-7 cells (Fig. 1b), the difference in CHIP expression may be inversely correlated to this invasive capacity. To determine whether CHIP inhibited the invasion of MDA-MB-231 cells, we overexpressed CHIP in these cells and analyzed the resulting cellular invasion in an ECMatrix-coated chamber. Compared with the cells transfected with the pcDNA control vector, CHIP-overexpressing MDA-MB-231 cells (pcDNA-CHIP) showed significantly reduced invasiveness (Fig. 1b). After transfection with 1 μg of pcDNA-CHIP, cell invasion decreased by 45% in the MDA-MB-231 cells (Fig. 1c).

UROKINASE-TYPE PLASMINOGEN ACTIVATOR AND MMP-9 ARE REDUCED IN CHIP-OVEREXPRESSING MDA-MB-231 CELLS

Extracellular matrix degradation-associated proteins, such as MMPs and uPA are responsible for breast cancer cell invasion [Rangaswami et al., 2004]. To investigate whether CHIP regulates the expression of uPA, the MDA-MB-231 cells were transfected with pcDNA-CHIP for 24 h, and the level of uPA was determined by immunoblot analysis. As shown in Figure 2a, the expression of uPA was significantly reduced in CHIP-overexpressing MDA-MB-231 cells, whereas the level of plasminogen activator inhibitor (PAI-1) was slightly increased. Furthermore, the activity of MMP-9 was remarkably decreased in the CHIP-transfected MDA-MB-231 cells (Fig. 2b). The gene expression levels of MMP-9 and uPA were also decreased when CHIP was overexpressed in MDA-MB-231 cells (Fig. 2c).

CHIP OVEREXPRESSION INHIBITS NF-κB PATHWAY IN MDA-MB-231 CELLS

As the expression of MMPs and uPA genes is regulated by the NF-κB transcription factor [Rangaswami et al., 2004], we sought to determine whether CHIP overexpression would affect the activity of NF-κB in the MDA-MB-231 cells. The level of the NF-κB p65

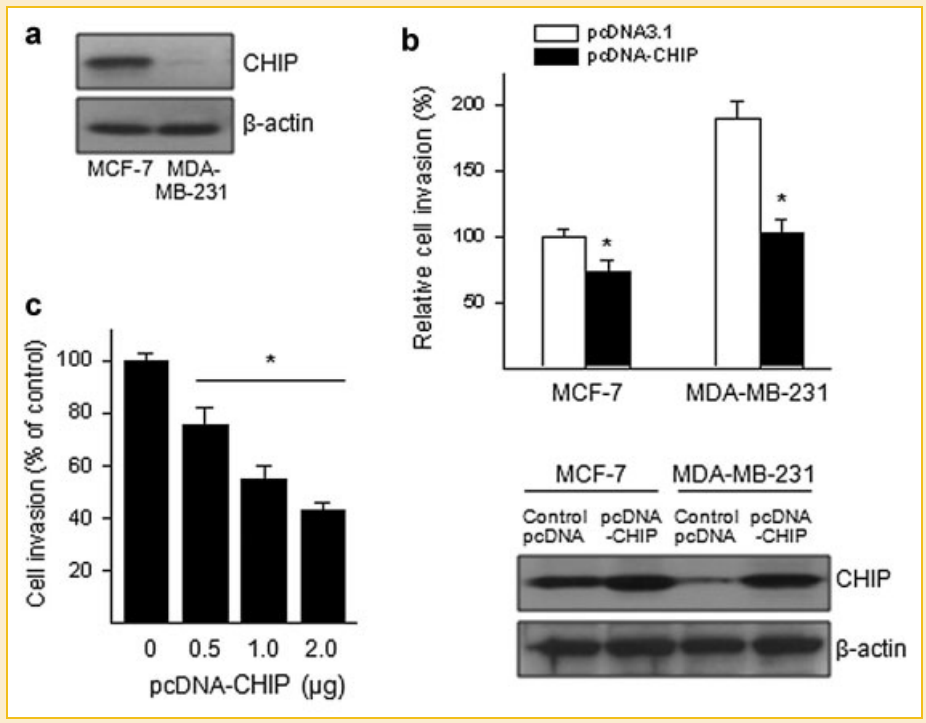

Fig. 1. Effect of CHIP overexpression on invasion of MDA-MB231 cells. a: Expression levels of CHIP in the MCF-7 cells and MDA-MB-231 cells. Equal amounts of protein were separated by SDS–PAGE gel, and immunoblot analysis was performed using anti-CHIP antibody. b: After transfection with 1 μg of pcDNA-CHIP for 24 h, cells were seeded to the upper portion of chamber and incubated for 16 h at 37°C. Invasive cells were then stained and quantified. The results present the means of three independent experiments. $^{*}P < 0.05$ compared to control (MCF-7 cells transfected with pcDNA3.1 vector). c: MDA-MB-231 cells transfected with the indicated concentrations of pcDNA-CHIP were added to the invasion assay chamber. After 16 h of incubation, invasive cells on the bottom of the invasion membrane were stained and quantified. The results present the means of three independent experiments. $^{*}P < 0.05$ compared to control.

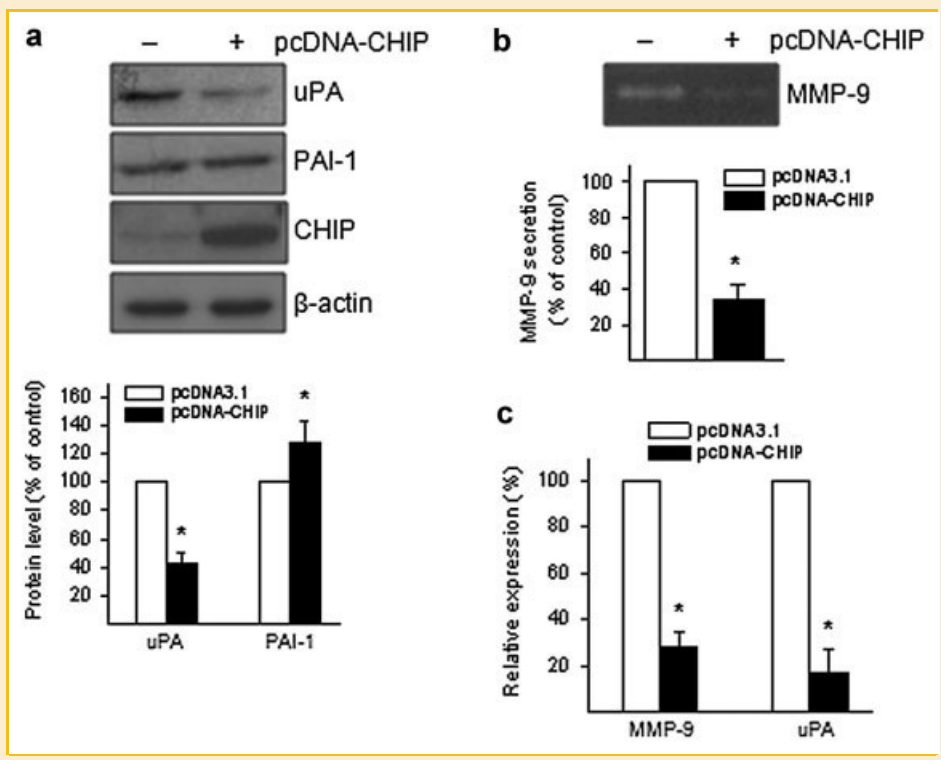

Fig. 2. Inhibition of uPA and MMP-9 in CHIP-overexpressing MDA-MB-231 cells. a: Cells were transfected with 1 μg of pcDNA-CHIP for 24 h. Equal amounts of protein were separated by SDS–PAGE gel, and immunoblot analysis was performed. b: After transfection with 1 μg of pcDNA-CHIP for 24 h, conditioned media were subjected to MMP zymography followed by densitometric analysis. The results present the means of three independent experiments. c: MMP-9 and uPA mRNA levels were analyzed by real-time PCR. GAPDH was used as an internal control. $^{*}P < 0.01$ compared to control.

subunit in nuclear and cytoplasmic fractions was analyzed by immunoblot assay. The nuclear translocation of p65 NF-κB was decreased in the MDA-MB-231 cells transfected with pcDNA-CHIP (Fig. 3a). An immunocytochemistry assay confirmed that pcDNA-CHIP transfection suppressed translocation of p65 NF-κB to the nucleus (Fig. 3b). The transcriptional activity of NF-κB was lower in cells transfected with pcDNA-CHIP in a concentration-dependent manner (Fig. 3c). In addition, the phosphorylation of IkappaB kinase (IKK)α/β, an upstream event associated with NF-κB activity, was also suppressed in CHIP-overexpressing cells (Fig. 3d). Moreover, as shown in Figure 3e, the activity of IKKα/β decreased in the MDA-MB-231 cells transfected with pcDNA-CHIP.

CHIP INTERACTS WITH TRAF2 AND INDUCES ITS UBIQUITINATION

TRAFs regulate the classical and alternative pathways of NF-κB activation, with TRAF2 and TRAF6 playing positive roles in the activation of the NF-κB pathway [Chen, 2005]. We found that CHIP overexpression resulted in a significant decrease in TRAF2 protein levels (Fig. 4a). No significant change in TRAF6 protein levels was observed (data not shown). However, treatment with the proteasome inhibitor MG-132 significantly restored TRAF2 protein levels (Fig. 4b). In addition, CHIP overexpression promoted the ubiquitination of TRAF2 (Fig. 4c). We also examined whether CHIP interacted with TRAF2. As shown in Figure 4d, CHIP was associated with endogenous TRAF2, and this interaction was enhanced by CHIP overexpression. Together, these findings strongly suggest that CHIP interacts with TRAF2 and promotes ubiquitin-proteasomal degradation of this protein in breast cancer cells. Figure 4e shows the effect of TRAF2 on the invasion of MDA-MB-231 cells using siRNA targeting TRAF2. Cell invasion was significantly decreased in the cells transfected with TRAF2 siRNA compared to the scrambled control siRNA transfected-cells. The invasion-related proteins, MMP-9 and uPA, were also significantly reduced by TRAF2 siRNA transfection (Fig. 4e).

CHIP KNOCK-DOWN INCREASES TRAF2 LEVELS AND PROMOTES NF-κB PATHWAY ACTIVATION AND CELLULAR INVASION IN MCF-7 CELLS

To further confirm the inhibitory function of CHIP in cell invasion, we used MCF-7 cells that downregulated CHIP expression by siRNA transfection. Transfection with CHIP siRNA markedly inhibited the expression of the CHIP protein compared to that of the control siRNA (Fig. 5a). The levels of MMP-9 and uPA were significantly increased in the cells transfected with CHIP siRNA compared to the

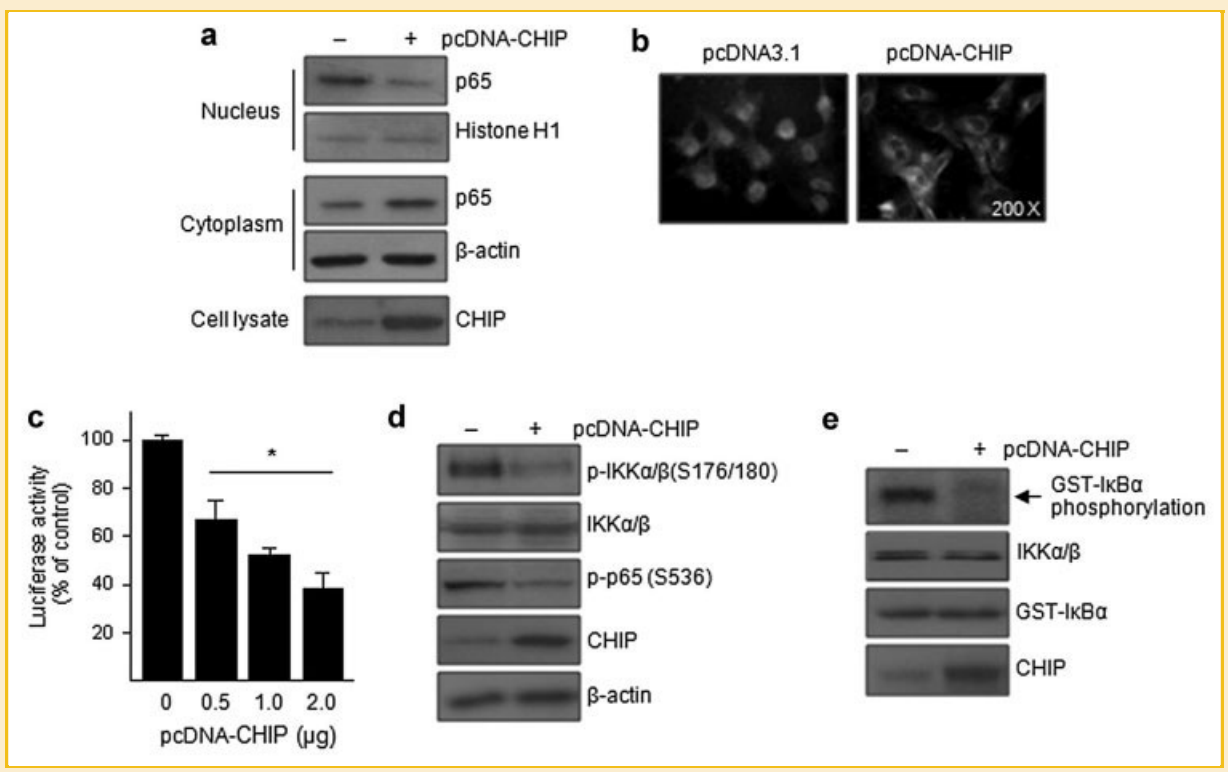

Fig. 3. Effect of CHIP overexpression on NF-κB activation in MDA-MB231 cells. a: Cells were transfected with 1 μg of pcDNA-CHIP for 24 h. Nuclear and cytoplasmic extracts were prepared and equal amounts of protein were separated by SDS–PAGE gel. The nuclear translocation of NF-κB was determined by immunoblot assay using anti-p65 NF-κB antibody. b: Cells were transfected with 1 μg of pcDNA-CHIP for 24 h. Immunocytochemistry assay was performed using anti-p65 NF-κB antibody. c: After transfection with the indicated concentrations of pcDNA-CHIP and 1 μg of pNF-κB/LUC luciferase reporter plasmid for 24 h, luciferase activity was determined using Luciferase Reporter Assay Kit. The results present the means of three independent experiments. $^{*}P < 0.05$ compared to control. d: Cells were transfected with 1 μg of pcDNA-CHIP for 24 h. Equal amounts of protein were separated by SDS–PAGE gel, and immunoblot analysis was performed using anti-phospho-IKKα/β or anti-phospho-p65 antibody. e: After transfection with 1 μg of pcDNA-CHIP for 24 h, cells lysates were immunoprecipitated with anti-IKKα/β antibody and analyzed for IKK activity using GST-IκBα and [γ-^{32}P] ATP.

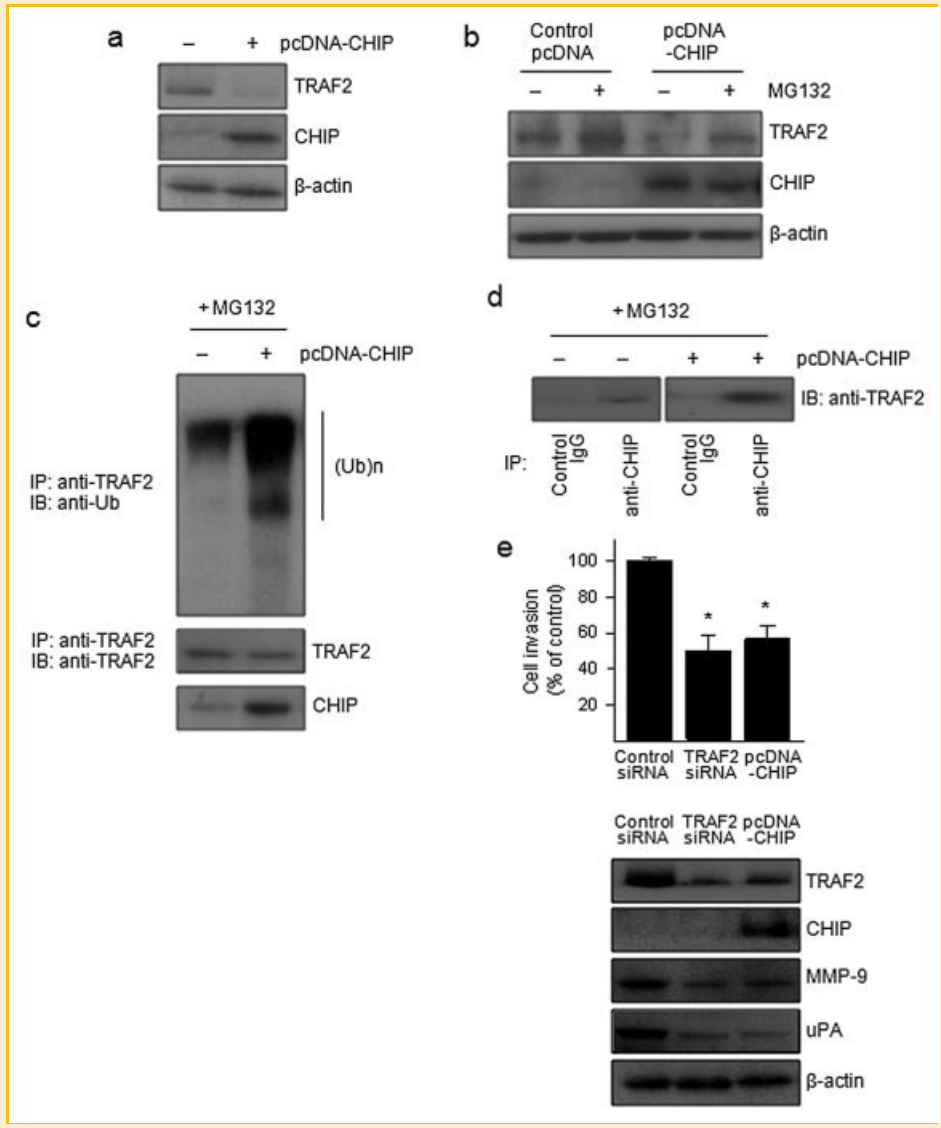

Fig. 4. Ubiquitination and proteasomal degradation of TRAF2 in CHIP-overexpressing MDA-MB-231 cells. a: Cells were transfected with 1 μg of pcDNA-CHIP for 24 h. Equal amounts of protein were separated by SDS–PAGE gel, and immunoblot analysis was performed using anti-TRAF2 antibody. b: Cells were pretreated with 10 μM of MG-132 for 6 h and then transfected with 1 μg of pcDNA-CHIP for 24 h. Immunoblot analysis was performed using anti-TRAF2 antibody. c: Cells were pretreated with 10 μM of MG-132 for 6 h and then transfected with 1 μg of pcDNA-CHIP for 24 h. Cells lysates were immunoprecipitated with anti-TRAF2 antibody, and immunoblot analyses were performed using anti-ubiquitin antibody. d: Cells pretreated with 10 μM of MG-132 were transfected with 1 μg of pcDNA-CHIP for 24 h. Cells lysates were immunoprecipitated with anti-CHIP antibody or control IgG, and immunoblot analyses were performed using anti-TRAF2 antibody. e: Cells transfected with TRAF2 siRNA for 48 h were added to the invasion assay chamber. After 16 h of incubation, invasive cells were then stained and quantified. The results present the means of three independent experiments. $^{*}P < 0.01$ compared to control (treatment with control siRNA). After 48 h of transfection with TRAF2 siRNA, the levels of TRAF2, MMP-9, and uPA were analyzed by immunoblot assay.

control siRNA transfected-cells (Fig. 5a). In an invasion assay, the knock-down of CHIP expression by siRNA significantly increased the invasiveness of MCF-7 cells (Fig. 5b). Interestingly, treatment of NF-κB inhibitor blocked the cell invasion increased by CHIP siRNA transfection, suggesting that the increased invasion by CHIP knock-down could be responsible for NF-κB activation. In addition, the nuclear translocation of p65 NF-κB was increased in cells transfected with the CHIP siRNA. The phosphorylation of IKKα/β was also increased in CHIP siRNA-treated cells (Fig. 5c). Furthermore, transfection with CHIP siRNA markedly increased the TRAF2 protein levels (Fig. 5c), suggesting that CHIP downregulation enhances TRAF2 stability in MCF-7 cells that show a relatively high basal expression of CHIP. The immunocytochemistry assay confirmed the increase of the nuclear translocation of NF-κB by CHIP knock-down (Fig. 5d).

DISCUSSION

Recent works have also revealed that CHIP is involved in the regulation of breast cancer cell growth through the ubiquitin-

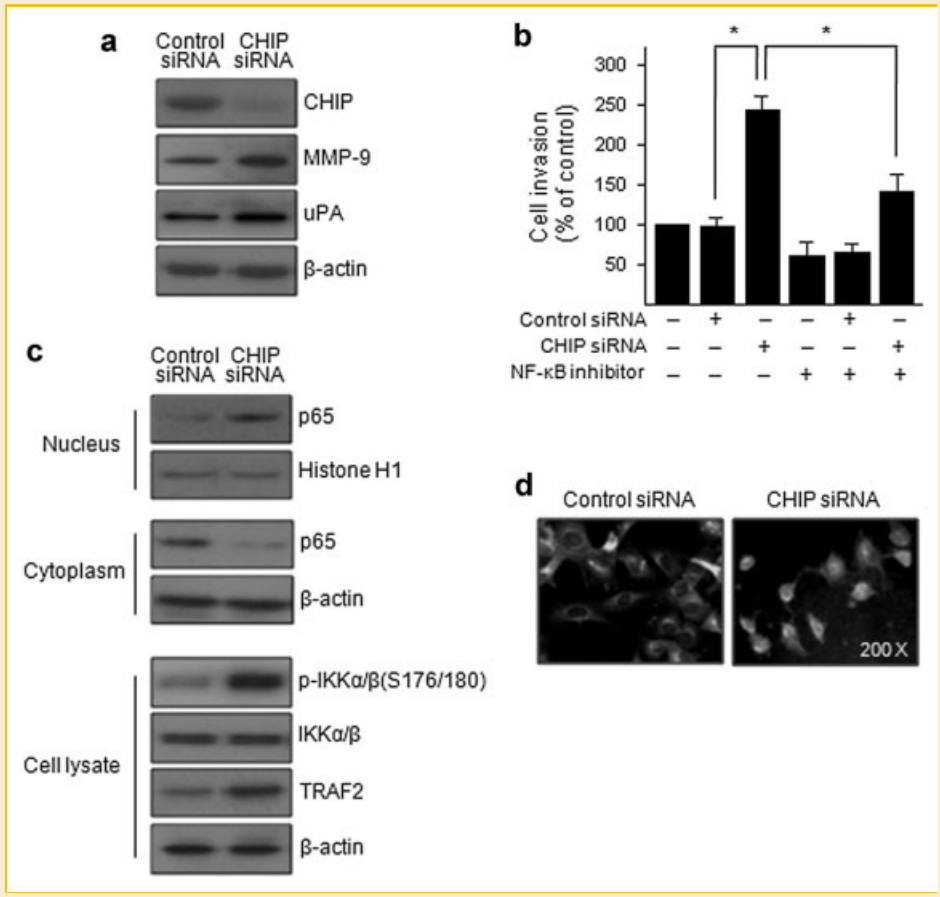

Fig. 5. Effects of CHIP knock-down on cellular invasion in MCF-7 cells. a: MCF-7 cells were transfected with CHIP siRNA for 48 h. The levels of CHIP, MMP-9 and uPA were analyzed by immunoblot assay. b: Cells were pretreated with a NF-κB inhibitor (Bay 11-7085, 10 μM), and transfected with CHIP siRNA for 48 h. Then, the cells were incubated in the invasion assay chamber for 16 h. Invasive cells on the bottom of the invasion membrane were stained and quantified. The results present the means of three independent experiments. $^{*}P < 0.01$ compared to control. c: After 48 h of transfection with siRNA, nuclear, and cytoplasmic fractions or cell lysate were analyzed by immunoblot assay. d: Immunocytochemistry assay was also performed using anti-p65 NF-κB antibody.

proteasome pathway [Fan et al., 2005; Yi et al., 2008; Kajiro et al., 2009]. CHIP stimulates ERα degradation through the ubiquitin-proteasome pathway in MCF-7 cells, an estrogen-responsive human breast cancer cell line [Fan et al., 2005; Yi et al., 2008]. It is still not clear, however, whether CHIP regulates the NF-κB pathway in tumor cell growth and metastasis. In the present study, we demonstrated that CHIP suppresses cellular invasion by blocking the TRAF2-NF-κB pathway in highly invasive human breast cancer cells.

Compared with CHIP expression in MCF-7 cells, the level of CHIP in the MDA-MB-231 cells was extremely low. Moreover, CHIP overexpression could more effectively suppress cell invasiveness in MDA-MB-231 cells than in MCF-7 cells (Fig. 1). Extracellular matrix degradation enzymes, which are highly expressed in aggressive breast cancers, were also decreased by CHIP overexpression in the MDA-MB-231 cells (Fig. 2). In contrast, the invasiveness of MCF-7 cells was significantly increased by CHIP knock-down (Fig. 5b). These results suggest that CHIP could serve as a therapeutic target in cancers with aggressive malignancies. Indeed, Kajiro et al. [2009] demonstrated that CHIP inhibits tumor growth and metastasis by degrading the target protein steroid receptor co-activator 3 (SRC-3). They also clarified that CHIP expression does not significantly correlate to ERα positivity in breast tumors. This evidence suggests that CHIP may regulate target proteins other than ERα in tumor progression. More importantly, we demonstrated that CHIP markedly inhibits the NF-κB activation pathway and regulates the level of TRAF in the MDA-MB-231 ERα-negative breast cancer cell line.

Numerous studies have reported that NF-κB regulates the expression of several genes involved in tumor cell invasion such as, uPA and MMP, thereby contributing to cancer aggressivity [Holst-Hansen et al., 1996; Balduyck et al., 2000; Sliva et al., 2002; Rangaswami et al., 2004]. Indeed, NF-κB is constitutively activated in the MDA-MB-231 cell line compared to the poorly invasive MCF-7 cells [Nakshatri et al., 1997]. However, thus far, the underlying regulatory mechanism of NF-κB signaling by CHIP has not been elucidated; a relationship between NF-κB signaling and CHIP has also yet to be demonstrated. In the present study, we found that CHIP overexpression reduced the nuclear translocation of NF-κB and its transcriptional activity in MDA-MB-231 cells. The activity of IKKα/β kinase was also inhibited by CHIP overexpression (Fig. 3). Moreover, CHIP knock-down by siRNA caused increased NF-κB activation in MCF-7 cells (Fig. 5c). These results suggest that CHIP

plays an important role in the upstream signaling pathways of NF-κB activation. We observed that CHIP does not interact with any upstream proteins of NF-κB, such as IKKα/β, Akt, or phosphatidylinositol-3-kinase (PI3K) (data not shown).

We showed that CHIP interacts with TRAF2 and mediates the degradation of this protein in MDA-MB-231 cells (Fig. 4). When CHIP expression was suppressed by siRNA in MCF-7 cells, the level of TRAF2 was also increased in conjunction with enhanced invasiveness (Fig. 5). However, cell invasion increased by CHIP knock-down was significantly reduced by the NF-κB inhibitor (Fig. 5b). These results suggest that CHIP is involved in the regulation of the TRAF2-NF-κB signaling pathway that promotes breast cancer cell invasion leading to metastasis. The TRAF protein family plays an important role in the classical and alternative pathways of NF-κB activation [Chen, 2005]. Activation of the NF-κB pathway requires TRAF2, which functions as an ubiquitin E3 ligase through the N-terminal RING domain [Li and Lin, 2008]. TRAF2 induces lysine-63 (K63)-linked ubiquitination of target proteins involved in the activation of the NF-κB pathway, such as receptor-interacting protein 1 (RIP1), NF-kB essential modulator (NEMO), and several signaling proteins [Li and Lin, 2008]. TRAF itself is also regulated by self-ubiquitination via the internal lysine residues, K48 and K63. Meanwhile, K48-linked poly-ubiquitin chains of TRAF2 lead to its degradation in a proteasome-dependent manner, whereas K63-linked auto-ubiquitination requires TRAF2 activation. The A20 and CYLD proteins have been shown to remove K63-linked ubiquitin from TRAF2, thereby negatively regulating TRAF2 and the NF-κB pathway [Kovalenko et al., 2003; Wertz et al., 2004]. It is also important to note that E3 ubiquitin ligases, such as the cellular inhibitor of apoptosis 1 (c-IAP1) [Wu et al., 2005] and seven in absentia homolog 2 (SIAH2) [Habelhah et al., 2002], regulate TRAF2 levels via K48-ubiquitination, leading to proteasomal degradation of TRAF2. Therefore, the role of CHIP in the regulation of cellular invasion and the inhibition of NF-κB signaling may be attributable to the induction of K48-linked ubiquitination of TRAF2. We also observed the significant effect of TRAF2 on cellular invasion in MDA-MB-231 cells (Fig. 4e). In summary, our work provides evidence that TRAF2 is a substrate for CHIP, an ubiquitin E3 ligase, and that, consequently, CHIP is capable of regulating breast cancer cell invasion via the inhibition of the NF-κB pathway.

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