INTRODUCTION

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor-suppressing lipid phosphatase that is frequently absent in breast tumors. Thus, the stability of PTEN is essential for tumor prevention and therapy. The ubiquitin-proteasome pathway has an important role in regulating the functions of PTEN. Specifically, carboxyl terminus Hsp70-interacting protein (CHIP), the E3 ubiquitin ligase of PTEN, can regulate PTEN levels. In this study, we report that BCL-2-associated athanogene 5 (BAG5), a known inhibitor of CHIP activity, reduces the degradation of PTEN and maintains its levels via an ubiquitylation-dependent pathway. BAG5 is identified as an antagonist of cell tumorigenicity. [BMB Reports 2013; 46(10): 490-494]

RESULTS

BAG5 significantly upregulates PTEN

A previous study (18) reported that BAG5 can inhibit the CHIP E3 ligase activity. In addition, CHIP was found to be an E3 ligase of PTEN. Thus, the level of endogenous PTEN in HEK293T cells was examined through the overexpression of Flag-BAG5 to explore the role of BAG5 in PTEN regulation. Western blot was conducted after 48 h. PTEN was found to be significantly upregulated by the overexpression of BAG5, whereas PTEN levels did not change in the HEK293T transfected with empty vectors. Therefore, the level of PTEN is influenced by the level of BAG5 in a dose-dependent manner (Fig. 1A).

The siRNAs that target BAG5 were used to knock down BAG5 in MCF-7 cells to further confirm the observed relationship between BAG5 and PTEN in the initial tests. After 48 h, PTEN level in the cells was examined using Western blot. After BAG5 was knocked down, the level of PTEN notably decreased, indicating that the decrease in BAG5 decreased the PTEN level (Fig. 1B). The positive correlation between the two gene products suggests that BAG5 positively regulates the PTEN level. We also transfected MCF-7 cells with other members of the BAG family; however, none of them stabilized PTEN protein, except for BAG5 (Fig. 1C). This result suggested that BAG5 specifically stabilizes PTEN.

BAG5 stabilizes PTEN by reducing its ubiquitination

The effect of BAG5 on the ubiquitination of PTEN in cells re-

Keywords: BAG5, CHIP, PTEN, Ubiquitin, Ubiquitination

PTEN is involved in other tumor-suppressor mechanisms, such as the maintenance of chromosomal integrity (12). As a crucial tumor-suppressor gene, pten is regulated by multiple factors. Previous studies (13, 14) have shown that pten is regulated at both transcriptional and post-transcriptional levels. Ubiquitylation is one of the PTEN regulatory mechanisms that occur after transcription (15, 16). A recent study (17) showed that the carboxyl terminus of Hsc70 interacting protein (CHIP) is an E3 ligase that mediates the proteasomal degradation of PTEN. BCL-2-associated athanogene 5 (BAG5) interacts with CHIP to inhibit E3 ubiquitin ligase activity; this process is mediated by Hsc70 (18). Therefore, the possible regulation of PTEN stability by BAG5 was explored in this study. This study established that BAG5 maintains PTEN levels by reducing PTEN degradation through CHIP inhibition.
Fig. 1. BAG5 stabilized PTEN. (A) HEK293T cells were transfected with Flag empty vector or Flag-BAG5. After 48 h, the cell lysates were detected by Western blot using anti-PTEN and anti-Flag antibodies. (B) MCF-7 cells were transfected with control siRNA or siRNAs against BAG5. PTEN protein levels were assessed by immunoblotting using anti-PTEN antibody. (C) MCF-7 cells were transfected with Flag empty vector, Flag-BAG1, Flag-BAG2, Flag-BAG3, Flag-BAG4 or Flag-BAG5, respectively. The protein levels of PTEN were assessed by immunoblotting using anti-PTEN antibody.

Fig. 2. BAG5 stabilized PTEN by decreasing PTEN polyubiquitination. (A) Flag-BAG5, Myc-CHIP, and HA-Ub were expressed in MCF-7 cells. At 24 h post-transfection, the cells were treated with MG132 (10 μM) for 6 h, and the PTEN ubiquitylation levels were evaluated by PTEN immunoprecipitation using anti-PTEN antibody followed by anti-HA immunoblotting. (B) MCF-7 cells transiently expressing Myc-PTEN were transfected with Flag-BAG5. At 24 h post-transfection, the cells were treated with cyclohexamide (CHX), and then collected at the indicated times. PTEN protein levels were determined by anti-Myc immunoblotting.

BAG5 regulates PTEN ubiquitination by inhibiting CHIP E3 activity

The interaction of Myc-PTEN and Flag-BAG5 was tested in HEK293T cells to validate whether BAG5 can interact with PTEN. The cells were co-transfected with Flag-BAG5 (or an empty Flag vector) and Myc-PTEN alone. Immunoprecipitation with the anti-Flag antibody was performed on extracts prepared from the cultured cells. The physiological interaction between BAG5 and PTEN was evaluated using Western blot with the respective antibodies (Fig. 3A). The results showed that the absence of interaction between BAG5 and PTEN. However, the results validated that an interaction exists between BAG5 and CHIP when the HEK293T cells were transfected with Flag-BAG5 (or an empty Flag vector) and Myc-CHIP under the same immunoprecipitation method. The combined results proved that BAG5 does not directly regulate PTEN levels. Instead, BAG5 performs its regulatory function by interacting with CHIP. MCF-7 cells were transfected with Flag-BAG5 and Myc-CHIP to confirm this hypothesis. The PTEN protein level was tested. We found that BAG5 could maintain the stability of PTEN well when the CHIP protein level was low; however, PTEN was less significant when the level was much higher (Fig. 3C). BAG5 suppresses the tumorigenicity of MCF-7 cells

After finding that BAG5 regulates PTEN protein stability, we posited that BAG5 may function as a tumor suppressor gene.
Fig. 3. Interaction between BAG5 and PTEN was mediated by CHIP. (A) HEK293T cells were co-transfected with Myc-PTEN and Flag-BAG5, and the interaction between PTEN and BAG5 was determined by immunoprecipitation and immunoblotting with the indicated antibodies. (B) HEK293T cells were cotransfected with Myc-PTEN and Flag-CHIP, and the interaction between PTEN and CHIP was determined by immunoprecipitation and immunoblotting with anti-Flag and anti-Myc antibodies. (C) MCF-7 cells were transfected with Flag-BAG5 and different doses of Myc-CHIP. After 48 h, the PTEN protein level was tested by immunoblotting.

Thus, we examined the characteristics of BAG5-depleted MCF7 cells to test this possibility.

Interestingly, we observed significant differences in the characteristics of the BAG5-depleted MCF7 cells and the control MCF-7 cells. The reduction of BAG5 promoted the proliferation and invasion (Fig. 4A, B) of MCF7 cells. Moreover, we tested the resistance to stress-induced cell death of BAG5-depleted MCF7 cells and control MCF-7 cells (Fig. 4C). BAG5-depleted MCF7 cells exhibited increased resistance to apoptosis. PTEN overexpression can suppress these effects. Disruption of PTEN leads to extensive centromere breakage and chromosomal translocations. We showed that knockdown of BAG5 regulated the expression of Rad51 by stabilizing PTEN. When BAG5 was absent, PTEN declined, thereby resulting in Rad51 deficiency (Fig. 4D). The same effect was observed in HCT116 cells (data not shown). We inferred that BAG5 could reduce the incidence of spontaneous DNA double-strand breaks. Thus, we collected breast cancer tissues and corresponding normal breast tissues from seven breast cancer patients to prove our inference. The protein levels of BAG5 and PTEN, as well as the ubiquitination level of PTEN in all of these tissues, were tested. The results showed that the level of BAG5 in the cancer tissues of most of the patients was less than that in the normal tissues, which led to higher ubiquitina-
tion level and lower protein level of PTEN (Fig. 4E). These results suggested that BAG5 suppresses the tumorigenicity of MCF-7 cells by stabilizing PTEN protein.

**DISCUSSION**

The tumor suppressor PTEN has critical roles in cell growth, migration, and death (19-21). The deletion of the pten gene in multiple tissues promotes tumorigenesis. The degradation of the stable protein PTEN is controlled by various pathways, the most important of which is the ubiquitin-proteasome pathway. In this study, BAG5 was identified as a positive regulator of PTEN. BAG5 can downregulate the polyubiquitination of PTEN by inhibiting CHIP E3 ligase activity (18), thereby protecting PTEN from proteasomal degradation. This finding provides a new direction for the future determination of BAG5 functions in PTEN regulation. CHIP is a known E3 ligase for PTEN (17), whereas BAG5 is identified as an inhibitor of CHIP. However, the exact mechanism by which BAG5 inhibits CHIP activity remains unclear. Furthermore, other E3 ligases of PTEN, which include NEDD4-1 and WWP2 (15,16), have similar functions that involve CHIP. However, the mechanisms that control their activity are still unknown. A previous study (17) suggested that CHIP may potentiate tumorigenesis because it could downregulate PTEN function. The present study elucidated the capability of BAG5 to inhibit CHIP activity. Thus, BAG5 may function as a tumor suppressor that maintains PTEN levels. Similar modes of regulation may also exist for Beclin1, USP10, and USP13 (22). PTEN can be phosphorylated, which reportedly influences its function and stability (23-26). Thus, PTEN phosphorylation may modulate its ubiquitination by CHIP or BAG5. However, the regulation of PTEN by BAG5 is fundamentally different from that of p53 by USP10 (27). The regulation of PTEN by BAG5 introduces a novel mechanism by which stable PTEN can resist proteasomal degradation. Collectively, BAG5 may be a tumor suppressor by maintaining PTEN stability.

**MATERIALS AND METHODS**

**Cell culture and agents**

The human embryonic kidney 293T (HEK293T) and breast cancer MCF-7 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% inactivated fetal bovine serum. All the cells were cultured with penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37°C with 5% CO₂.

**Plasmids and antibodies**

The following plasmids were used for the experiments: Myc-PTEN, Flag-BAG1, Flag-BAG2, Flag-BAG3, Flag-BAG4, Flag-BAG5, Myc-BAG5, Myc-CHIP, and HA-Ubiquitin (HA-Ub). Antibodies against CHIP, BAG5, Rad51, and GAPDH were obtained from Santa Cruz Biotechnology. Antibodies against Flag and PTEN were obtained from Sigma-Aldrich and Abcam, respectively.

**RNA interference and transfection**

Control siRNA, siRNA against CHIP (si-Chip), PTEN (si-PTEN), and BAG5 (si-BAG5) were purchased from Santa Cruz Biotechnology. The si-CHIP targets the following sequence: 5’-AAT GAAATTCTGAGGAGTGAAGGGCAAGAGAGACG-3’ (18). DNA and siRNA transfections were performed with Lipofectamine 2000 (Invitrogen).

**Co-immunoprecipitation**

Protein immunoprecipitation from HEK293T and MCF-7 cell lysates was performed under nondenaturing conditions. The cultured cells were lysed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer composed of 50 mM HEPES (pH 7.2), 250 mM NaCl, 10% glycerol, 1% NP-40, 1.0 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, and Complete ULTRA Protease Inhibitor Cocktail Tablets (Roche). The cell lysates were incubated for 8 h at 4°C on agarose with conjugated anti-BAG5 or control IgG antibodies (Santa Cruz Biotechnology), and then washed according to the manufacturer’s protocol. The samples were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot.

**In vitro proliferation assay**

MCF-7 cells were assayed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) reduction, a measure of mitochondrial viability. Cells were harvested from exponential-phase cultures grown in a culture medium, counted, and then plated in 96-well flat-bottomed microtiter plates (200 μl cell suspensions, 5×10³ cells/ml). After 24 h, the cells were transfected with control siRNA, si-BAG5, and si-PTEN or co-transfected with si-BAG5 and Myc-PTEN. After 48 h, the cells were incubated with MTT substrate (20 mg/ml) for 4 h, the culture medium was removed, and dimethyl sulfoxide was added. The optical density was spectrophotometrically measured at 550 nm. Each experiment was repeated at least three times.

**Invasion assay**

Invasion assays were conducted using a cell transwell system (Corning) according to the manufacturer’s instructions. The assay was performed in an invasion chamber consisting of a 24-well tissue culture plate with 12 cell culture inserts. The MCF-7 cells transfected with control siRNA, si-BAG5 or si-PTEN and suspended in serum-free culture medium were added to the inserts, and each insert was placed in the lower chamber containing 10% FBS culture medium. After 72 h of incubation in a cell culture incubator, invasiveness was evaluated by staining the cells that migrated through the extracellular matrix layer and clung to the polycarbonate membrane at the bottom of the insert.

**Apoptosis assays**

Cells were transfected with Myc-BAG5, Myc-PTEN, Myc-CHIP or Myc empty vector. After 48 h, the transfected cells were treated
with cisplatin (10 μM, 24 h, Sigma). The apoptotic cells were then washed with PBS and stained with fluorescein isothiocyanate-Annexin V and propidium iodide (PI) according to the manufacturer’s protocol (Promega). Apoptotic cells (Annexin V positive, PI negative) were then determined by flow cytometry.

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