Deleted in Breast Cancer 1 (DBC1) is a dynamically regulated protein

J. E. KIM, S. SUNG

Department of Pharmacology, Kyung Hee University, School of Medicine, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea, e-mail: jekim@khu.ac.kr

Deleted in breast cancer 1 (DBC1, KIAA1967, p30 DBC) is a novel protein that has been suggested to be a critical regulator of tumorigenesis. DBC1 modulates the activities of various proteins, including SIRT1, ERα, RARα, and AR. The functions of DBC1 with target molecules have been investigated, but little is known of its molecular characteristics. We found that DBC1 is homo-oligomerized and exhibits a dynamic expression pattern during cell cycle. In addition, DBC1 expression is higher in lung cancer cells than in normal lung fibroblasts and the protein recognized by anti-DBC1 antibodies in normal cells differs from that in lung and breast cancer cells. Further examination of the oligomerization and the dynamic expression of DBC1 during the cell cycle will help us to understand the mechanisms of DBC1 function. Moreover, the elucidation about differential expression of DBC1 between normal cells and cancer cells will aid the development of DBC1 as a diagnostic marker for cancers.

Key words: DBC1, SIRT1, tumor suppressor, tumor promoter, cancer

Deleted in breast cancer 1 (DBC1, KIAA1967, p30 DBC) has been shown to regulate cell survival or death through different pathways. First, DBC1 acts as a negative regulator of SIRT1 (sirtuin 1) deacetylase, thereby attenuating SIRT1-dependent resistance to cellular stresses [1, 2]. DBC1 also stabilizes estrogen receptor α (ERα) protein via a direct interaction in an estrogen-independent manner, and thus, plays a role as a pro-survival factor in breast cancer cells [3]. In contrast, DBC1 augments tumor necrosis factor-α (TNF-α)-induced apoptotic signaling via targeting of the DBC1 C-terminal fragment into mitochondria following caspase activation [4]. In addition, several reports suggest that DBC1 modulates cell growth through transcriptional activation of the retinoic acid receptor α (RARα) in breast cancer cells, or the androgen receptor (AR) in prostate cancer cells [5, 6]. Although the precise role of DBC1 in the regulation of cancer cell growth is still unknown, these studies suggest possibility that DBC1 acts as either a tumor promoter or a tumor suppressor [7]. Furthermore, it has been shown that DBC1 inhibits the SUV39H1 methyltransferase that mediates methylation at histone H3 Lys9 and possibly regulates heterochromatin formation [8]. Overall, DBC1 is a multifaceted protein regulating diverse cellular functions.

In addition to the role of DBC1 in cell survival, the level of DBC1 expression is also still controversial. In an early study,

breast cancer specimens were found to contain homozygous deletions of the gene encoding DBC1 on chromosome 8p21. In contrast to this initial report, some microarrays have revealed that DBC1 mRNA is increased in breast cancer patients [9, 10]. Increased levels of DBC1 protein have also been detected in gastric carcinoma patients and are correlated with shorter survival times [11]. Expression of DBC1 mRNA is higher in lung adenocarcinomas than lung squamous cell carcinomas [12]. In contrast, Fu et al. reported that no significant increases in DBC1 were detected in prostate cancer (5). Meanwhile, other groups have suggested that genomic loss of *DBC1* is responsible for tumor progression and metastasis in colon cancer [13, 14].

As described above, the functions and expression of DBC1 are various. This diversity may be dependent on cancer type or cancer-inducing signals. The next important question is the mechanism by which DBC1 regulates cellular functions and by which DBC1 expression is regulated under specific circumstances. Most current studies have focused on determining how DBC1 functions through a specific modulator or target. However, studies of the molecular characteristics of DBC1 itself are limited. Here, we report that the DBC1 protein is oligomerized and cell cycle-regulated. Surprisingly, the expression pattern of DBC1 differs between normal cells and cancer cells. These aspects of DBC1 will provide

important clues regarding the regulatory mechanism of DBC1 functions.

Materials and methods

Cell culture and synchronization. The 293T, T24, MRC-5, A549, and Sum52PE cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% (vol/vol) CO_2. HMEC (human mammary epithelial cell) was maintained in Mammary Epithelial Growth Medium (MEGM, Lonza.) Synchronization of T24 cells at G0 was achieved by contact inhibition as described [15].

Antibodies. The anti-DBC1 A and B antibodies were raised by immunizing rabbits with human DBC1 peptides (amino acid 2~25 and 900~923) or with a GST-fused human DBC1 protein fragment (amino acids 600~923), respectively.
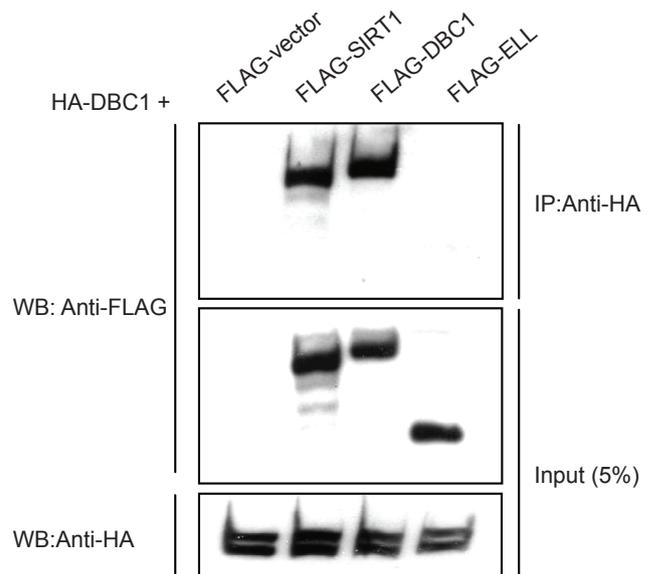
Immunoprecipitation and Western blotting. Cell lysates were incubated with anti-HA antibody (Sigma) and protein G sepharose beads (GE Healthcare Life Sciences) at 4°C for 1 hour. After washing the immunocomplex, beads were collected by centrifugation. The beads were eluted with Laemmli buffer at 95°C for 5 minutes. The eluted immunocomplexes were loaded onto 10% SDS-polyacrylamide gel and separated by electrophoresis. The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked with 10% (w/v) non-fat milk in TBST (Tris-buffered saline containing 0.2% (vol/vol) Tween20) for 1 hour and then incubated with an anti-FLAG antibody (Sigma) at 4°C with shaking overnight. The membrane was then incubated with HRP-conjugated rabbit anti-mouse antibody at room temperature for 1 hour. The immunocomplex was detected using the SuperSignal West Pico chemiluminescent substrates (Pierce).

Cell cycle analysis. The cells were fixed with 70% ethanol and stained with propidium iodide (PI). The percentage of cells in each phase of cell cycle was determined by fluorescence-activated cell sorting (FACS).

Results

DBC1 is oligomerized. The functions of some proteins depend on oligomerization. To examine whether DBC1 is oligomerized, 293T cells were co-transfected with DNA constructs encoding DBC1 with a HA and a FLAG tag. Immunoprecipitates using anti-HA antibody were blotted with anti-FLAG antibody (Fig. 1). FLAG-tagged SIRT1 and FLAG-tagged ELL (mRNA accession number U16282) were co-transfected with HA-DBC1 as positive and negative controls, respectively. HA-DBC1 did not bind to FLAG-ELL, but strongly bound to FLAG-SIRT1, a previously indentified binding partner of DBC1 [1, 2]. In addition, HA-DBC1 also interacted with FLAG-DBC1, indicating that DBC1 is oligomerized in vivo via an intermolecular interaction.

DBC1 expression is cell cycle-regulated. We examined whether the expression of DBC1 changes during the cell

Figure 1. DBC1 is oligomerized in vivo. 293T cells were transfected with HA-tagged DBC1 and FLAG-tagged SIRT1, DBC1, or ELL. Cell lysates were immunoprecipitated with anti-HA antibody and the immunocomplexes were subjected to western blotting with an anti-FLAG antibody.

cycle. T24 bladder carcinoma cells were arrested at G0 by contact-inhibition and then replated to a low density, which allows them to re-enter the cell cycle. Cells were harvested at different cell cycle phases and the distribution of cells in each phase of the cell cycle was determined by FACS analysis (Fig. 2A). In addition, expression of DBC1 during the cell cycle was examined by Western blotting. We observed that the pattern of DBC1 bands on Western blot changed during the cell cycle. In unsynchronized cells, both the A and B anti-DBC1 antibodies recognized an upper and a lower band. At G0 phase, the lower band of DBC1 is predominant. However, when cells were released to enter the cell cycle, the upper band of DBC1 became more predominant and the level of protein in this band finally peaked at the G2/M phase (Fig. 2B). In order to confirm that both bands represent DBC1, immunoprecipitation was performed using the anti-DBC1 B antibody and the immuno-complexes were blotted with the anti-DBC1 A antibody (Fig. 2C). The antigens for the A and B antibodies are described in the Materials and Methods. Overall, these results indicate that DBC1 is a cell cycle-regulated protein.

DBC1 expression differs between normal and cancer cells. The expression of many tumor suppressors or tumor promoters is dysregulated in cancer cells. To gain knowledge regarding the dysregulation of DBC1 expression, DBC1 protein levels in normal lung fibroblast MRC-5, normal breast HMEC (human mammary epithelial cell), lung carcinoma A549 and breast cancer Sum52PE cell lines were compared using an anti-DBC1 antibody. As shown in Fig. 3A and 3B, the expression of DBC1
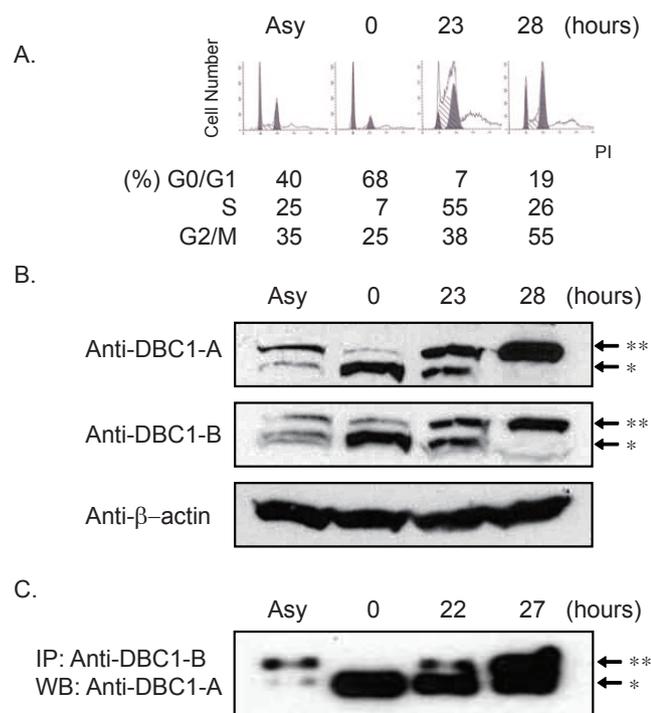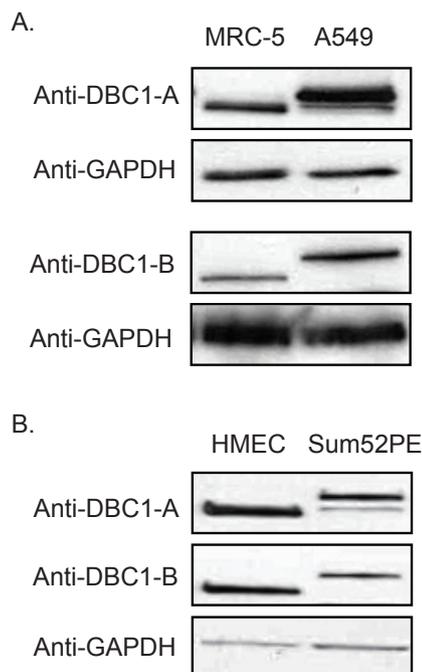
Figure 2. The expression of DBC1 protein is dynamic during the cell cycle. T24 cells were arrested at G0 and then released to re-enter the cell cycle. (A) The percentage of cells in each phase of the cell cycle was determined by FACS analysis. The two different anti-DBC1 antibodies were used for western blotting (B) and immunoprecipitation (C). **, upper band; *, lower band; Asy, Asynchronous

Figure 3. The DBC1 protein migrates more slowly in SDS-PAGE in cancer cells than in normal cells. The western blotting was performed by either anti-DBC1 A antibody or anti-DBC1 B antibody using either lung (A) or breast cell lines (B).

was higher in the A549 cancer cells than in the MRC-5 normal cells whereas their expression in normal HMEC and cancer Sum52PE is not significantly changed. Normal MRC-5 and MHEC cells possess the lower band of DBC1 whereas A549 and Sum52PE cancer cells possess the upper band (Fig. 3A, 3B). Taken together, the migration of DBC1 protein in normal cells is faster than that in cancer cells, suggesting that DBC1 expression in cancer is modified.

Discussion

DBC1 has been suggested to be a multifunctional protein that inhibits the activities of SIRT1 and Suv39H1, regulates the stability of ERα, and activates transcription through RARα and AR. While a number of functional targets of DBC1 have been elucidated, the molecular characteristics of DBC1 are still unknown. The present study demonstrates that DBC1 is homo-oligomerized and its expression changes during the cell cycle. In addition, the present study shows the overexpression of DBC1 in lung cancer cell lines and its expression pattern appears to differ between normal and cancer cells.

Our data show that DBC1 forms an intermolecular oligomer. However, whether DBC1 oligomerization occurs via direct interactions between DBC1 molecules or is mediated by a DBC1 binding partner, such as SIRT1, is unknown. In addition, the motif or domain responsible for DBC1 oligomerization needs to be determined. DBC1 contains an N-terminal S1-like RNA-binding domain, an N-terminal coiled-coil (leucine zipper) region, and a C-terminal EF-hand motif. DBC1 also possesses a Nudix hydrolase (MutT) domain, which is located to the C-terminal side of coiled-coil region. The MutT domain is likely to bind NAD metabolites such as ADP-ribose, thereby possibly regulating SIRT1 activity [16]. Lastly, whether oligomerization affects other functions of DBC1 remains to be determined.

We found that the expression pattern of DBC1 determined by anti-DBC1 antibodies is dynamic during the cell cycle. A lower band disappears upon release from quiescence (G0), and an upper band becomes the major form as cells reach G2/M. The differences between the upper and lower bands of DBC1 may result from post-translational modification of DBC1 during the cell cycle. Strikingly, the different migration of DBC1 also appears in normal versus cancer cell lines. Similar to the pattern detected during the cell cycle, the DBC1 antibodies recognize a lower band in normal cell lines and an upper band in cancer cell lines. The two different DBC1

bands in normal and cancer cell lines may be different iso-forms or could be differentially post-translational modified forms. Interestingly, our group have recently reported that the interaction between DBC1 and SIRT1 is dysregulated in most breast cancer cell lines [17]. Therefore, it is also interesting to determine which of the DBC1 bands is capable of interacting with SIRT1.

As described in the Introduction, the role of DBC1 in the growth of cancer cells is still controversial. Given that SIRT1, a negatively regulated target of DBC1, acts as either a tumor promoter or a tumor suppressor, DBC1 may function as either a tumor suppressor or a tumor promoter in a SIRT1-dependent manner in a cancer type where DBC1 interacts with SIRT1. The inducers and/or signals that determine the functional direction of DBC1 and SIRT1 in tumorigenesis also remain to be investigated. Furthermore, the identification of differential expression of DBC1 in normal and cancer cells may be useful in determining whether DBC1 expression could be used as a diagnostic marker for cancer.

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