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DBC2 Resistance is Achieved by Enhancing 26S Proteasome-mediated Protein Degradation

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Abstract

Tumor suppressor gene DBC2 stops growth of tumor cells through regulation of CCND1. Interference of CCND1 down-regulation prevented growth arrest caused by DBC2 [1]. It was also noted that DBC2 resistant cells eventually arose after repeated induction of DBC2 with muristerone A treatment [2]. In order to elucidate the mechanism of resistance acquisition, we analyzed DBC2 sensitive and resistant cells derived from the same progenitor cells (T-47D). We discovered that DBC2 protein was abundantly expressed in the sensitive cells when DBC2 was induced. In contrast, it was undetectable by western blot analysis in the resistant cells. We confirmed that the inducible gene expression system was responsive in both cells by detecting induced GFP. Additionally, inhibition of 26S proteasome by MG132 revealed production of DBC2 protein in the resistant cells. These findings indicate that the resistant T-47D cells survive DBC2 induction by rapid destruction of DBC2 through 26S proteasome-mediated protein degradation.

Keywords

DBC2; CCND1; 26S proteasome; protein degradation; T-47D; ecdysone; inducible gene expression system

Introduction

DBC2 was isolated from a chromosomal region (8p22) that is frequently deleted in breast cancer. Additionally, it was found to be inactive in a majority of breast cancers that retained the DBC2 locus. Reactivation of DBC2 in tumor cells resulted in growth arrest, indicating its tumor suppression capability [2]. Subsequent studies revealed that DBC2 negatively regulated CCND1 in a posttranscriptional manner and that the tumor cells continued to grow when CCND1 down-regulation was interfered [1]. These discoveries indicate that DBC2 suppresses growth of tumor cells through CCND1 regulation.

DBC2 belongs to the RHOBTB family, which consists of proteins that contain a distinct RHO domain and BTB domains. DBC2's RHO domain is different from canonical RHO proteins because it does not bind to GTP, GDP or ATP [3]. Another unique feature of DBC2 is that it lacks the carboxyl terminal lipid-binding sites that anchor the typical G proteins to membranes. Instead, it has two well-conserved BTB domains that are known to play a role in protein-protein interaction. Indeed, CUL3 was demonstrated to interact with DBC2 through the BTB domain

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[4]. DBC2 also contains a YXXXXLφ motif that exists in eIF4E binding proteins including HHEX and BP1 [5,6]. The C-terminus of DBC2 exhibits a weak homology to the RING finger domain but its significance is yet to be clarified. DBC2 mainly distributes in the cytoplasm, but its transport into the nucleus has not been confirmed.

A number of functional studies suggested that DBC2 was a multi-functional protein. Microarray analysis implied that DBC2 possibly participated in cell cycle control, apoptosis, membrane trafficking and cytoskeleton regulation [7]. DBC2's role in cell cycle control was confirmed by the discovery of its negative regulation of CCND1 [1]. DBC2's involvement in protein transport was verified by demonstrating its necessity in the ER-to-Golgi transport of viral protein, VSVG [3]. Association of DBC2 with CUL3 implied DBC2's role in ubiquitination [4]. However, regulation of DBC2 such as transcriptional regulation, translational control and protein degradation has not been studied.

Although DBC2 was demonstrated to possess tumor suppressor activity, nearly half of breast cancer cells express DBC2 [2]. Many tumor cell lines including 293 cells continue growing even when exogenous DBC2 is abundantly expressed [1]. So what defines DBC2 sensitivity? In order to answer this question, we conducted comparative analyses of DBC2 sensitive and resistant cells derived from the same T-47D progenitor cells.

Materials and Methods

Cell lines and cell culture

Cell culture media and reagents were purchased from Invitrogen. T-47D cells were obtained from American Tissue Type Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (ICN Biomedicals Inc., Costa Mesa, CA) supplemented with 15 % FBS and antibiotics (100 μg/ml hygromycin, 50 units/ml penicillin and 50 μg/ml streptomycin) at 37 °C in a 5 % CO_2 incubator. The cell lines with the inducible DBC2 gene were established by previously described methods [8]. Briefly, ecdysone receptors were transfected into the host cells using retroviral vectors. Green fluorescent protein (GFP) under ecdysone response elements was also transfected as a marker. The host cells were selected twice: first, GFP positive cells were winnowed out by fluorescent activated cell sorter (FACS). Then, GFP was induced in the remaining cells and GFP-positive cells were collected. DBC2 under the ecdysone response elements was transfected into the host cells. For induction, the host cells were cultured for 4 hours or longer in medium containing muristerone A at a final concentration of 2 – 5 μM. Proteasome inhibition was achieved by adding 50μM of MG132 (Sigma-Aldrich) 6 hours prior to harvesting the cells.

Reagents

All restriction enzymes were purchased from New England Biolabs. Muristerone A, formaldehyde and other chemicals were purchased from Sigma Aldrich unless otherwise stated.

Western blot analysis

Cell lysates were prepared with CelLytic MT (Sigma-Aldrich). Proteins were separated by SDS-PAGE and blotted to a Hybond P membrane (Amersham) using a Mini Protean III System (Bio-Rad). HRP-conjugated anti-flag M2 antibody was purchased from Sigma-Aldrich and used with a dilution of 1:2000. HRP-conjugated goat anti-β actin (I-19) antibody was purchased from Santa Cruz Biotech and used with a dilution of 1:7000. Anti-cyclin D1 antibody (Santa Cruz Biotech) was used with a dilution of 1:1500. HRP-conjugated goat-anti-rabbit antibody (Santa Cruz Biotech) was used with a dilution of 1:25000. Antibody detection was performed with ECL Plus Blotting Reagent (Amersham).

Microscopic analysis

The cells were plated on 22 mm cover glass (0.17 mm thick) 24 hours prior to fixing. The cells were fixed by incubating in 4% paraformaldehyde for 10 minutes at room temperature and permeabilized by incubating in 0.5 % Triton X-100 and 0.1 % SDS in PBS for 5 minutes at room temperature. The cells were observed with a TS100-F microscope (Nikon, Melville, NY) using a CFI Plan Apochromat (oil, 60x, 1.4 NA) objective. Excitation light was obtained from T1-FM Epi Fluorescence Module through a HYQ-FITC filter (Ex 470/40, Dm 495, Bar 525/50). Images were captured with an Insight-QE Color Digital camera and Act-2U Imaging Software.

Results and Discussion

Generating DBC2 resistant T-47D cells

T-47D cells do not express endogenous DBC2. DBC2 transcripts and proteins were below the detectable threshold level by RPA and western blot analysis, respectively [1,2]. When T-47D cells were cultured in medium containing muristerone A to induce DBC2, a significant number of cells detached and died. The surviving cells stopped growing [1,2]. After 24-hour induction, the surviving cells were cultured in DMEM without muristerone A for 48 hours. T-47D cells that experienced 3 cycles of this induction challenge became resistant to DBC2 induction. After culturing the resistant T-47D (R cells) in DMEM containing muristerone A, much fewer floating cells were observed (Table 1). A series of comparative analyses using the original cells (S cells) and R cells were conducted.

DBC2 protein was not detected in R cells

Western blot analysis of surviving and floating cells was performed (Figure 1). A substantial amount of DBC2 was observed in the floating cells while no detectable DBC2 was found in the surviving cells, indicating that T-47D cells were not permissive for DBC2 expression. This result led us to question how the surviving T-47D cells shut down DBC2 expression. There were 3 possible mechanisms: (1) The ecdysone-mediated inducible gene expression system was not functional in the surviving cells and DBC2 was not transcribed; (2) Translation of DBC2 mRNA was being inhibited in the R cells; (3) DBC2 proteins were degraded rapidly. Each possibility was tested by examining the responsiveness of the R cells to muristerone A (mechanism 1), verifying the production of DBC2 protein in the R cells (mechanism 2) and studying the consequences of protein degradation inhibition (mechanism 3).

The inducible gene expression system is functional

The T-47D host cells contained the inducible GFP gene as a marker. The responsiveness to muristerone A was assessed by checking GFP expression in S and R cells cultured in DMEM containing 5 μM of muristerone A. Control cells (S and R cells cultured in DMEM containing ethanol) did not glow. In contrast, both S and R cells exhibited bright GFP signals after induction, indicating that they retained ecdysone receptors (Figure 2). The proportion of the brightly glowing cells was approximately 60% in both S and R cells whereas this proportion was less than 0.1 % in control cells. It is unlikely that the cells had lost the DBC2 transgene containing a hygromycin phosphotransferase gene, since the cells had been cultured with 100 μg/ml hygromycin. As long as the host cells have both the ecdysone receptors and the DBC2 transgene, DBC2 is conceivably transcribed. Nevertheless, DBC2 expression in the R cells was verified next.

DBC2 protein is produced in R cells

In order to clarify whether translational control or protein degradation plays an important role in depleting DBC2 protein, the R cells were treated with MG132 to inhibit 26S proteasome

and then analyzed by western blot analysis. If DBC2 protein is produced upon induction but degraded quickly by the 26S proteasome in the R cells, MG132 treatment will enable detection of DBC2 protein by western blot analysis. If R cells decrease DBC2 protein levels by hindering translation or by promoting degradation using a system not mediated by 26S proteasome, then DBC2 protein will not be detected in MG132-treated R cells. Indeed, a considerable amount of DBC2 was observed in MG132-treated R cells after induction, indicating that DBC2 transcription and translation were activated by muristerone A. Since no DBC2 was detected in non-induced cells with MG132 treatment, the observed DBC2 was induced DBC2 not endogenous DBC2 (Figure 3). The R cells responded to the induction and produced DBC2 protein, but maintained DBC2 protein below the detectable level through expeditious DBC2 degradation. We concluded that the R cells accomplish DBC2 depletion by enhancing the 26S proteasome-mediated protein degradation system to survive.

Clarifying DBC2 function is important

DBC2 exhibits selective toxicity against breast cancer cells. DBC2 expression is observed in many normal tissues including lymphocytes, mammary glands and brain tissue, while a number of breast cancer cells are sensitive to DBC2 expression [2]. Elucidating the molecular mechanism of DBC2's tumor suppressor function is crucial for a better understanding of breast cancer development. Studies on DBC2 sensitivity or resistance would provide further insight into DBC2 function. We report here how a DBC2 sensitive cell can acquire resistance to DBC2 induction. However, there are tumor cells that keep growing with DBC2 proteins in the cell. We postulate that certain cancer cells are not dependent on CCND1, one of the major targets of DBC2. T-47D cells have CCND1 over expression and may be more sensitive to CCND1 down-regulation than 293 cells that do not have CCND1 amplification. One of the immediate future tasks is to isolate what makes cells permissive to DBC2 protein expression.

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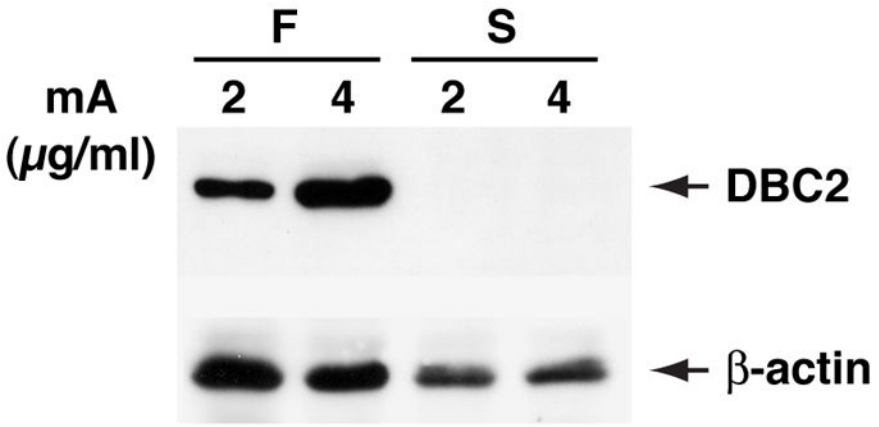

Figure 1.

Western blot analysis of T-47D cells. Floating and surviving cells were collected separately and lysed with CelLytic MT buffer. The letters “F” and “S” indicate floating and surviving cells, respectively. Muristerone A, denoted as mA in the figure, was administered at two different concentrations (2 and 4 μg/ml), which are indicated above the lanes. β-actin was used as a control. DBC2 protein was not detected in surviving cells. In contrast, DBC2 protein was observed in the floating cells and the amounts were proportional to the concentration of muristerone A.

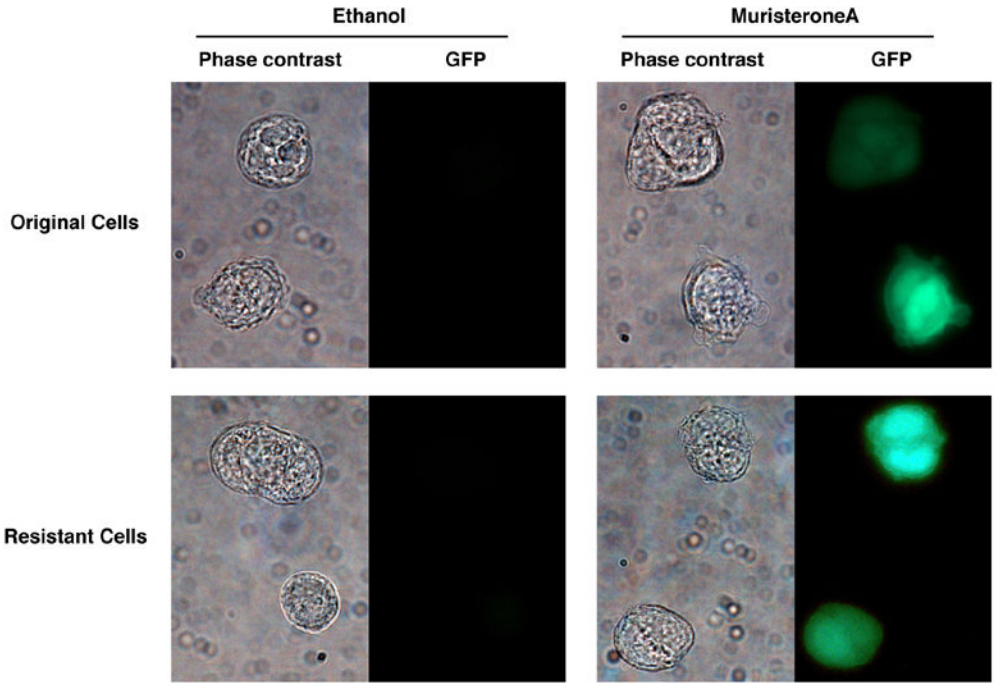

Figure 2.

GFP expression in T-47D cells. GFP signals and equivalent phase contrast images are shown side by side. Top panes are photos of S cells and bottom panes are those of R cells. Cells in the left panes were cultured in DMEM containing ethanol and those in the right panes, were cultured in DMEM containing muristerone A. The proportion of the glowing cells among R cells was indistinguishable from that among S cells.

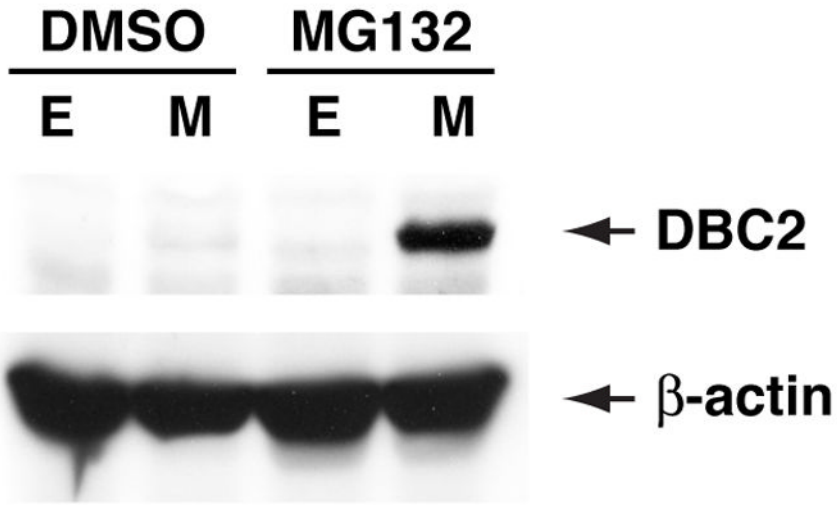

Figure 3.
Screening of R cells for DBC2 expression. Western blot analysis was performed on T-47D cells with 4 different treatments. E and M represent the cells cultured in DMEM with ethanol and those cultured in DMEM with muristerone A, respectively. MG132 and DMSO at the top indicate that MG132 and DMSO were added to the media before the harvest, respectively. A substantial amount of the DBC2 protein was observed in MG132-treated T-47D cells after induction.

Table 1

Ratios of dead and surviving cells observed after DBC2 induction. Approximately 2×10^5 cells were plated and, 24 hours later, muristerone A was administered. The number of floating cells and attaching cells were counted. The experiment was performed with sextuplicates. The ratios of the S and R cells were analyzed by Student's *t*-test and found to be significantly different ($p < 0.05$).

Measurements	S cells	R cells
1	39 %	5.9 %
2	20 %	5.9 %
3	14 %	0.0 %
4	14 %	0.0 %
5	11 %	0.0 %
6	4.0 %	0.0 %
Average	17 %	3.0 %
SD	12 %	2.0 %