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CREB represses p53-dependent transactivation of *MDM2* through the complex formation with p53 and contributes to p53-mediated apoptosis in response to glucose deprivation

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ABSTRACT

Recently, we have described that CREB (cAMP-responsive element-binding protein) has the ability to transactivate tumor suppressor *p53* gene in response to glucose deprivation. In this study, we have found that CREB forms a complex with p53 and represses p53-mediated transactivation of *MDM2* but not of $p21^{WAF1}$. Immunoprecipitation analysis revealed that CREB interacts with p53 in response to glucose deprivation. Forced expression of CREB significantly attenuated the up-regulation of the endogenous MDM2 in response to p53. By contrast, the mutant form of CREB lacking DNA-binding domain (CREBΔ) had an undetectable effect on the expression level of the endogenous MDM2. During the glucose deprivation-mediated apoptosis, there existed an inverse relationship between the expression levels of MDM2 and p53/CREB. Additionally, p53/CREB complex was dissociated from *MDM2* promoter in response to glucose deprivation. Collectively, our present results suggest that CREB preferentially down-regulates MDM2 and thereby contributing to p53-mediated apoptosis in response to glucose deprivation.

1. Introduction

Tumor suppressor p53 which acts as a nuclear sequence-specific transcription factor plays an important role in cell fate determination in response to various cellular stresses [1]. Upon cellular stresses, p53 is induced to be converted from the latent form to the active one and transactivates a set of p53-target genes implicated in the promotion of cell cycle arrest and apoptosis. Under physiological conditions, p53 is a short-lived protein and its expression is largely regulated at the protein level. For example, MDM2 which is one of p53-target gene products, targets p53 for ubiquitin/proteasome-dependent degradation [2–4]. Furthermore, it has been shown that MDM2 has the ability to recruit ubiquitinated p53 to proteasome to facilitate proteasomal degradation of p53 [5]. In addition, MDM2 binds to NH_2-terminal transactivation domain of p53 and inhibits its transcriptional as well as its pro-apoptotic activity [6]. Pro-apoptotic activity of p53 is tightly linked to its sequence-specific transactivation function [7]. Thus, p53 regulates its own activity and stability by a negative feedback loop in which p53 up-regulates its own negative inhibitor MDM2. This auto-regulatory feedback loop escapes cells from p53-dependent inappropriate apoptosis. In response to cellular stresses such as DNA damage, p53 is rapidly phosphorylated at multiple Ser and Thr residues. Among them, NH_2-terminal phosphorylation of p53 including Ser-15, Ser-20 and Ser-46, promotes the dissociation of MDM2 from p53 and thereby enhancing its stability as well as its activity [1,8,9].

Recently, we have found that *p53* is transcriptionally up-regulated during glucose deprivation-mediated apoptosis [10]. Further studies demonstrated that the promoter region of human *p53* gene contains a putative CREB (cAMP-responsive element-binding protein)-binding element and CREB is required for the transcriptional induction of *p53* in response to glucose deprivation [11]. In the present study, we sought to examine whether there could exist the functional interaction between p53 and CREB. Based on our present results, CREB was associated with p53 in cell nucleus and participated in the repression of p53-dependent transactivation of *MDM2* but not of $p21^{WAF1}$ in response to glucose deprivation. Therefore, CREB might contribute to glucose deprivation-mediated apoptosis through down-regulation of MDM2.

2. Materials and methods

2.1. Cell culture

Human osteosarcoma-derived U2OS cells bearing wild-type *p53* were cultivated in Dulbecco's modified Eagle's medium (Invitrogen)

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supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. Transfection

For transient transfection, cells were transfected with the indicated combinations of the expression plasmids using LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

2.3. FACS analysis

At the indicated time points after the glucose deprivation, floating and attached cells were collected, washed in ice-cold PBS and fixed in 70% ethanol at −20 °C. The cells were washed in ice-cold PBS and resuspended in phosphate-citrate buffer (4 mM citric acid and 200 mM Na_2HPO_4) and kept at room temperature for 15 min. Nuclear DNA was stained with propidium iodide (40 μg/ml) in the presence of RNase A (10 μg/ml) and the reaction mixture was incubated in the dark for 30 min. After the incubation with propidium iodide, DNA content of cells was examined by FACS can flow cytometer (Beckton Dickinson) using CellQuest software.

2.4. Rt-PCR

Total RNA was extracted from the indicated cells using the RNeasy Mini Kit (Qiagen), and the quality of the extracted RNA was confirmed by electrophoresis on 1.2% denaturing agarose gels. For RT-PCR analysis, total RNA (5 μg) was reverse transcribed using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The resultant first-strand cDNA was amplified by PCR-based strategy to monitor the expression levels of genes of interest. The list of primer sets used will be provided upon request. *GAPDH* was used as an internal control.

2.5. Immunoblotting

Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in SDS-sample buffer. Equal amounts of cell lysates (50 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis, electro-transferred onto Immobilon-P membrane filters (Millipore) and blocked with 0.5% non-fat milk in tris-buffered saline (TBS) containing 0.1% Tween 20 at room temperature. The membranes were incubated with monoclonal anti-p53 (DO-1: Oncogene Research Products), monoclonal anti-MDM2 (SMP14: Santa Cruz Biotechnology), polyclonal anti-CREB (48H2, Cell Signaling Technology), polyclonal anti-PARP (Cell Signaling Technology), polyclonal anti-$p21^{WAF1}$ (H164: Santa Cruz Biotechnology) or with anti-actin (20–33: Sigma) antibody at room temperature for 1 h followed by incubation with horseradish peroxidase-conjugated appropriate secondary antibodies (Cell Signaling Technology) at room temperature for 1 h. Immunoreactive bands were visualized by using ECL system (Amersham Biosciences) according to the manufacturer's instructions.

2.6. Immunoprecipitation

U2OS cells were lysed in the lysis buffer containing 25 mM Tris–HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 and a commercial protease inhibitor mixture (Sigma) for 30 min on ice, and subjected to a brief sonication for 10 s at 4 °C followed by centrifugation at 15,000 rpm at 4 °C for 10 min to remove insoluble materials. Equal amounts of cell lysates (1 mg) were precleared with 30 μl of protein G-Sepharose beads and used for immunoprecipitation with monoclonal anti-p53 antibody. After the addition of 30 μl of protein G-Sepharose beads, incubations were continued for additional 1 h at 4 °C. The beads were then collected by centrifugation and washed three times with the lysis buffer. The immunoprecipitates were analyzed by 10% SDS–polyacrylamide gel electrophoresis followed by immunoblotting with polyclonal anti-CREB antibody.

2.7. In vitro pull-down assay

A series of p53 deletion mutants were generated *in vitro* in the presence of [^{35}S] methionine using the quick-coupled *in vitro* transcription and translation system (TNT) according to the procedure suggested by the manufacturer (Promega). The quality of the synthesized proteins was verified by electrophoresis through 10% SDS–polyacrylamide gel and autoradiography. For the *in vitro* pull-down assay, radio-labeled p53 derivatives were incubated with cell lysates prepared from U2OS cells transfected with the expression plasmid for CREB and immunoprecipitated with anti-CREB antibody. The immunoprecipitates were analyzed by 10% SDS–polyacrylamide gel and autoradiography.

2.8. Luciferase reporter assay

U2OS cells were plated for transfection at a density of 5×10^4 cells/well in a 12-well tissue culture dish for 24 h. U2OS cells were then co-transfected with 100 ng of the indicated p53-responsive reporter plasmid (*MDM2* or $p21^{WAF1}$), 10 ng of pRL-TK *Renilla* luciferase cDNA and 25 ng of the expression plasmid for p53 together with or without the increasing amounts of the expression plasmid for CREB (50, 100 or 200 ng). The total amount of DNA was kept constant (510 ng) with pcDNA3 (Invitrogen) per transfection. Forty-eight hours after transfection, cells were lysed and luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. The transfection efficiency was standardized against *Renilla* luciferase.

3. Results

3.1. CREB forms a complex with p53

To examine whether CREB could form a complex with p53 in cells, we performed the immunoprecipitation analysis. To this end, human osteosarcoma-derived U2OS cells bearing wild-type *p53* were transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cell lysates were prepared and immunoprecipitated with anti-p53 antibody followed by immunoblotting with anti-CREB antibody. As shown in Fig. 1A, the exogenously expressed CREB was co-immunoprecipitated with p53. To further confirm this issue, U2OS cells were subjected to glucose deprivation. At the indicated time points after glucose deprivation, cell lysates were prepared and processed for immunoblotting. In accordance with our recent observations [11], the endogenous CREB as well as p53 was induced in response to glucose deprivation (Fig. 1B). Under these experimental conditions, we performed the immunoprecipitation experiments. As shown in Fig. 1B, the endogenous CREB was co-immunoprecipitated with the endogenous p53, suggesting that CREB interacts with p53 in cells. In addition, CREB co-localized with p53 in cell nucleus in response to glucose deprivation (Supplementary Fig. S1).

To map the region(s) of p53 required for complex formation with CREB, we carried out the *in vitro* pull-down assay. [^{35}S]-labeled p53 deletion mutants as well as wild-type p53 (Fig. 1C) were