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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

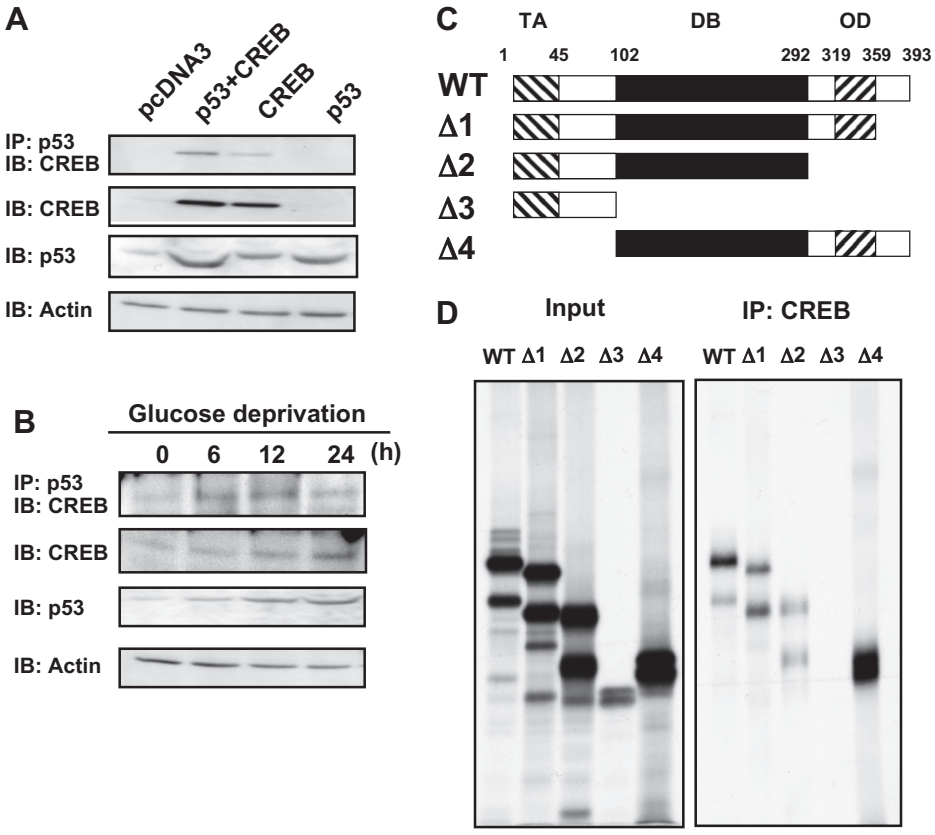

Fig. 1. Complex formation between CREB and p53. (A) Immunoprecipitation of the exogenously expressed CREB and p53. U2OS cells were transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cell lysates were prepared and immunoprecipitated with anti-p53 antibody followed by immunoblotting with anti-CREB antibody (upper panel). 1/20 of inputs are also shown (lower panels). (B) Endogenous interaction between CREB and p53. At the indicated time points after glucose deprivation, cell lysates were prepared and processed for immunoprecipitation with anti-p53 antibody followed by immunoblotting with anti-CREB antibody (upper panel). 1/20 of inputs are also shown (lower panels). (C) Schematic drawing of the structures of p53 and its deletion mutants. TA, transactivation domain; DB, DNA-binding domain; OD, oligomerization domain. (D) *In vitro* pull-down assay. ^{35}S-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 anti-CREB immunoprecipitates were analyzed by SDS–PAGE followed by autoradiography (right panel). 1/20 of inputs of the radio-labeled p53 derivatives are also shown (left panel).

incubated with cell lysates prepared from U2OS cells transfected with the expression plasmid for CREB and then immunoprecipitated with anti-CREB antibody. The anti-CREB immunoprecipitates were analyzed by SDS–PAGE followed by autoradiography. As shown in Fig. 1D, wild-type p53, Δ1, Δ2 and Δ4 were co-immunoprecipitated with CREB, whereas Δ3 was not. These results indicate that the central core DNA-binding domain of p53 is required for the interaction with CREB.

3.2. CREB attenuates p53-mediated transactivation of MDM2

To examine the possible effect of CREB on p53-mediated transcriptional activation, we performed luciferase reporter assay. U2OS cells were co-transfected with the constant amount of the expression plasmid for p53 and luciferase reporter construct carrying p53-responsive element derived from *MDM2* or $p21^{WAF1}$ promoter together with or without the increasing amounts of CREB expression plasmid. Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured. Intriguingly, CREB significantly inhibited p53-dependent transcriptional activation of *MDM2*, whereas CREB had a negligible effect on $p21^{WAF1}$ promoter in response to p53 (Fig. 2A and B). To confirm this issue, U2OS cells were transfected with the constant amount of the expression plasmid for p53 along with or without the increasing amounts of the expression plasmid encoding CREB. Forty-eight hours after transfection, total RNA and cell lysates were prepared and subjected to RT-PCR and immunoblotting, respectively. Consistent with the results obtained from luciferase reporter assay, forced expression of CREB reduced p53-mediated transcriptional activation of *MDM2* in a dose-dependent manner, whereas p53-mediated transcriptional activation of $p21^{WAF1}$ was unaffected in the presence of the exogenous CREB (Fig. 2C). As described in [11], CREB up-regulated the transcription of *p53*. Similar results were also obtained from immunoblotting analysis (Fig. 2D).

3.3. Mutant form of CREB lacking DNA-binding domain has an undetectable effect on p53-mediated transactivation of MDM2

According to our recent observations [11], mutant form of CREB lacking DNA-binding domain (CREBΔ) failed to transactivate *p53*. We then sought to examine the possible effect of CREBΔ on p53-mediated transcriptional activation of *MDM2*. For this purpose, we carried out luciferase reporter assay. As shown in Fig. 3A, luciferase reporter assay demonstrated that, in contrast to CREB, CREBΔ has an undetectable effect on p53-mediated transactivation of *MDM2* promoter. Like CREB, p53-mediated transactivation of $p21^{WAF1}$ was not affected by CREBΔ (Fig. 3B). In accordance with these results, forced expression of CREBΔ in U2OS cells displayed a marginal effect on p53-mediated up-regulation of MDM2 as examined by RT-PCR and immunoblotting (Fig. 3C and D). Next, we examined whether CREBΔ could interact with p53. To this

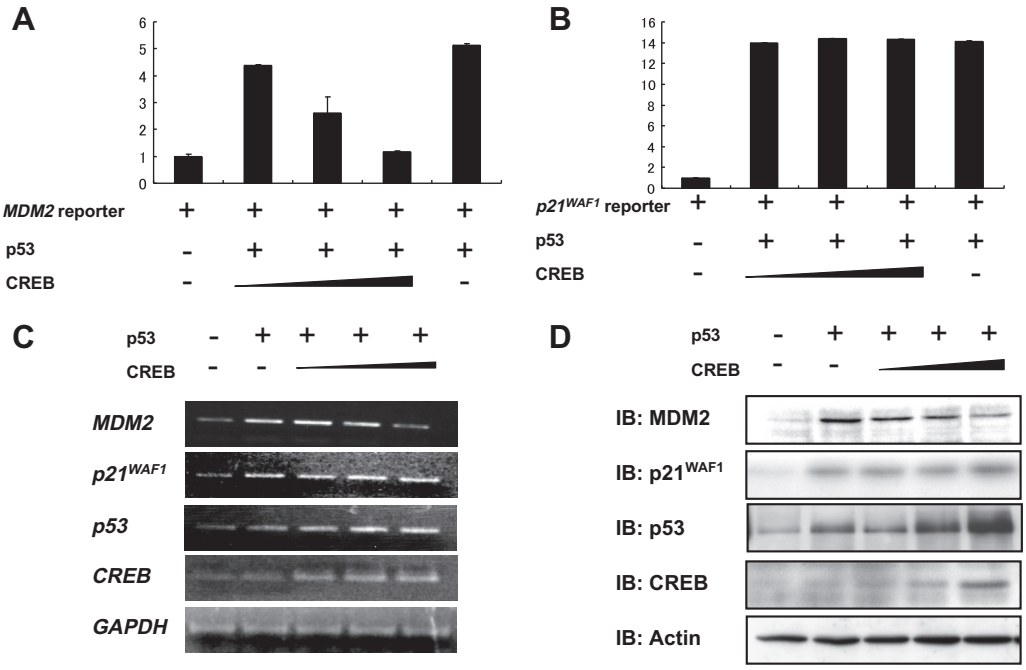

Fig. 2. CREB inhibits p53-dependent transactivation of MDM2. (A, B) Luciferase reporter assay. U2OS cells were transfected with the constant amount of the expression plasmid for p53, *Renilla* luciferase plasmid (pRL-TK), and luciferase reporter construct carrying human *MDM2* (A) or human $p21^{WAF1}$ (B) promoter together with or without the increasing amounts of the expression plasmid for CREB (50, 100, or 200 ng). Total amount of plasmid DNA was kept constant per transfection (510 ng) with pcDNA3. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured by Dual Luciferase Reporter System. Shown are averages of three replicates ±S.D. (C) RT-PCR. U2OS cells were transfected with the constant amount of the expression plasmid for p53 (0.5 μg) together with or without the increasing amounts of CREB expression plasmid (0.5, 1.0 or 1.5 μg). Forty-eight hours after transfection, total RNA was prepared and subjected to RT-PCR. *GAPDH* was used as an internal control. (D) Immunoblotting. U2OS cells were transfected as in (C). Forty-eight hours after transfection, cell lysates were extracted and processed for immunoblotting with the indicated antibodies. The expression level of actin was examined as a loading control.

end, U2OS cells 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. The anti-p53 immunoprecipitates were analyzed by immnoblotting with anti-CREB antibody. As clearly shown in Fig. 3E, CREBΔ was not co-immunoprecipitated with p53, suggesting that DNA-binding domain of CREB is required for the complex formation with p53 and the interaction between CREB and p53 plays an important role in the down-regulation of MDM2. Additionally, CREB but not CREBΔ had an ability to enhance pro-apoptotic activity of p53 (Supplementary Fig. S2).

3.4. Down-regulation of MDM2 in response to glucose deprivation

We then asked whether MDM2 could be reduced during apoptosis mediated by glucose deprivation. To this end, U2OS cells were cultured in the absence of glucose. At the indicated time periods after glucose deprivation, the attached and floating cells were collected and subjected to FACS analysis. As shown in Fig. 4A, the number of cells with sub-G1 DNA content increased in a time-dependent manner, suggesting that U2OS cells undergo apoptosis in response to glucose deprivation. Under these experimental conditions, total RNA and cell lysates were prepared and processed for RT-PCR and immunoblotting, respectively. Consistent with our recent observations [11], *p53* and *CREB* were transcriptionally induced in response to glucose deprivation (Fig. 4B). On the other hand, the expression level of *MDM2* was reduced in response to glucose deprivation in a time-dependent manner. Similarly, immunoblotting analysis showed that glucose deprivation results in an increase in the expression levels of p53 as well as CREB, whereas a remarkable reduction of MDM2 is detectable (Fig. 4C). In addition, the proteolytic cleavage of PARP was observed in a time-dependent manner, suggesting that U2OS cells undergo apoptosis in response to glucose deprivation.

To clarify the molecular mechanism(s) behind CREB/p53-mediated down-regulation of MDM2, we performed ChIP assay. At the indicated time points after glucose deprivation, U2OS cells were cross-linked with formaldehyde and immunoprecipitated with normal rabbit IgG or with polyclonal anti-CREB antibody. Genomic DNA was purified from the immunoprecipitates and subjected to PCR to amplify *MDM2* and $p21^{WAF1}$ promoters. As shown in Fig. 4D, CREB was dissociated from *MDM2* promoter but not from $p21^{WAF1}$ promoter in a time-dependent manner. Similarly, the recruitment of p53 onto *MDM2* promoter decreased, whereas the amount of p53 recruited onto $p21^{WAF1}$ promoter remained unchanged (Fig. 4E). These results strongly suggest that CREB preferentially induces the dissociation of p53 from *MDM2* promoter through the complex formation with p53 and contributes to the promotion of p53-dependent apoptosis in response to glucose deprivation.

4. Discussion

In the present study, we found for the first time that CREB has an ability to preferentially repress p53-dependent transactivation of *MDM2* through the complex formation with p53 in response to glucose deprivation. Our present findings might provide a novel insight into understanding the molecular mechanisms behind the stress-induced stabilization and activation of p53.

It has been well-established that oncogenic MDM2 displays a dominant-negative behavior toward p53 as well as its family members such as p73 and p63 [12]. MDM2 acts as an E3 ubiquitin protein ligase for p53 and thereby promoting its proteolytic degradation through ubiquitin/proteasome pathway [2–4]. Additionally,

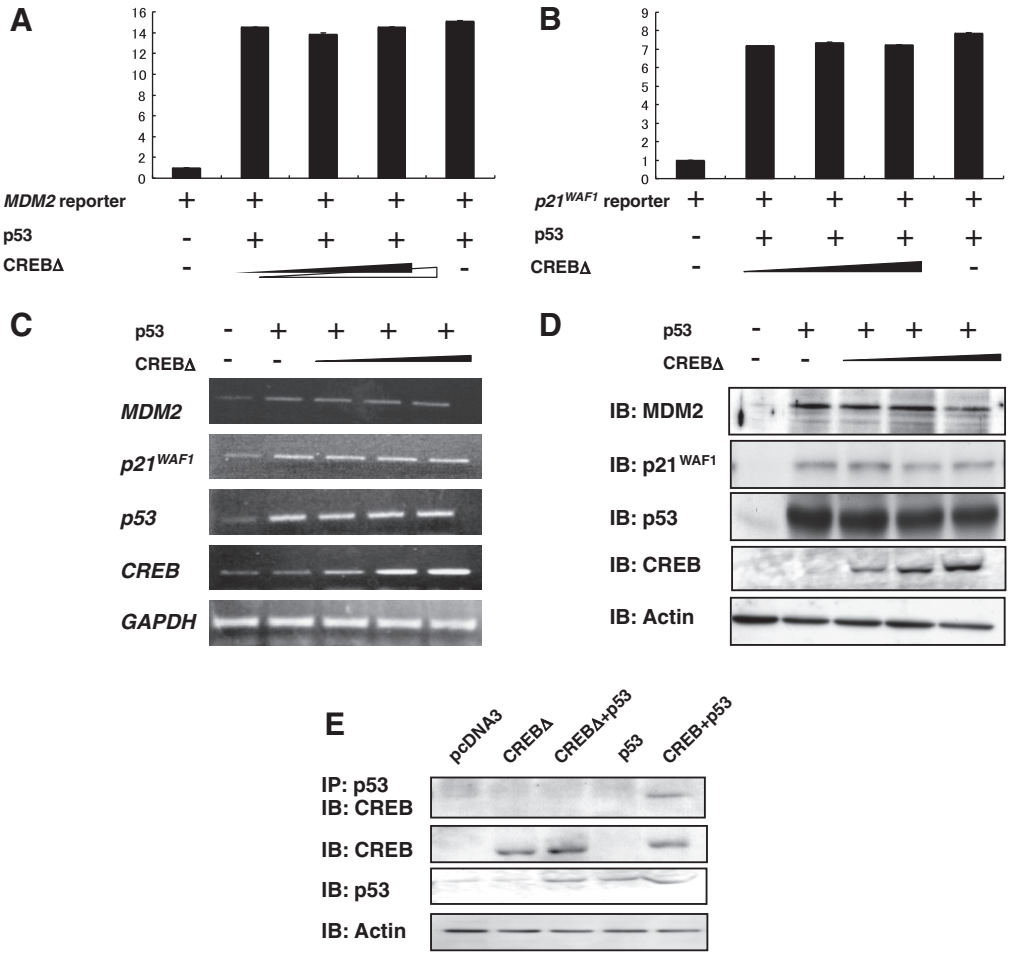

Fig. 3. CREBΔ does not interact with p53 and has a marginal effect on p53-mediated transactivation of *MDM2*. (A, B) Luciferase reporter assay. U2OS cells were transfected with the constant amount of pRL-TK, the expression plasmid for p53 and luciferase reporter construct bearing *MDM2* (A) or $p21^{WAF1}$ (B) promoter along with or without the increasing amounts of the expression plasmid encoding CREBΔ. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured. (C, D) Expression analysis. U2OS cells were transfected with the expression plasmid for p53 (0.5 μg) along with or without the increasing amounts of CREBΔ expression plasmid (0.5, 1.0 or 1.5 μg). Forty-eight hours after transfection, total RNA and cell lysates were prepared and subjected to RT-PCR (C) and immunoblotting (D), respectively. (E) Immunoprecipitation. U2OS cells were transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cells lysates were prepared and immunoprecipitated with anti-p53 antibody followed by immunoblotting with anti-CREB antibody. 1/20 of inputs are also shown.

MDM2 masks NH_2-terminal transactivation domain of p53 and blocks its transcriptional activity [6]. MDM2 had an undetectable effect of the protein stability of p73 and p63, whereas their transcriptional activity was strongly inhibited by MDM2 [12]. In response to cellular stresses, p53 is induced to be activated and thereby exerting its pro-apoptotic function to eliminate cells with seriously damaged DNA [1,8,9]. Although MDM2 is a dominant-negative inhibitor of p53, the activated form of p53 transactivates *MDM2* [13]. This p53-mediated induction of MDM2 might contribute to suppress the overactive p53 and attenuate the inappropriate apoptosis.

Upon serious cellular stresses, however, the efficient induction of apoptosis is required to maintain the genomic integrity. The dysregulation of an appropriate stress response leads to genomic instability and tumor formation. For this purpose, it is necessary to remove the negative effect of MDM2 on p53. Accumulating evidence strongly suggests that stress-induced phosphorylation at Ser-15, Ser-20 and Ser-46 of p53 disrupts the complex formation between p53 and MDM2 and thereby enhancing its stability as well as its pro-apoptotic activity [1,8,9]. Alternatively, several lines of evidence indicate that MDM2 is destabilized in response to DNA damage [14,15]. Watson et al. described that NEDD8 conjugation promotes MDM2 stabilization, and DNA damage-dependent MDM2 destabilization is mediated through the increased NEDP1-dependent deneddylation of MDM2 [16].

According to our present results, CREB formed a transcription complex with p53 and promoted the dissociation of p53 from *MDM2* promoter in response to glucose deprivation as examined by ChIP analysis. Thus, CREB-mediated down-regulation of *MDM2* at mRNA level contributes to glucose deprivation-dependent apoptosis through the activation of p53 pro-apoptotic pathway. In support with this notion, forced expression of CREB in U2OS cells enhanced p53-dependent apoptosis, whereas siRNA-mediated knocking down of the endogenous CREB resulted in the suppression of p53-dependent apoptosis in response to glucose deprivation [11]. Since CREB has an ability to transactivate *p53* [11], there might exist a positive feedback loop in which CREB regulates pro-apoptotic activity of p53. As described previously [17], ASPP1 and ASPP2 significantly enhance p53-mediated transactivation of pro-apoptotic target genes such as *BAX* and *PIG-3* (p53-inducible gene 3), whereas ASPP1 and ASPP2 enhance p53-dependent transactivation of p53-target genes implicated in cell cycle arrest to a lesser degree. Considering that CREB/p53 complex repressed the transcription of *MDM2*, the promoter selection by p53 could be dependent on the content of the transcriptional complex including p53. Further study should be required to address this issue.

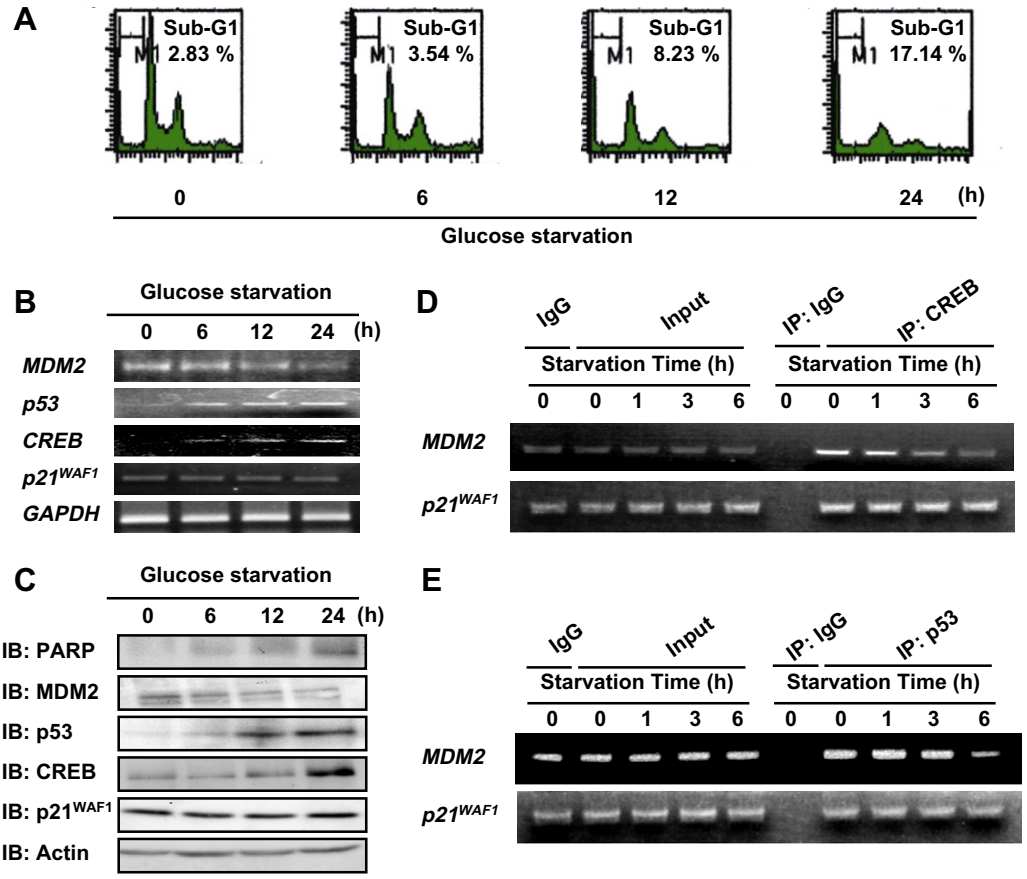

Fig. 4. Down-regulation of MDM2 in response to glucose deprivation. (A) FACS analysis. At the indicated time periods after glucose deprivation, the attached and floating U2OS cells were collected, stained with propidium iodide (PI) and number of cells with sub-G1 DNA content was measured by FACS. (B, C) Expression of p53-related genes in response to glucose deprivation. At the indicated time points after glucose deprivation, total RNA and cell lysates were prepared from U2OS cells and subjected to RT-PCR (B) and immunoblotting (C), respectively. (D, E) ChIP assay. At the indicated time points after glucose deprivation, U2OS cells were cross-linked with formaldehyde and cross-linked chromatin was sonicated followed by immunoprecipitation with anti-CREB (D) or with anti-p53 (E) antibody. Genomic DNA was purified from the immunoprecipitates and subjected to PCR to amplify the genomic region containing *MDM2* or *p21*^{WAF1} promoter. Input corresponds to 5% of genomic DNA used in this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.114.

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