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SIRT1 interacts with and protects glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from nuclear translocation: Implications for cell survival after irradiation

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ABSTRACT

Upon apoptotic stimulation, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a cytosolic enzyme normally active in glycolysis, translocates into the nucleus and activates an apoptotic cascade therein. In the present work, we show that SIRT1 prevents nuclear translocation of GAPDH *via* interaction with GAPDH. SIRT1 depletion triggered nuclear translocation of cytosolic GAPDH even in the absence of apoptotic stress. Such translocation was not, however, observed when SIRT1 enzymatic activity was inhibited, indicating that SIRT1 protein *per se*, rather than the deacetylase activity of the protein, is required to inhibit GAPDH translocation. Upon irradiation, SIRT1 prevented irradiation-induced nuclear translocation of GAPDH, accompanied by interaction of SIRT1 and GAPDH. Thus, SIRT1 functions to retain GAPDH in the cytosol, protecting the enzyme from nuclear translocation *via* interaction with these two proteins. This serves as a mechanism whereby SIRT1 regulates cell survival upon induction of apoptotic stress by means that include irradiation.

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1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme of glycolysis. GAPDH converts glyceraldehyde-3-phosphate derived from glucose to 1,3-bisphosphoglycerate in the presence of NAD^+ [1]. Apart from this basic function, GAPDH has been suggested to play multifunctional roles in biological processes including apoptosis [2], endocytosis [3], DNA replication [4], and DNA repair [5].

Although GAPDH is usually located in the cytosol, the protein can become translocated to the nucleus upon induction of stress triggering apoptosis. Translocation is stimulated by a rise in cellular nitric oxide content and subsequent S-nitrosylation of GAPDH [6,7]. Such modification promotes interaction of GAPDH with the E3-ubiquitin ligase Siah; this, in turn, stimulates nuclear translocation of GAPDH [7,8]. The GAPDH–Siah interaction requires lysine 227 of GAPDH [7]. Upon translocation of GADPH, the protein interacts with and is acetylated by the acetyltransferase p300/CBP; GAPDH is required for activation of p300/CBP [2]. In this manner, the nuclear GAPDH–p300/CBP complex regulates the acetylation and subsequent activities of proteins, including p53, that are substrates of p300/CBP [9,10].

Over the past decade, SIRT1 has been shown to deacetylate various proteins [11], with nicotinamide adenine dinucleotide (NAD^+) as a required cofactor. This activity links the functions of NAD^+-dependent metabolic pathways to signals evoked under various physiological and disease conditions [12]. SIRT1 activation *via* calorie restriction extends the lifespan of many organisms, from yeast to mammals [13], and influences the activities of cellular metabolic pathways in a NAD^+-dependent manner [14]. Indeed, SIRT family members directly control the activities of certain enzymes of metabolism, including glutamate dehydrogenase [15] and isocitrate dehydrogenase 2 [16]. Metabolic changes involving SIRT1 activity are also evident during apoptosis triggered by genotoxic agents including irradiation [17]. When apoptosis is underway, SIRT1 plays an important role in sensing the extent of cellular damage and in delivery of repair messages to appropriate transcription factors [17,18].

The connections between SIRT1 activity, metabolism, and stress prompted us to investigate the possible involvement of SIRT1 in regulation of GAPDH. In the present work, we show that SIRT1 protects cytosolic GAPDH from nuclear translocation upon irradiation; binding of SIRT1 to GADPH appears to retain the latter protein in the cytosol. This serves as a mechanism by which SIRT1 enhances cell survival after irradiation.

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2. Materials and methods

2.1. siRNAs and plasmids

The sequences of siRNA oligonucleotides were: si-control (Invitrogen, Carlsbad, CA, USA); and si-SIRT1: (#1 5′-ACU UUG CUG UAA CCC UGU A-3′) and (#2 5′-AGA GUU GCC ACC CAC ACC U-3′). The Sh–SIRT1 plasmid was constructed by annealing forward (5′-cacca CAC CAG ATT CTT CAG TGA TTG TCA tctcTGA CAA TCA CTG AAG AAT CTG GTG G-3′) and reverse (5′-aaaaCCA CCA GAT TCT TCA GTG ATT GTC AgagaTGA CAA TCA CTG AAG AAT CTG GTG G-3′) primers, followed by cloning into the pENTP^{TM}/H1/TO vector. A plasmid encoding SIRT1 tagged with Myc has been previously described.

2.2. Cell culture, transfection, and reagents

HeLa and 293T cells were maintained in minimal essential medium (Welgene, Daegu, Korea) and Dulbecco's modified Eagle's medium (Welgene), respectively, both supplemented with 10% (v/v) fetal bovine serum (FBS) (JRS, Woodland, CA, USA) and 1% (w/v) penicillin/streptomycin (Welgene). To deplete SIRT1, cells were transfected with either siRNAs targeting SIRT1 or control siRNAs, with the aid of the RNAi MAX reagent (Invitrogen). To create cell lines overexpressing SIRT1, cells were transfected with a plasmid encoding wild-type SIRT1 [19] or empty vector, using Turbofect (Fermentase, Ontario, Canada). Reagents employed included nicotinamide (Sigma, St. Louis, MO), BML-210 (BIOMOL, Plymouth Meeting, PA, USA), Ex-527 (Tocris, Bristol, UK), and hydrogen peroxide (Sigma).

2.3. Western blotting

Cells were lysed in the RIPA immunoprecipitation assay buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% [v/v] Nonidet P-40, 10 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM EDTA; pH 7.7) that included protease inhibitors (Roche, Penzberg, Germany). Proteins of cell extracts were separated by SDS–PAGE, transferred to nitrocellulose membranes, and the membranes were next immunoblotted with primary antibodies detecting SIRT1, GAPDH, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HSP90 (BD Biosciences, Flanklin, NJ, USA), or Flag (Sigma), followed by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Both anti-mouse IgG (H^+L)–HRP and anti-rabbit IgG (H^+L)–HRP (Koma, Seoul, Korea) were used in this context. Detection of HRP-conjugated secondary antibodies employed a luminal regent (Santa Cruz Biotechnology).

2.4. Immunofluorescence and confocal microscopy

Cells grown on glass coverslips were fixed in cold methanol, blocked with 3% (w/v) bovine serum albumin containing 0.1% (w/v) Tween, and incubated with antibodies detecting SIRT1 and GAPDH, diluted in TBST buffer (25 mM Tris–HCl, 150 mM NaCl, 2 mM KCl, pH 7.4) with 3% [w/v] bovine serum albumin, and 0.1% [w/v] Tween; pH 7.0), for 2 h. After washing with TBST, the cells were incubated with anti-rabbit or anti-mouse antibodies conjugated with Alexa Fluor 488 (Invitrogen) for 1 h and next counterstained using Vectashield mounting medium of the hardest grade, and DAPI (Vector Laboratories, Burlingame, CA). Images were collected with the aid of a laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and analyzed using inbuilt browser software.

2.5. Preparation of nuclear and cytosolic fractions

Nuclear and cytoplasmic extracts were prepared employing a commercial reagent (Thermo, Rlckford, IL, USA) according to the manufacturer's instructions. Briefly, cells were washed with PBS and lysed with extraction buffer. Lysates were centrifuged at 16,000*g* for 10 min and the supernatants constituted the cytosolic fractions. The precipitates were resuspended in nuclear extraction buffer and the extracts were centrifuged at 16,000*g* for 10 min. The supernatants constituted the nuclear fractions.

2.6. Immunoprecipitation

To detect interaction of SIRT1 and GAPDH, lysates prepared from cells transfected with Myc–SIRT1 and/or Flag–GAPDH were incubated with anti-Flag (Sigma) or anti-Myc antibody (Santa Cruz Biotechnology). Antibody-bound materials were precipitated with the aid of Protein G microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The beads were collected by centrifugation and boiled in sample buffer for 5 min. Proteins thus solubilized were characterized by Western blotting as described above.

2.7. Colony formation assay

Cells transfected with either siRNAs targeting SIRT1, or control siRNAs, and with either wild-type SIRT1 or empty vector, were resuspended in medium supplemented with 10% (v/v) FBS. Transfected cells were diluted with pre-warmed medium and identical amounts of cells, at 1,500 cells per dish, were plated onto dishes 60 mm in diameter and grown for 48 h. The cells were next exposed to irradiation (2 or 4 Gy) and grown for another 8 days. Colonies formed were fixed in 70% (v/v) ethanol for 10 min, and next stained with 1% (w/v) crystal violet. Colonies were counted using a colony counter (BioLogics, Manassas, VA, USA).

3. Results

3.1. SIRT1 depletion induces nuclear translocation of GAPDH

To determine whether SIRT1 was involved in nuclear translocation of GAPDH, we measured GAPDH protein levels in nuclear and cytosolic fractions when SIRT1 was depleted. As expected, GAPDH protein was present principally in the cytosol of HeLa cells when control siRNA was transfected (Fig. 1A). Upon SIRT1 depletion induced by SIRT1–siRNA (siRNA #1), significant amounts of GAPDH protein were detected in both the nucleus and cytosol. An increase in nuclear GAPDH protein level was also apparent when another siRNA directed against *SIRT1* (siRNA #2) was transfected. SIRT1 depletion caused a concomitant fall in cytosolic GAPDH protein levels. Nuclear translocation of GAPDH, and the concomitant cytosolic decrease in GAPDH levels, triggered by SIRT1 depletion, were also evident in 293T cells (Fig. 1B). Immunofluorescence revealed that GAPDH was present in the nucleus when SIRT1 was depleted (Fig. 1C). The GAPDH nuclear translocation triggered by SIRT1 depletion was accompanied by an increase in the expression levels of PUMA, a downstream target of nuclear GAPDH and the p300/CBP complex [4], and PARP cleavage, a molecular indicator of early-stage apoptosis (Supplementary Fig. 1). The amount of GAPDH protein in whole-cell lysates (thus prior to fractionation) did not change upon SIRT1 depletion (Fig. 1B), and our present findings thus indicate that, when SIRT1 is depleted, GAPDH undergoes nuclear translocation even in the absence of any apoptotic stimulus. Thus, SIRT1 is required to retain GAPDH in the cytosol; SIRT1 prevents nuclear translocation of the protein.

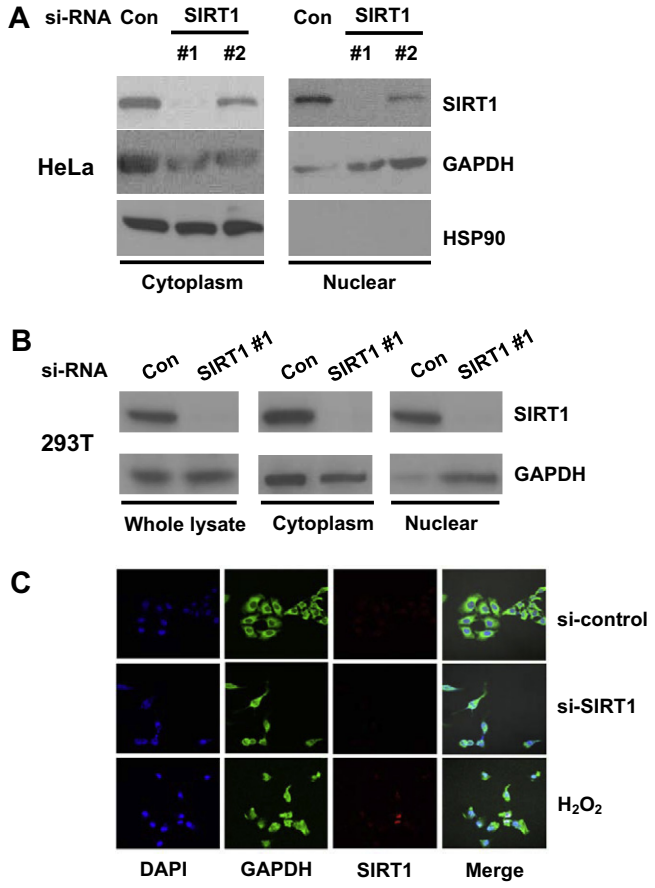

Fig. 1. SIRT1 depletion induces nuclear translocation of GAPDH. (A and B) The effect of SIRT1 on nuclear GAPDH levels. SIRT1 levels were reduced *via* transfection with SIRT1–siRNAs (#1 or #2) recognizing different regions of the *SIRT1* gene. The levels of endogenous GAPDH protein were next determined in the cytosolic and nuclear fractions of HeLa (A) and 293T cells. (B) HSP90 proteins served as cytosolic controls, respectively. (C) Immunofluorescence microscopy localizing GAPDH. Antibodies against SIRT1 or GAPDH were added to HeLa cells subjected (or not) to SIRT1 depletion, and antibody binding was visualized by fluorescence microscopy (40×). H_2O_2 (50 μM) was used as a control agent inducing nuclear translocation [29]. si-Con: si-control.

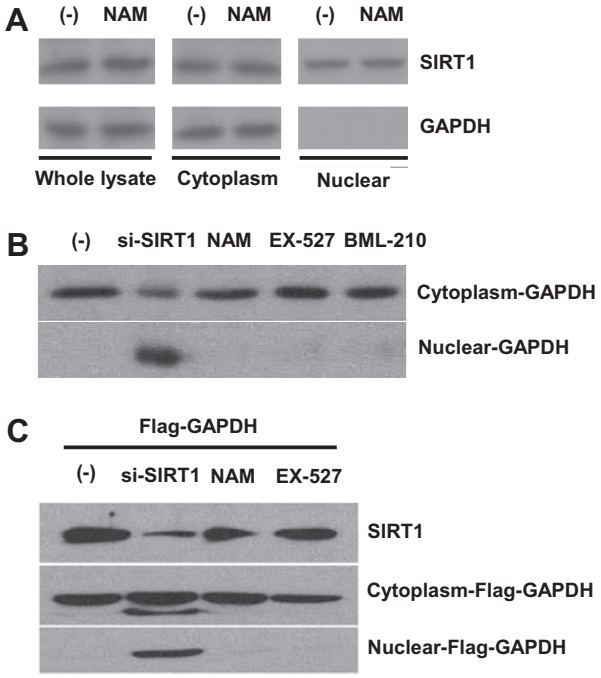

Fig. 2. SIRT1 protein, but not SIRT1 enzymatic activity, is required for nuclear translocation of GAPDH. (A) HeLa cells were treated with NAM (20 mM), an inhibitor of SIRT family enzymes, for 12 h, and the levels of GAPDH in the cytosolic and nuclear fractions were next determined. (B and C) Control HeLa cells, (B) and cells transfected with Flag–GAPDH, (C) were treated with various SIRT1 inhibitors [NAM (20 mM); EX-527 (1 μM), or BML-210 (10 μM)] for 12 h and GAPDH protein levels were next measured in both the cytosolic and nuclear fractions. SIRT1–siRNA was included as a positive control stimulating nuclear translocation of GAPDH. (–): no addition.

The SIRT1 enzyme promotes deacetylation of substrate proteins in a NAD^+-dependent manner [14]. To determine whether SIRT1-mediated translocation of GAPDH was enzymatically controlled by SIRT1, we monitored GAPDH translocation after addition of a SIRT1 inhibitor. Unlike what was observed upon SIRT1 depletion, NAM, an inhibitor of the actions of all SIRT family members including SIRT1, did not induce nuclear translocation of GAPDH (Fig. 2A and B). Other SIRT1 inhibitors, EX527 and BML-210, also failed to trigger translocation, even though SIRT1 depletion caused cytosolic GAPDH to become localized to the nucleus (Fig. 2B). To extend these observations, we next examined the cellular distribution of transfected Flag–GAPDH in HeLa cells. As was true of endogenous GAPDH, exogenously introduced GAPDH was also translocated into the nucleus upon SIRT1 depletion but not when SIRT1 enzymatic activity was inhibited (Fig. 2C). Our present findings thus indicate that SIRT1 presence *per se* rather than SIRT1 enzymatic activity is essential to prevent nuclear translocation of GAPDH.

3.2. SIRT1 depletion-mediated nuclear translocation of GAPDH attenuates sensitivity toward irradiation

Loss of SIRT1 function *via* either inactivation or depletion of the protein attenuates the ability of cells to overcome stresses elicited by genotoxic agents, including irradiation [19,20]. Although cytosolic GAPDH moves into the nucleus in response to apoptotic stress, we found that such translocation was evident when SIRT1 was depleted, even in the absence of any stress. Thus, both earlier data reported by others, and our present observations, suggest that nuclear translocation of GAPDH caused by SIRT1 depletion may render cells susceptible to apoptotic stress, thus compromising survival. We explored this assumption further by examining the relationship, when SIRT1 was depleted, between cell survival and GAPDH nuclear translocation upon exposure to irradiation. Under such conditions, addition of SIRT1–siRNA significantly compromised cell survival (Fig. 3A). Specifically, the control survival rate of 90% was reduced to 62% after exposure of cells to 2 Gy of irradiation (left panel of Fig. 3A). Reduced survival was also evident in HeLa clones in which endogenous SIRT1 was stably depleted upon introduction of SIRT1–shRNA (right panel of Fig. 3A). Two SIRT1-deficient clones, #7 and #9, exhibited much lower survival rates (68 and 32%, respectively) than did controls (96%) (Supplementary Fig. 2). Conversely, cells overexpressing SIRT1 showed enhanced survival, compared to that of cells transfected with empty vector (70% vs. 32%, respectively) after irradiation with 4 Gy (Fig. 3B).

After establishing the conditions under which cell survival was regulated by SIRT1, we next examined nuclear translocation of GAPDH. We explored whether irradiation damage induced nuclear GAPDH translocation (exposure to nitric oxide is known to induce such translocation [21]). We found that cells responded to irradiation by translocating a proportion of cytoplasmic GAPDH into the nucleus (Fig. 3C). Addition of hydrogen peroxide, a reactive oxygen species that is generated upon irradiation, caused a dramatic increase in the extent of translocation of GAPDH (Fig. 1C). SIRT1 overexpression increased the survival rate of irradiated cells

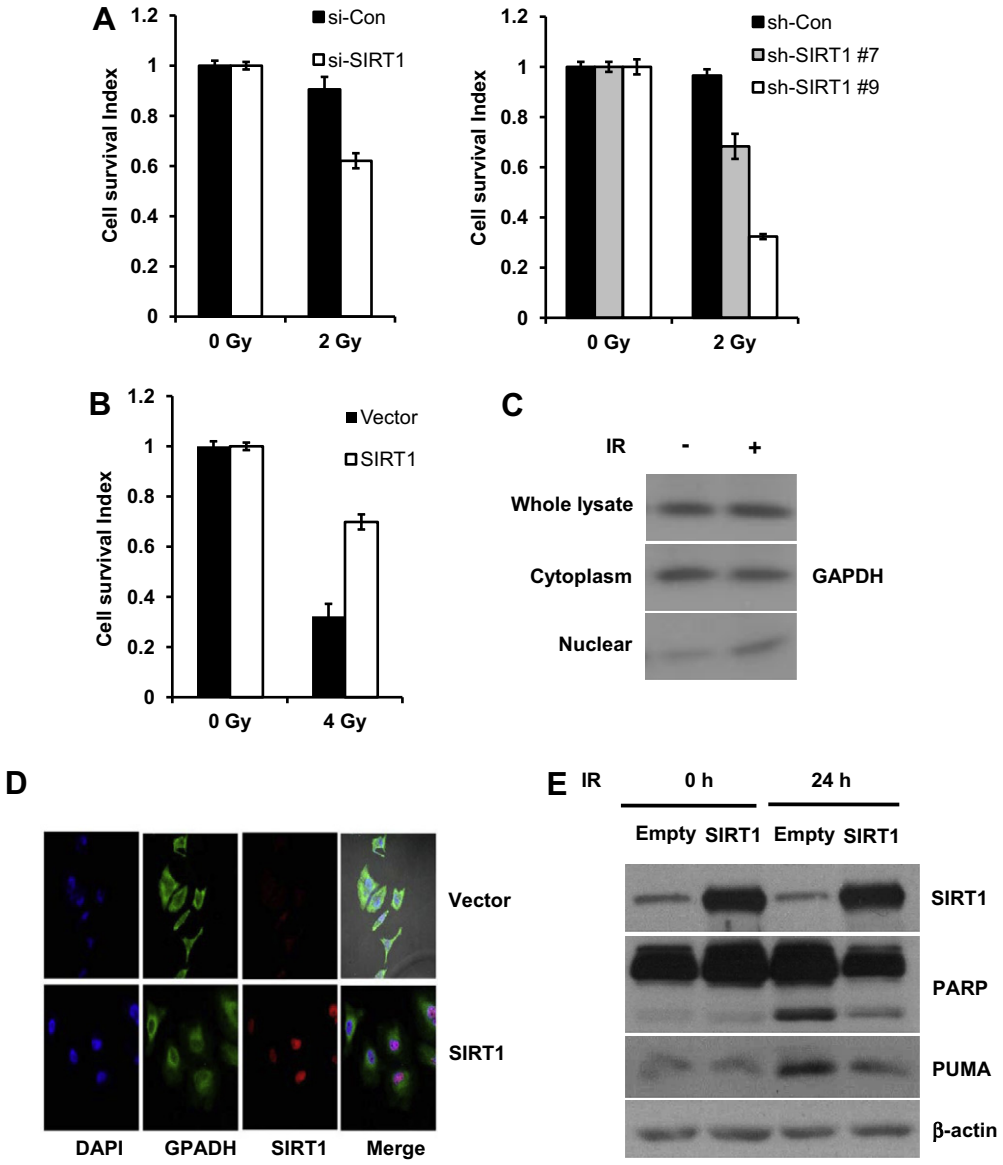

Fig. 3. Nuclear translocation of GAPDH upon SIRT1 depletion sensitizes cells to irradiation. (A) HeLa cells were depleted of SIRT1, either transiently (by using siRNAs directed against *SIRT1*, and control siRNAs; left panel), or stably by using sh–SIRT1 (#7 and #9) and an sh-control plasmid (right panel). The cells were exposed to 2 Gy of irradiation and survival rates were determined by analysis of colony formation. (B, D and E) HeLa cells transfected with SIRT1 or empty vector were irradiated and survival rates, (B) GAPDH localization, (D) and levels of PUMA expression, (E) were determined by enumeration of colony counts, immunofluorescence, and Western blotting, respectively. (C) GAPDH levels in the cytosol and nucleus were determined by Western Blotting of lysates from HeLa cells exposed to 4 Gy of irradiation.

(Fig. 3B), and reduced the extent of irradiation-induced nuclear translocation of GAPDH (Fig. 3D). The levels of PUMA expression and PARP cleavage, both of which were increased by irradiation, fell upon SIRT1 overexpression (Fig. 3E); the reverse was apparent when SIRT1 was depleted (Supplementary Fig. 1). Under the latter conditions, nuclear translocation of GAPDH was evident (Fig. 1). Our findings thus suggest that nuclear translocation resulting from SIRT1 depletion, and subsequent nuclear GAPDH accumulation prior to induction of apoptosis, attenuated the ability of the cell to deal with genotoxic stress.

3.3. SIRT1 interacts with GAPDH, and this interaction is facilitated by irradiation

Our present finding that SIRT1 prevents GAPDH nuclear translocation upon irradiation, thus causing GAPDH to be retained in the cytoplasm, suggested that the SIRT1 and GAPDH proteins might be closely associated. In an effort to understand the possible functional interactions between SIRT1 and GAPDH, we co-transfected HeLa cells with Myc-tagged SIRT1 and Flag-tagged GAPDH, and employed immunoprecipitation to monitor interactions between these proteins. Lysates of co-transfected cells were mixed with anti-Flag-antibody and the presence of SIRT1 in immune complexes was analyzed *via* immunoblotting with anti-Myc antibody. Immunoprecipitation of GAPDH from the co-transfected lysates co-precipitated SIRT1 (Fig. 4A); thus indicating that SIRT1 bound to GAPDH.

Such protein–protein interaction encouraged us to speculate that the interaction might protect cells from damage induced by irradiation. Thus, we monitored the interactions of endogenous GAPDH upon irradiation, and found that binding of GADPH and SIRT1 increased under such conditions (Fig. 4B). This indicated that SIRT1 forms a physical complex with GAPDH, and that complex formation was triggered by irradiation. This, and the fact that SIRT1

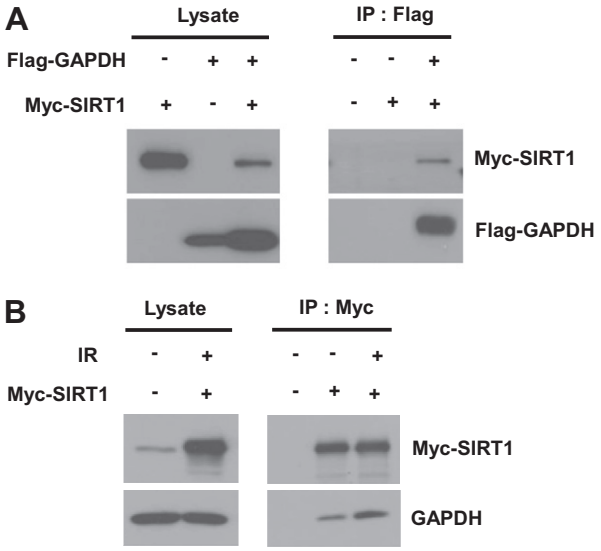

Fig. 4. SIRT1 interacts with GAPDH and such interaction is promoted by irradiation. (A) HeLa cells were transiently co-transfected with Myc–SIRT1 and Flag–GAPDH. Cell lysates were immunoprecipitated with antibodies against Flag, and the resulting immune complexes probed with antibodies against Flag or Myc. (B) HeLa cells transfected with Myc–SIRT1 were exposed to 6 Gy of irradiation, and lysates were immunoprecipitated with antibody against Myc. SIRT1 and GAPDH proteins in Myc immunoprecipitates were detected by probing with antibodies detecting Myc and GAPDH, respectively.

prevents irradiation-induced nuclear translocation of GAPDH (Fig. 3D), further suggest that physical interaction between the two proteins, upon irradiation, may inhibit nuclear translocation of GAPDH and that even a transient interaction of this nature enhances the survival of irradiated cells.

4. Discussion

Recently, the nuclear function of GAPDH has attracted a great deal of attention. GAPDH was conventionally considered to be an enzyme of the glycolytic pathway, functioning exclusively in the cytoplasm. Upon induction of apoptotic stress, GAPDH, which lacks any nuclear localization signal, is nonetheless translocated into the nucleus; a transient interaction of GADPH with Siah is an essential feature of this process [7,8]. In the present study, we have shown that SIRT1 prevents GAPDH nuclear translocation in response to apoptotic stresses including irradiation; SIRT1 thus contributes to cell survival. Further, inhibition of GAPDH translocation by SIRT1 may be attributable to an irradiation-induced SIRT1/GAPDH interaction.

Nuclear translocation of GAPDH is important in terms of cell signaling processes that precede apoptosis [2]. Nuclear GAPDH mediates apoptosis by complexing with, and activating, the p300/CBP acetyltransferase, and subsequently stimulating the synthesis of proteins required for expression of the stress response [2]. Herein, we have shown that SIRT1 protein *per se*, rather than SIRT1 activity, is required for nuclear translocation of GAPDH; we have demonstrated that SIRT1 depletion, but not SIRT1 inactivation, induced nuclear translocation of GAPDH. Over the past decade, many authors have focused on the deacetylase activity of SIRT1, rather than SIRT1 expression *per se*, when exploring the role played by SIRT1 in the cellular responses to various forms of stress [22]. Indeed, stress-induced cellular damage is reduced by the activation of SIRT1 and subsequent deacetylation of substrate proteins playing roles in apoptosis, DNA repair, and metabolism [23]. These earlier findings, together with our present data, suggest that, enzymatic activity aside, SIRT1 expression *per se* significantly enhances survival in the face of apoptotic stress; SIRT1 inhibits translocation of GAPDH to the nucleus prior to induction of apoptosis.

To date, only the interaction between Siah and GAPDH has been recognized as essential for GAPDH translocation. Thus, much research effort has sought to understand how the interaction is regulated [7,9]. S-nitrosylation of GADH is a major trigger facilitating interaction of GAPDH and Siah [7,8]. This modification is regulated by nitric oxide levels [6–8]. Therefore, either intra- or inter-cellular changes that stimulate or inhibit generation of nitric oxide can control GAPDH/Siah interaction and the consequent nuclear translocation of GAPDH. Similarly, the activities of signaling pathways stimulating nuclear translocation have been explored at various nitric oxide concentrations, such as those generated upon macrophage activation [2,7,24]. In the present work, we show that, under certain circumstances, SIRT1 prevents nuclear translocation of GAPDH. The fact that GAPDH was translocated into the nucleus when SIRT1 was depleted, even in the absence of stress, indicates that SIRT1 inhibits such translocation; GAPDH is retained in the cytosol and is active in glycolysis. Further, SIRT1 depletion seemed to render the cellular environment favorable for entry of GAPDH into the nucleus, even in the absence of apoptotic stress, by inhibition GAPDH modifications including S-nitrosylation [6–8], and the interaction of GAPDH with Siah [7,8]. Upon irradiation, interaction of SIRT1 and GAPDH was enhanced; nuclear translocation of GAPDH was thus further inhibited, as was the expression of PUMA, a downstream target of GAPDH activated post-translocation. Therefore, we suggest that, upon irradiation, the SIRT1/GAPDH interaction may inhibit nuclear translocation of GAPDH. SIRT1 and Siah compete to bind to GAPDH. Also, glyceraldehyde-3-phosphate competitively (with respect to Siah) binds to GAPDH [25]; such binding prevents nuclear translocation of GAPDH.

We have shown herein that SIRT1 prevents translocation of cytosolic GAPDH into the nucleus. SIRT1 was originally identified as a member of a class of telomere-binding proteins that serve to repair DNA after damage is incurred [22]. Loss of telomeric functions as a result of either shortening of telomeric repeats or depletion of telomere-binding proteins attenuates the effects of apoptotic stress and eventually sensitizes cells to genotoxic anticancer agents [26–28]. Our present work suggests that inhibition of the SIRT1/GAPDH interaction may be a valuable therapeutic approach toward development of anticancer drugs. Future studies should seek to define the mode by which the SIRT1–GAPDH interaction is regulated upon SIRT1 depletion or induction of apoptotic stress. Such work would increase our understanding of the processes underlying nuclear translocation of GAPDH.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.006.

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