B23 Regulates GADD45α Nuclear Translocation and Contributes to GADD45α-induced Cell Cycle G2-M Arrest*

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Gadd45α is an important player in cell cycle G2-M arrest in response to genotoxic stress. However, the underlying mechanism(s) by which Gadd45α exerts its role in the control of cell cycle progression remains to be further defined. Gadd45α interacts with Cdc2, dissociates the Cdc2-cyclin B1 complex, alters cyclin B1 nuclear localization, and thus inhibits the activity of Cdc2/cyclin B1 kinase. These observations indicate that Gadd45α nuclear translocation is closely associated with its role in cell cycle G2-M arrest. Gadd45α has been characterized as a nuclear protein, but it does not contain a classical nuclear localization signal, suggesting that Gadd45α nuclear translocation might be mediated through different nuclear import machinery. Here we show that Gadd45α associates directly with B23 (neuramin), and the B23-interacting domain is mapped at the central region (61–100 amino acids) of the Gadd45α protein using a series of Myc tag-Gadd45α deletion mutants. Deletion of this central region disrupts Gadd45α association with B23 and abolishes Gadd45α nuclear translocation. Suppression of endogenous B23 through a short interfering RNA approach disrupts Gadd45α nuclear translocation and results in impaired Gadd45α-induced cell cycle G2-M arrest. These findings demonstrate a novel association of B23 and Gadd45α and implicate B23 as an important regulator in Gadd45α nuclear import.

The cell cycle checkpoint is one of the major genomic surveillance systems in mammalian cells. Inactivation of such a system results in genomic instability and malignant transformation of cells (1, 2). The tumor suppressor gene p53 is implicated in the control of both cell cycle G1-S and G2-M arrests in response to genotoxic stress (3, 4). In addition to the well-characterized p53-p21waf1/cip1 pathway in regulating DNA damage-activated cell cycle checkpoints (5–7), the p53-Gadd45α pathway has been shown to primarily play a role in the control of G2-M arrest following certain DNA-damaging agents or growth arrest signals such as ionizing radiation, UV radiation, methyl methanesulfonate (MMS), hydroxyurea (12), growth factor withdrawal, and serum starvation (13, 14). The ionizing radiation induction of Gadd45α is transcriptionally regulated by p53 via a p53-binding site in the third intron of the Gadd45α gene (3) and strictly depends on normal cellular p53 function (15). Gadd45α has also been shown to be a downstream gene of BRCA1 (16–18), a breast cancer-associated gene that plays roles in the control of cell cycle progression, apoptosis, DNA repair, and gene regulation. The regulation of Gadd45α by BRCA1 does not require normal p53 function (17). Gadd45α physically interacts with several important cellular proteins, including proliferating cell nuclear antigen, p21, Cdc2 (9), core histones, and MTK1/MEKK4 (9, 19–24). The presence of Gadd45α in these protein complexes suggests that Gadd45α may play important roles in cell cycle control, DNA repair, and the regulation of signaling pathways. The role of Gadd45α in maintaining genomic stability has been demonstrated by the findings that mouse embryonic fibroblasts (MEFs) derived from Gadd45α-null mice exhibit aneuploidy, chromosomal aberrations, gene amplification, and centrosome amplification. Additionally, Gadd45α knock-out mice display increased ionizing radiation- or UV radiation-induced carcinogenesis (25–27).

Multiple lines of evidence indicate that Gadd45α is an important regulator of the cell cycle G2-M checkpoint in response to certain genotoxic stressors. Gadd45α interacts with Cdc2, dissociates Cdc2-cyclin B1 complexes, and suppresses Cdc2/cyclin B1 kinase activity. Overexpression of Gadd45α results in the alteration of cyclin B1 subcellular localization and reduces nuclear distribution of cyclin B1 protein (8–11). Gadd45α is a nuclear protein (20, 21), and its nuclear translocation might be critical for its role in the control of the cell cycle G2-M checkpoint. However, Gadd45α protein has no classical nuclear localization signal sequence (NLS) and may utilize different mechanism(s) for its nuclear import. The machinery controlling nuclear translocation of Gadd45α protein is currently unclear.

It has been well accepted that all nuclear proteins are synthesized in the cytoplasm and need to be imported through the nuclear pore complexes into the nucleus. Active nuclear import is energy-dependent and is mediated by import receptors. Import into the nucleus can be conferred by several distinct import signals. The classical NLS consists of one or more clusters of basic amino acids and is present in a large number of pro-

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1 The abbreviations used are: MMS, methyl methanesulfonate; NLS, nuclear localization signal; siRNA, short interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; MEFs, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; RT, reverse transcription; BrdUrd, bromodeoxyuridine; GST, glutathione S-transferase; GFP, green fluorescent protein.

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B23 Regulation of Gadd45α Nuclear Translocation

Plasmid Clones—The following plasmid clones were used. All Myc-tagged Gadd45α deletion expression vectors were constructed as described previously (9, 10). These Myc-tagged clones harbor different regions of Gadd45α protein. GST-Cds2, GST-p53, GST-p21, and GST-EP1 were constructed by inserting their open reading frames into the EcoRI-XhoI sites of pGEX-5X-1 plasmid. GST-Gadd45α was made by cloning Gadd45α cDNA containing the open reading frame into the XhoI site of the pGEX-5X-1 vector. pEGFP-Gadd45α was constructed by inserting the open reading frame of Gadd45α cDNA into the pEGFP vector. Similarly, pEGFP-(61–100) was constructed by inserting Gadd45α cDNA containing 61–100 amino acids into the pEGFP plasmid. The pEGFP-D (Δ60–100)-Gadd45α was made to express the truncated GFP-Gadd45α fusion protein with deletion of 61–100 amino acids. GST-B23 was provided by Dr. Kenji at the University of Cincinnati.

Cell Cultures and Treatment—The human colon carcinoma line HCT116 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Wild-type and Gadd45α knock-out MEFs were kindly provided by Albert Fornace of the National Institutes of Health and maintained in DMEM with 10% FBS. HCT116 Gadd45α-inducible cells were established previously. Cells were grown in DMEM containing tetracycline at a concentration of 2 μg/ml. Following withdrawal of tetracycline, Gadd45α protein exhibits high expression.

For cell transfection with Myc tag-Gadd45α expression vectors, 5 × 10⁵ HCT116 cells were seeded onto 10-mm plates before transfection. In each plate, 5 μg of DNA and 15 μl of Lipofectamine (Invitrogen) were added in 300 μl of Opti-MEM (Invitrogen) in separate tubes. Solutions were mixed gently, allowed to sit 15 min at room temperature, diluted with 2.4 ml of Opti-MEM, and added to the plates for 6 h at 37°C. Equal volumes of media with 10% FBS were added, and plates were incubated overnight. Fresh medium was then added the following day, and cells were harvested 48 h later.

For radiation treatment, cells plated in 100-mm dishes were rinsed with PBS and irradiated with UV to a dose of 10 J/m². Following UV radiation treatment, fresh medium was added to plates, and cells were cultured in the incubator until harvest. For MMS treatment, cells were exposed to MMS (Aldrich) at 100 μg/ml for 4 h, and media were then removed and replaced with fresh media. Cells were then collected at the indicated time points.

siRNA Transfection—The B23 siRNA sequence was designed as UGA UGA AAA UGA CCA CCA G. The nonspecific siRNA sequence was designed as GAC CAC GAG UAA AG UAG UU. siRNA transfection with siRNA, HCT116 cells were placed onto 6-well plates 16 h prior to transfection and grown at 2 × 10⁵. HCT116 cells were seeded onto 6-well plates. In one tube, 10 μl of 20 μl siRNA was added to a tube containing 50 μl of Opti-MEM. In a separate tube, 1 μl of Lipofectamine was mixed with 50 μl of Opti-MEM. The two tubes were next mixed, allowed to sit at room temperature for 30 min, and then added to each well in the plate, which contained 1 ml of medium. 4 h later, 4 ml of fresh medium was added, and transfected cells were incubated for 24–72 h until they were ready to assay for gene knock-down analysis.

RT-PCR—HCT116 cells were grown in RPMI 1640 supplemented with 10% FBS. Total RNA was isolated using RNAeasy mini kit (Qiagen) according to the manufacturer’s protocol. RT-PCR was carried out using an Applied PCR core kit (PerkinElmer Life Sciences). 0.5 μg total RNA in 1 μl of RNase-free water was used in 20 μl of RT mix containing the following: 4 μl of 25 mm MgCl₂, 2 μl of 10× PCR buffer, 2 μl of dNTP, 2 μl of dNTP mix (2.5 mM each of dATP, dCTP, dGTP, and dTTP), 1 μl of RNAse inhibitor (20 units/ml), 1 μl of random hexamers (50 μM), and 1 μl of murine leukemia virus reverse transcriptase (50 units/ml). The mixture was subjected to cDNA synthesis using Taq DNA polymerase (5 units/μl). The reaction was then collected at the indicated time points.

For cell transfection with Myc tag-Gadd45α expression vectors, 5 μg of DNA and 15 μl of Lipofectamine (Invitrogen) in separate tubes. Solutions were mixed gently, allowed to sit 15 min at room temperature, diluted with 2.4 ml of Opti-MEM, and added to the plates for 6 h at 37°C. Equal volumes of media with 10% FBS were added, and plates were incubated overnight. Fresh medium was then added the following day, and cells were harvested 48 h later.

For immunoblotting analysis, 100 μg of proteins were loaded onto SDS-polyacrylamide gels for electrophoresis and then transferred to Protran membranes. Membranes were blocked in 5% milk, washed with PBST (PBS with 0.1% Tween), and incubated with anti-B23 or actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Following washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at 1:4000 in 5% milk, membranes were washed and detected by ECL (Amersham Biosciences) and exposed to x-ray film.

For immunofluorescence, cellular lysates were incubated with 10 μl of antibody and 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 4 h. Immunocomplexes were washed four times with lysis buffer, loaded onto 12% SDS-polyacrylamide gels, and analyzed as described above.

Antibodies to Gadd45α, c-Myc, Cdc2, actin, and actin were commercially provided by Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody to cyclin B1 was provided by Pharmingen.

GST fusion protein expression was induced in Escherichia coli with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Bacterial pellets were washed with PBS and resuspended in cold STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA 150 mM NaCl). Following incubation with freshly prepared lysosome solution and Sarkosyl (0.7%), bacterial mixtures were subjected to sonication for 1 min. Following centrifugation at
Histone H1 kinase assays were then performed in the presence of 10 μg/ml aphidicolin for 24 h. On release from the aphidicolin block, cells were treated with UV radiation and incubated for an additional 20 h in the presence of BrdUrd. Cells were collected, and the BrdUrd-positive cells were subjected to FACS analysis. In the case of the HCT116 Gadd45a-inducible line, cells were plated into 100-mm dishes at a density of 5 × 10^5 and grown in DMEM containing 2 μg/ml tetracycline. 16 h later, medium was removed, and plates were washed four times followed by addition of fresh medium. After incubation for 36 h, cells were collected, washed with PBS, and fixed with 70% ethanol for 2 h at 4°C. Cells were then incubated with RNase (10 μg/ml) for 30 min and stained with propidium iodide (Sigma; 50 μg/ml). Cell cycle analysis was performed by using a BD Biosciences fluorescence-activated cell analyzer. At least 10,000 fluorescein isothiocyanate-positive cells were analyzed using Cellquest and Modfit programs.

Cdc2 kinase Analysis—500 μg of cellular lysate isolated from MEFs treated with UV radiation at 10 J/m^2 and MMS at 50 μg/ml or Gadd45a-inducible line, was incubated with 10 μl of cyclin B1 antibody (Pharmpingen) and 20 μl of protein A/agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 6 h. Immunocomplexes were washed four times with lysis buffer and followed by kinase buffer. Histone H1 kinase assays were then performed in the presence of 10 μg of histone H1 (Upstate Biotechnology, Inc., Lake Placid, NY), 15 mM MgCl_2, 7 mM β-glycerolphosphate, 1.5 mM EDTA, 0.25 mM sodium orthovanadate, 0.25 mM dithiothreitol, and 10 μCi of [γ-32P]ATP in a 30-μl volume. After 15 min at 30 °C, the reactions were mixed with an equal amount of standard 2× SDS protein denaturing loading buffer and then size-separated on a 12% SDS-polyacrylamide gel.

Measurement of Mitotic Index—HCT116 Gadd45a-inducible cells were seeded at a density of 5 × 10^5 and grown in DMEM containing 2 μg/ml tetracycline and 0.4 μg/ml nocodazole. Following withdrawal of tetracycline, cells were transfected with 3 μg siRNA and washed with methanol:acetic acid (3:1), spread on glass microscope slides, air-dried, and stained with 5% Giemsa. Nuclei exhibiting condensed, evenly staining chromosomes were scored as mitotic. At least 1000 cells were counted in each determination.

RESULTS

Disruption of Endogenous Gadd45a Results in Impaired Cell Cycle G2-M Arrest following Certain DNA-damaging Agents—Several lines of evidence indicate that Gadd45a is one of the important components involved in the control of the cell cycle G2-M checkpoint. We have demonstrated previously that disruption of endogenous Gadd45a via an antisense approach results in a perturbed G2-M delay following DNA damage (8). To confirm further the role of Gadd45a in G2-M arrest after genotoxic stress, cell cycle analysis was performed using MEFs in which both Gadd45a alleles had been disrupted by homologous recombination to demonstrate that disruption of endogenous Gadd45a was sufficient to abrogate the G2-M checkpoint following treatment with UV radiation. A modified double labeling (PI and BrdUrd) protocol, which requires no cell cycle inhibitors, was employed in this experiment (8). Cell cycle progression was evaluated at very early passages to minimize effect because of the passage of cells in culture. As shown in the first panel of Fig. 1A, wild-type MEFs displayed a clear G2-M arrest after treatment with UV radiation. In contrast, the MEFs derived from Gadd45a knockout mice exhibited a less stringent G2-M arrest after UV radiation.

As described earlier, the Cdc2-cyclin B1 complex is a key regulator of the transition from G2 to mitosis (41, 42). Generally, Cdc2/cyclin B1 activity is inhibited following DNA damage, and this inhibition acts to block the G2-M transition (43).

In the second panel of Fig. 1B, the wild-type MEFs displayed a strong reduction in Cdc2 kinase activity following UV radiation or MMS, whereas the reduction was significantly attenuated in the Gadd45a−/− MEFs. This result correlates with the attenuated G2-M arrest after UV radiation described above.

In addition, HCT116 Gadd45a-inducible cells, where Gadd45a-inducible expression is controlled by a tetracycline system, were employed. Following the withdrawal of tetracycline, HCT116 exhibited a highly induced expression of the Gadd45a protein (Fig. 1C, 1st panel). The cell cycle distribution analyses were conducted in the HCT116 Gadd45a-inducible cell line. Following removal of tetracycline, Gadd45a-inducible cells were collected at 36 h and subjected to flow cytometric analysis. As shown in Fig. 1D, inducible expression of Gadd45a resulted in a substantial accumulation of the G2-M fraction, and ~38% of the cells were halted at the G2-M phase of the cell cycle following removal of tetracycline. In contrast, about 14% population of cells presented at the G2-M phase in the presence of tetracycline. This Gadd45a-induced G2-M arrest was coupled with altered nuclear localization of cyclin B1 by Gadd45a induction (Fig. 1C, 2nd panel). Collectively, Gadd45a is an important component required for cell cycle G2-M arrest following certain DNA-damaging agents.

Gadd45a Localizes to Both Nuclear and Cytosol Compartments—Several reports have suggested that Gadd45a is predominantly a nuclear protein (20, 21). Overexpression of Gadd45a has been shown to result in dissociation of the Cdc2-cyclin B1 complex and reduction of the nuclear distribution of cyclin B1 (11). Therefore, nuclear localization of Gadd45a protein might be associated with its biological function in the control of cell cycle G2-M arrest. In Fig. 2A, HCT116 cells were treated with UV radiation and assayed for expression of Gadd45a. In addition to the observation that Gadd45a was clearly induced by UV radiation, this protein was shown to localize to both the nuclear and cytosol compartments. To exclude protein leakage during preparations of nuclear or cytosol proteins, the detection of actin (cytosol protein) and p53 (nuclear protein) was included. The localization of these two (actin and p53) proteins confirms that no cross-contamination occurred during the fractionation. In addition, we transfected Gadd45a(1–165), a full-length Myc tag-Gadd45a expression vector, into HCT116 cells, and we examined the Myc tag-Gadd45a fusion protein using anti-Myc antibody. In Fig. 2B, Myc tag-Gadd45a was seen to distribute in both nuclear and cytoplasmic fractions. Taken together with previous observations by others, Gadd45a is a nuclear protein but remains in both the nuclear and cytosol compartments.

The Central Region of the Gadd45a Protein Is Required for Nuclear Translocation—To rule out the possibility that Gadd45a protein is diffused into the nucleus due to its small molecular size (21 kDa), the Myc-tagged Gadd45a deletion mutants, Gadd45a-(61–100), Gadd45a-(1–71), Gadd45a-(91–165), and Gadd45a-(48–165) were co-transfected with Gadd45a(1–165, full length) into HCT116 cells, and an immunoblotting assay with anti-Myc antibodies was performed. As illustrated in Fig. 3A, Gadd45a-(1–71) and Gadd45a-(91–165) were only seen to localize at the cytoplasmic compartment (Fig. 3A, labeled as C). In contrast, Gadd45a-(48–165) clearly exhibited nuclear localization (Fig. 3A, labeled as N). To examine further the Gadd45a domains involved in its nuclear translocation, six Gadd45a deletion mutants, each of which spans 40 residues with a 10-residue overlap between each contiguous peptide, were co-introduced with Gadd45a-(1–165) into HCT116 cells and subjected to immunoblotting analysis. As shown in Fig. 3B, only Gadd45a(61–100) displayed nuclear localization, but the rest of the five deletion mutants showed exclusive cytoplasmic localization. Next, we constructed a new Gadd45a deletion mutant, Δ(61–100)-Gadd45a, in which the region between 61 and 100 amino acids was truncated, and we examined its subcellular localization. Consistent with the results described in Fig. 3A and B, when the amino acids from 61 to 100...
were deleted, the Gadd45a protein remained in the cytoplasm (Fig. 3C), further indicating that the central region of the Gadd45a protein from amino acids 60 to 100 is required for Gadd45a nuclear translocation.

Gadd45a Interacts with B23 (Nucleophosmin), Which Transports Certain Proteins into the Nucleus—Despite its nuclear localization, Gadd45a does not have a classical NLS sequence, suggesting that Gadd45a nuclear translocation might be mediated through different nuclear import machinery. It is speculated that Gadd45a protein nuclear translocation is possibly conducted via certain carrier proteins that can act as “nuclear delivery vehicles,” to which the Gadd45a protein is able to physically bind. We used the two-hybrid system to identify Gadd45a-associated proteins. The Gadd45a protein was divided into four different baits, and in total, 32 cellular proteins were characterized, of which 12 proteins were confirmed to interact with Gadd45a using in vitro biochemical methods. By using a GST pull-down assay, several cellular proteins were defined to physically associate with Gadd45a. Those proteins include Cdc2 kinase, EF-1α, B23 (nucleophosmin), and p21WAF/CIP (Fig. 4A). In Fig. 4B, the Gadd45a(1–165) fusion protein expression vector or unfused Myc tag vector were transfected into HCT116 cells, and cell lysates were prepared for immunoblotting analysis with antibodies to Gadd45a, cyclin B1, and Cdc2. In addition, 200 μg of nuclear protein was immunoprecipitated with anti-cyclin B1 antibody, and histone H1 kinase assays were performed as described under “Experimental Procedures.” In Fig. 4C, altered nuclear localization of cyclin B1 and inhibition of Cdc2 activity after induction of Gadd45a. HCT116 Gadd45a-inducible cells were collected at the indicated time points for preparation of nuclear protein. 50 μg of nuclear protein was used for immunoblotting analysis with antibodies to Gadd45a, cyclin B1, and Cdc2.
lated from HCT116 Gadd45a-inducible cells (see Fig. 1C), which were collected 24 h after tetracycline withdrawal and were confirmed to highly express the Gadd45a protein. Cellular lysates were incubated with anti-Gadd45a, anti-B23, anti-Cdc2, anti-actin, or anti-GFP antibodies and immunoprecipitated with protein A/G-agarose beads. The immunocomplexes were then analyzed by Western blotting assay using antibodies to Gadd45a and B23. As shown in Fig. 4C, Gadd45a protein was present in the immunocomplexes precipitated by the antibodies against Cdc2 and B23. Similarly, B23 protein was detected in the immunocomplexes with both anti-Gadd45a and anti-Cdc2 antibodies. In contrast, no Gadd45a or B23 proteins were present in the anti-actin- or anti-GFP-immunoprecipitated complexes. Therefore, the results presented in Fig. 4, A–C, indicate an association of Gadd45a with B23.

To map the B23-interacting motif of Gadd45a, GST or GST-B23 was incubated with cell lysates prepared from the HCT116 cells transfected with different Gadd45a deletion mutants as follows: Gadd45a-(1–165), Δ(60–100)-Gadd45a, Gadd45a-(1–100), and Gadd45a-(91–165). Following immunoprecipitation, GST complexes were analyzed with anti-Myc antibody. As shown in Fig. 4D, full-length Gadd45a and Gadd45a-(1–165) were pulled down by GST-B23. In contrast, Δ(60–100)-Gadd45a and Gadd45a-(91–165) were unable to interact with B23, suggesting that the central region is required for the interaction of Gadd45a and B23 protein. By using the same approach, we examined the interaction of GST-B23 with a series of Myc tag-Gadd45a deletion mutants, and we found that the central region was also required for Gadd45a nuclear import (results not shown). When pEGFP-Gadd45a or pEGFP-Gadd45a-(61–100) expression vectors were transfected into HCT116 cells, the green fluorescent protein (GFP) was primarily seen in nuclei. In contrast, the GFP mainly stayed in the cytoplasm in cells transfected with pEGFP-Δ(60–100)-Gadd45a or pEGFP empty vector (results not shown). Therefore, disruption of the central region abolishes Gadd45a nuclear translocation.
FIG. 4. Physical interaction of Gadd45a with B23. A, GST or GST-cyclin B1, GST-p53, GST-EP-1α, GST-Cdc2, GST-B23, and GST-p21 proteins were prepared (see "Experimental Procedures") and incubated with cell lysates isolated from HCT116 cells transfected with the full-length Myc tag-Gadd45a expression vector. The GST protein pull-down complexes were washed three times with lysis buffer, analyzed by SDS-PAGE, and immunoblotted with anti-Myc antibody. B, Myc tag-Gadd45a was expressed in HCT116 cells. Whole cell extracts were prepared and immunoprecipitated with anti-actin, anti-cyclin B1, anti-Cdc2, anti-Myc, and anti-B23 antibodies. Following SDS-PAGE, immunoblotting assays were carried out with anti-Myc antibody. C, nuclear protein from HCT116 cells was prepared and immunoprecipitated with anti-actin, anti-Gadd45α, anti-B23, anti-Cdc2, and anti-GFP antibodies. The immunocomplexes were analyzed by SDS-PAGE and immunoblotted with antibodies against B23 and Gadd45α, respectively. The visualized bands are shown. Their estimated masses were 38 kDa for B23 and 21 kDa for Gadd45α. D, Gadd45α(1–165) and Gadd45α deletion mutants, Δ(60–100)-Gadd45α, Gadd45α(1–100), and Gadd45α(91–165), were transfected into HCT116 cells. 48 h later, cell lysates were prepared and incubated with GST or GST-B23 proteins. Following GST protein pull-down and SDS-PAGE, an immunoblotting assay was carried out with anti-Myc antibody.

Suppression of B23 Protein Levels Using siRNA Approach Substantially Affects Gadd45α Nuclear Translocation—As discussed earlier, B23 is able to deliver certain important cellular or viral proteins from the cytoplasm into the nucleus (36–40). To investigate if the B23 protein contributes to Gadd45α nuclear translocation, the siRNA approach was used to inhibit endogenous B23 expression and was followed by examination of Gadd45α nuclear localization. B23 siRNA and nonspecific siRNA were added into HCT116 cells in culture at a concentration of 40 pmol. 48 h later, cells were collected for preparation of poly(A)+, and an RT-PCR assay was performed to examine the levels of B23 mRNA. As shown in Fig. 5A, endogenous B23 mRNA was substantially suppressed by B23 siRNA but not by nonspecific siRNA. Endogenous B23 protein was also examined following B23 siRNA treatment. Consistent with the results in the RT-PCR, addition of B23 siRNA was shown to greatly knock down cellular B23 expression (Fig. 5B), whereas actin protein expression remained unaltered. In contrast, nonspecific siRNA did not affect cellular B23 protein levels.

Next, Gadd45α nuclear localization following suppression of B23 endogenous protein was evaluated. In Fig. 5C, HCT116 cells were transfected with B23 siRNA or nonspecific siRNA for 48 h, treated with UV radiation, and harvested at 0, 4, and 8 h. Both cytosol and nuclear proteins were extracted and assayed for subcellular distribution of endogenous Gadd45α protein. The results in Fig. 5C show that nuclear localization of Gadd45α protein was greatly reduced following the suppression of B23 protein expression by B23 siRNA. The addition of B23 siRNA suppressed more than 70% of the nuclear Gadd45α protein, compared with that seen in the cells treated with nonspecific siRNA. To rule out any nonspecific effect of B23 siRNA, the nuclear localization of ATF3 protein, a stress-inducible transcription factor, was also examined and did not show any alterations after B23 siRNA treatment. Additionally, Myc tag-Gadd45α was co-introduced with B23 siRNA into the HCT116 line, and cells were collected for examination of Myc tag-Gadd45α fusion protein. As shown in Fig. 5D, Myc tag-Gadd45α protein in either control cells or cells treated with nonspecific siRNA exhibited substantial nuclear accumulation. However, the cells treated with B23 siRNA displayed a weak accumulation of endogenous Gadd45α. Taken together, these results indicate that disruption of endogenous B23 protein abrogates Gadd45α nuclear localization.

Gadd45α Nuclear Localization Is Required for Gadd45α-induced Cell Cycle G2-M Arrest—Previous findings demonstrate that Gadd45α interacts with Cdc2 and dissociates Cdc2-cyclin B1 complexes, inhibiting Cdc2 kinase activity. Recently, induction of Gadd45α has been shown to result in alterations of cyclin B1 subcellular distributions, as reflected by reduced nuclear accumulation of cyclin B1, suggesting that the place for Gadd45α targeting on the Cdc2-cyclin B1 complex should be in the nucleus. If so, Gadd45α nuclear translocation is critical for its function in cell cycle G2-M arrest. Therefore, experiments were carried out to determine whether Gadd45α nuclear translocation is required for Gadd45α-mediated G2-M arrest. To do so, HCT116 Gadd45α-inducible cell lines via the Tet-Off system were employed, and cell cycle distributions of Gadd45α-inducible cells in the presence of B23 siRNA were analyzed. In Fig. 6A, after transfection of 21-nucleotide siRNA duplexes that target B12 mRNA transcripts into HCT116 Gadd45α-inducible cells, nuclear proteins were prepared and assayed for Gadd45α-
inducible expression. Similar to the observations presented in Fig. 5, nuclear accumulation of Gadd45a protein in HCT116 Gadd45a-inducible cells was significantly reduced by B23 siRNA compared with that seen in cells treated with nonspecific siRNA. Next, the B23 siRNA-treated cells were subjected to flow cytometric analysis, and the results are shown in Fig. 6B. Clearly, disruption of Gadd45a nuclear localization via employment of B23 siRNA greatly affected Gadd45a-induced cell cycle G2-M arrest. The cell populations of G2-M phase were reduced from 31 to 16% following B23 siRNA treatment.

The mitotic index was also measured in HCT116 Gadd45a-inducible cells in the presence of B23 siRNA. To facilitate the

![Diagram](image)

**FIG. 5.** Knock-down of endogenous B23 expression results in reduced nuclear translocation of Gadd45a protein. A, 5 × 10^5 HCT116 cells were plated into 100-cm dishes and grown in RPMI 1640 medium with 10% FBS. After incubating overnight, the cells were transfected with 21-nucleotide siRNA duplexes that target B23 mRNA transcript via Oligofectamine (see “Experimental Procedures”). As a control, nonspecific 21-nucleotide siRNA duplexes were included in the experiments. Cells were collected at the indicated times for preparation of poly(A) RNA. The levels of B23 mRNA in cells treated with B23 siRNA were analyzed by RT-PCR assays. B, HCT116 cells treated with B23 siRNA or nonspecific siRNA were harvested at 0, 24, or 48 h post-transfection. Cell lysates were isolated and assayed for measurement of endogenous B23 protein. C, HCT116 cells were plated onto 100-mm dishes and transfected with B23 siRNA or nonspecific siRNA as described under “Experimental Procedures.” 48 h later, cells were rinsed with PBS and irradiated with UVC to a dose of 10 J/m^2^. Following UV radiation treatment, fresh medium was added to the plates, and cells were cultured in an incubator until harvesting at the indicated time points. Both cytosol and nuclear proteins were prepared, and immunoblotting assays were conducted to detect the levels of Gadd45a. As the controls, actin (cytosol protein) and ATF3 (nuclear protein) were examined in the same experiments. D, a Myc-tag-Gadd45a expression vector was co-transfected into HCT116 cells with B23 siRNA and nonspecific siRNA. Cells were collected 48 h later for preparation of cytosol and nuclear proteins, and Western analysis was carried out using antibodies to c-Myc and actin.

![Diagram](image)

**FIG. 6.** Suppression of endogenous B23 expression abrogates Gadd45a-induced cell cycle G2-M arrest. A, effect of B23 siRNA on Gadd45a-inducible expression. HCT116 Gadd45a-inducible cells were established as described previously. Cells were placed in 100-mm dishes at a density of 4 × 10^5 and grown in DMEM containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were transfected with either B23 siRNA or nonspecific RNA and harvested at the indicated time points. Whole cell lysates were isolated and followed by immunoblotting analysis with antibody to B23 and Gadd45a. B, knockdown of B23 protein results in abrogated Gadd45a-induced cell cycle G2-M arrest. HCT116 Gadd45a-inducible cells were growing in DMEM with 10% fetal bovine serum in the presence of tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were transfected with B23 siRNA, collected 36 h later, and subjected to flow cytometric analysis as described under “Experimental Procedures.” C, inhibition of B23 affects mitotic entry after inducible expression of Gadd45a protein. HCT116 Gadd45a-inducible cells were grown in medium with tetracycline (2 μg/ml) and treated with nocodazole for 24 or 36 h. Upon the withdrawal of tetracycline, cells were treated with B23 siRNA and followed by determination of mitotic indices at the indicated time points.
measurement of the mitotic index, nocodazole, a microtubule disrupter, was included in the experiments. In Fig. 6C, high mitotic indices were observed in HCT116 cells treated with nocodazole for 24 or 36 h. After withdrawal of tetracycline, mitotic indices substantially decreased, indicating that inducible expression of Gadd45a protein arrests cells in the G2-M transition. Addition of B23 siRNA at a concentration of 40 pmol was shown to greatly attenuate the Gadd45a-induced G2-M arrest. In contrast, nonspecific siRNA did not present a significant effect on Gadd45a-induced cell cycle G2-M arrest. Taken together with the results obtained from flow cytometric analysis, disruption of cellular B23 expression affected the G2-M accumulation by Gadd45a, suggesting that nuclear localization of Gadd45a protein be required for Gadd45a-induced cell cycle arrest.

DISCUSSION

The studies presented in this report further demonstrate that the stress-inducible gene Gadd45a is a nuclear protein despite the fact that Gadd45a does not contain a classical NLS. Most interestingly, Gadd45a nuclear localization was shown to be enhanced by DNA damage and is mediated through the interaction of Gadd45a with B23 proteins (nucleophosmin), which act as carrier proteins for nuclear import of certain proteins, indicating that Gadd45a nuclear translocation utilizes a distinct pathway from classical nuclear import machinery. By using Myc-tagged Gadd45a deletion fusion proteins, the B23-binding domain was mapped at the central region of the Gadd45a protein. This B23-binding motif was required for Gadd45a nuclear translocation because disruption of this domain abolished Gadd45a nuclear localization. In support of these observations, suppression of endogenous B23 proteins via employment of B23 siRNA substantially affected Gadd45a nuclear localization and greatly abrogated Gadd45a-induced cell cycle G2-M arrest.

Gadd45a is one of the p53-regulated genes (3, 15) and has been implicated in maintaining genomic fidelity (25). Deletion of the endogenous Gadd45a gene results in severe genomic instability, including aneuploidy, chromosomal aberrations, gene amplification, centrosome amplification, and increased carcinogenesis induced by DNA damage such as ionizing radiation and UV radiation (25–27). Previous reports from our laboratory have demonstrated that Gadd45a acts as a critical player in DNA damage-induced cell cycle arrest after treatment with certain DNA-damaging agents such as UV radiation or MMS (8). Induction of Gadd45a by either microinjection or via the Tet-Off system causes cells to arrest at G2/M phases (8, 11). In further support of these findings, MEFs derived from Gadd45a knock-out mice exhibited a defect in UV-activated G2-M arrest. These Gadd45a-deficient MEFs also presented a weak inhibition of Cdc2/cyclin B1 kinase activity after UV radiation treatment (Fig. 1, A and B). Taken together with the observations that inducible expression of Gadd45a resulted in accumulation of G2/M population (Fig. 1, C and D), Gadd45a acts as a critical player in DNA-damage-activated cell cycle G2-M arrest.

With regard to the molecular mechanism(s) by which Gadd45a functions in the control of the G2-M checkpoint, we have previously reported that Gadd45a interacts with Cdc2, dissociates the Cdc2/cyclin B1 complex, and inhibits Cdc2/cyclin B1 activity (9). Induction of Gadd45a leads to a reduction of nuclear cyclin B1 protein, whose nuclear localization is necessary for the completion of the G2-M transition (11). The Gadd45a-altered cyclin B1 nuclear localization correlates with suppression of Cdc2/cyclin B1 activity (10). These findings strongly suggest that Gadd45a nuclear translocation is critical for its role in cell cycle G2-M arrest. Because of the lack of a nuclear localization signal, the mechanism that mediates Gadd45a nuclear translocation remains unknown. In the current study, Gadd45a was demonstrated as a nuclear protein. After exposure to DNA-damaging agents, HCT116 cells displayed Gadd45a protein accumulation in both cytosol and nuclear compartments (Fig. 2A). Transfection of exogenous Myc tag-Gadd45a expression vectors into HCT116 cells also exhibited nuclear localization of Gadd45a (Fig. 2B). Additionally, Gadd45a nuclear localization was further confirmed by the evidence that green fluorescent Gadd45a fusion protein was observed primarily in the nucleus after introduction of a pGFP-Gadd45a vector into HCT116 cells (results not shown).

As discussed earlier, nuclear translocations of a large number of proteins are mediated through their NLS or M9 domains. However, there are NLS-independent and importin-independent nuclear import mechanisms for certain nuclear proteins such as β-catenin, a key member in the Wnt signaling pathway (31, 32). Additionally, B23 protein has been found to play an important role in carrying some proteins into the nucleus (36–40). Most interestingly, Gadd45a nuclear localization was also found to be mediated via its physical interaction with B23 protein (Fig. 5). These findings demonstrate that B23 is also involved in the nuclear translocation of certain DNA damage-inducible proteins, suggesting a role for B23 protein in cellular response to genotoxic stress. Most importantly, B23-mediated Gadd45a nuclear localization was clearly shown to be required for Gadd45a-induced cell cycle G2-M arrest. In the presence of B23 siRNA, which strongly suppresses endogenous B23 expression, HCT116 cells exhibited a disrupted cell cycle G2-M arrest after inducible expression of Gadd45a protein, as reflected by reduced G2/M accumulations and increased mitotic index (Fig. 6).

In addition to its association with B23, Gadd45a has been found to interact with several important cellular proteins such as Cdc2 kinase, proliferating cell nuclear antigen, core histone protein, p21<sup>WAF1/CIP1</sup>, and MTK/MEKK4 (9, 19–24), which play important roles in the control of cell cycle progression, DNA repair, and the regulation of signaling transduction pathways. The B23-binding domain was mapped at the central region of the Gadd45a protein. Most interestingly, this region is also critical for the interaction of Gadd45a with Cdc2 and for inhibition of Cdc2/cyclin B1 kinase activity (10). Deletion of the central region substantially abolished the capability of Gadd45a for inducing cell cycle arrest and suppressing cell growth. In addition, the p21<sup>WAF1/CIP1</sup>-interacting motif is localized at the central part of the Gadd45a protein as well. Collectively, the central region of Gadd45a appears to mediate the cross-talks between Gadd45a and other cell cycle regulators and coordinates the function of Gadd45a in the cellular response to genotoxic stress. In conclusion, we have made a novel observation that B23 protein, as a carrier protein for nuclear import of certain proteins, is involved in Gadd45a nuclear translocation and that B23-mediated Gadd45a nuclear translocation is required for Gadd45a-activated cell cycle G2-M arrest after DNA damage.

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