Gadd45a Interacts with Aurora-A and Inhibits Its Kinase Activity*

Shujuan Shao†,‡, Yang Wang‡,†, Shunqian Jin†,‡, Yongmei Song‡, Xiaoxia Wang§, Wenhong Fan**, Zhiying Zhao†,⁎, Ming Fu†, Tong Tong†, Lijia Dong†, Feiyue Fan‖, Ningzhi Xu§, and Qimin Zhan§,†

From the †State Key Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China, ‡Department of Radiation Oncology, Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, §Dalian Medical University, Dalian 116027, China, ¶Institute of Radiation Medicine, Tianjing 300192, China, and ‖Department of Neurobiology, Institute of Basic Medical Sciences, Beijing 100850, China

Centrosome stability is required for successful mitosis in mammalian cells. Amplification of the centrosome leads to chromosomal missegregation and generation of aneuploidy, which are closely associated with cell transformation and tumorigenesis (Doxsey, S. J. (2001) Nat. Cell Biol. 3, E105–E108; Hinchcliffe, E. H., and Sluder, G. (2001) Genes Dev. 15, 1167–1181; Pihan, G. A., Purohit, A., Wallace, J., Malhotra, R., Liotta, L., and Doxsey, S. J. (2001) Cancer Res. 61, 2212–2219). However, there are currently limited insights into mechanism(s) for this critical biological event. Here we show that Gadd45a, a DNA damage-inducible protein that is regulated by tumor suppressors p53 and BRCA1, participates in the maintenance of centrosome stability. Mouse embryonic fibroblasts derived from gadd45a-null mice exhibit centrosome amplification (designated as increased centrosome numbers). Introduction of exogenous Gadd45a into mouse embryonic fibroblasts isolated from gadd45a-null mice substantially restored the normal centrosome profile. In contrast to p21waf1/cip1, which ensures coordinated initiation of centrosome, Gadd45a had no significant effect on centrosome duplication in S phase. Interestingly Gadd45a was found to physically associate with Aurora-A protein kinase, whose deregulated expression results in centrosome abnormality. Furthermore Gadd45a was demonstrated to strongly inhibit Aurora-A kinase activity and to antagonize Aurora-A-induced centrosome amplification. These findings identify a novel mechanism for Gadd45a in the maintenance of centrosome stability and broaden understandings of p53- and BRCA1-regulated signaling pathways in maintaining genomic fidelity.

The essence of successful mitosis in mammalian cells is the generation of two genetically identical daughter cells. This requires the assembly of a strictly bipolar mitotic apparatus that will ensure that all daughter chromosomes are segregated to opposite sides of the cell before the completion of mitosis. This process is controlled by the centrosomes that organize the spindle poles during mitosis. In a manner similar to the chromosomal DNA of a cell, centrosomes replicate only once per cell cycle, generating two centrosomes, which then form the two poles of the mitotic spindle. If the centrosome duplicates more than once in a cell cycle then a multiple spindle may be assembled, and the chromosomes may be unequally distributed to the daughter cells, leading to genetic imbalances that generate genomic instability and produce cells with aggressive growth characteristics. Therefore, centrosome abnormalities may result in chromosomal missegregation and generation of aneuploidy, which are closely associated with cell transformation and tumorigenesis (1–4).

Centrosome amplification has been linked to numerous genetic aberrations (5, 6), including the loss of the tumor suppressor protein p53 (7, 8) or disruption of its downstream target genes such as p21 (9, 10) and GADD45a (11). Mouse embryonic fibroblasts (MEFs) derived from gadd45a-null mice display aneuploidy, chromosomal aberrations, gene amplification, and centrosome amplification (11). Other genetic alterations reported to affect centrosome numbers concern proteins involved in the response to DNA damage, including BRCA1 (12), BRCA2 (13), and ATR (14). In addition, several protein kinases have been implicated in the centrosome cycle, particularly in centrosome amplification. These include Aurora-A kinase, Aurora-B kinase (15–17), Polo kinase 1 (18–20), and NIMA-related kinase 2 (21–23). It has been reported that over-expression of Aurora-A kinase results in centrosome hypertrophy (16). The precise function of Aurora-A is not known, but it may be involved in centrosome separation, spindle assembly, and spindle maintenance. Furthermore Aurora-A is considered as an oncogene and is capable of conferring transformed phenotypes to non-oncogenic cells (16). Interestingly absence of p53 appears to exacerbate the Aurora-A-induced centrosome amplification, providing a plausible explanation for why cells

* This work was supported in part by National Fundamental Research Program of China Grant 2002CB513101, National Natural Science Foundation of China Grants 30225018 and 30400074, and National Institutes of Health Grant R01 CA93640. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These authors made equal contributions to this work.

‡ To whom correspondence should be addressed: State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences and Peking Union Medical College, Cancer Inst. and Cancer Hospital, Beijing 100021, China. Tel: 86-10-67762694; Fax: 86-10-67715058; E-mail: Zhanqimin@pumc.edu.cn.

3 The abbreviations used are: MEF, mouse embryonic fibroblast; MMS, methyl methanesulfonate; GFP, green fluorescent protein; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; MBP, myelin basic protein; p21, p21waf1/cip1.
with inactivated p53 function display increased centrosome numbers (8, 24).

GADD45a is a p53- and BRCA1-regulated stress gene and is highly induced by a variety of genotoxic agents, such as methyl methanesulfonate (MMS), UV radiation, and hydroxyurea (25). In addition to the response to base-damaging agents and certain other stress-related growth arrest treatments including hypoxia and growth factor withdraw, the human GADD45a gene is induced by ionizing radiation (26). The ionizing radiation induction of GADD45a is transcriptionally dependent on cellular p53 function. Recently GADD45a has been characterized as a downstream gene of BRCA1 (27, 28), a breast cancer-associated gene that plays roles in the control of cell cycle progression, apoptosis, DNA repair, and gene regulation. Gadd45a is an acidic and nuclear protein. It physically binds to multiple important cellular proteins, including proliferating cell nuclear antigen, p21waf1/cip1 (p21), Cdc2 protein kinase, core histones, and cellular p53 function. Recently GADD45a has been implicated in maintenance of genomic fidelity, although the precise mechanism(s) remains to be further defined. Previous findings have demonstrated that the MEFs derived from gadd45a knock-out mice exhibit aneuploidy, chromosomal aberrations, gene amplification, abnormal cytokinesis, and centrosome amplification. In addition, gadd45a-null mice are susceptible to DNA damage-induced tumors, including carcinogenesis induced by ionizing radiation, UV radiation and dimethylbenzanthracene. Interestingly Gadd45a has been shown to contribute to tumor progression and malignancy. In this study, we report that MEFs with disrupted Gadd45a exhibit centrosome amplification as manifested by increased centrosome copies. Gadd45a was found to physically associate with Aurora-A kinase. The interaction between Gadd45a and Aurora-A was shown to strongly inhibit Aurora-A kinase activity. These observations provide a tight connection between Gadd45a and centrosome stability, further supporting a role for Gadd45a in the maintenance of genomic fidelity.

EXPERIMENTAL PROCEDURES

Plasmid Clones and Antibodies—Myc-tagged Gadd45a expression vectors were described previously (29, 30). pCMV45 and pCMV-p21 are the expression vectors for Gadd45a. They were constructed by PCR cloning of the open reading frame regions from human cDNA clones for GADD45a (pHu145B2) and p21waf1/cip1 (pZL-WAF1). The PCR products were inserted into the HindIII/XbaI sites in pCMV3 downstream from cytomegalovirus promoter. PEGFP-Gadd45a, pEGFP-p21, and pEGFP-p53 were made by inserting open reading frames of GADD45a, p21, and p53 into BglII/KpnI sites of pEGFP-C1 vector, respectively. These constructs express green fluorescent proteins (GFPs). pEGFP-Aurora-A was constructed by inserting the open reading frame region of Aurora-A into Xhol/BamHI sites of pEGFP-C1 vector. Myc-tagged Aurora-A was made by inserting the open reading frame of Aurora-A into NocI/Xhol sites of pCS2-MT vector.

GST-Gadd45a was made by cloning GADD45a cDNA into the Xhol site of the pGEX-5X-1 vector. GST-Cdc2, GST-p53, GST-p21, and GST-ERK were constructed by inserting their open reading frames into the EcoRI/Xhol sites of pGEX-5X-1 plasmid. GST-B23 was provided by Dr. Kenji at the University of Cincinnati.

The following antibodies were used in the experiments. Antibodies against Gadd45a, Cdc2, c-Myc, γ-tubulin, and Actin were commercially provided by Santa Cruz Biotechnology (Santa Cruz, CA). Aurora-A was purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture and Transfection—MEFs derived from both normal and gadd45a knock-out mice were maintained in minimum Eagle’s medium supplemented with 15% fetal bovine serum (FBS). Normal human fibroblasts were maintained in Dulbecco’s modified Eagle’s medium with 10% FBS. Human cervical carcinoma (HeLa) cells and Chinese hamster ovary (CHO) cells were grown in F-12 medium (Invitrogen) with 10% FBS. For cell transfection, 5 × 10^4 HeLa or CHO cells were seeded onto 10-mm plates 1 day prior to transfection. In each plate, 5 μg of plasmid DNA and 15 μl of Lipofectamine (Invitrogen) were added to 300 μl of Opti-MEM (Invitrogen) in separate tubes. The solutions were mixed gently, allowed to sit for 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 medium with 10% FBS were added, and plates were incubated overnight. Fresh medium was added the following day, and cells were harvested 48 h later.

Cell Treatment—For UV treatment, cells plated in 100-mm dishes were rinsed with PBS and irradiated with UV radiation at a dose of 10 J/m². After cell exposure to UV radiation, fresh medium was added in plates, and the cells were cultured in the incubator until harvest. For MMS treatment, cells were grown in medium containing MMS at a concentration of 50 μg/ml for 4 h. Medium was replaced, and cells were collected at the indicated time.

Cellular Protein Preparation, Immunoprecipitation, and Immunoblotting Analysis—Following treatment, cells were harvested, rinsed with PBS, and lysed in PBS containing 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% Nonidet P-40 (lysis buffer). Lysates were collected by scraping and cleared by centrifugation at 4 °C. 100 μg of cellular protein were resolved by 12% SDS-PAGE and transferred to Protran membranes. Membranes were blocked for 1 h at room temperature in 5% milk, washed with PBST (PBS with 0.1% Tween 20), and incubated with the indicated antibodies for 2 h. Membranes were washed four times in PBST, and horseradish peroxidase-conjugated anti-mouse antibody was added at 1:4000 in 5% milk. After 1 h, membranes were washed and detected by ECL (Amersham Biosciences) and exposed to x-ray film (Eastman Kodak Co.). For immunoprecipitation, cellular lysates were incubated with 10 μl of the indicated antibodies and 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) at 4 °C for 6 h. Immunocomplexes were analyzed as described above.
GST Preparations—GST fusion protein expression was performed as follows. *Escherichia coli* were treated with 0.1 mM isopropyl 1-thio-β-p-galactopyranoside for GST protein induction. Bacteria were collected by centrifugation, and bacterial pellets were resuspended in cold STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl). After incubation with lysozyme (100 μg/ml), bacteria were treated with dithiothreitol (10 mM) and Sarkosyl (0.7%) and subjected to sonication. Following centrifugation, supernatants were treated with Triton X-100 at a final concentration of 2%. Next the glutathione-agarose beads were mixed with supernatant solution at 4 °C overnight. After washing several times with PBS, glutathione-agarose bead-conjugated GST fusion proteins were ready for use in the designed experiments.

Immunofluorescent Staining of Centrosome—Normal human fibroblasts were placed in 100-mm dishes. 16 h later, cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature and washed with PBS. Cells were treated with cold methanol at −20 °C followed by 0.5% Triton X-100 and 0.5% bovine serum albumin in PBS for 30 min. After incubation with antibodies to γ-tubulin and Gadd45a, plates were washed and incubated with Cy3- or Cy4-conjugated goat anti-mouse IgG. Following washing with PBS, cells were visualized using an Olympus fluorescence microscope, and photographs were generated using a Kodak digital camera. Similarly MEFs were placed onto Lab-tek (Nunc) tissue culture slides. After incubation, the slides were stained with monoclonal anti-γ-tubulin antibody and rhodamine-conjugated goat anti-mouse IgG. After washing with PBS, cells were examined under a fluorescence microscope.

Aurora-A Kinase Assay—HeLa cells were grown in F-12 medium with 10% FBS and transfected with 5 μg of Myc-tagged Aurora-A expression vector. 48 h later, cells were collected for preparation of cellular protein. 400 μg of whole cell lysates were incubated with 15 μl of anti-Myc antibody at 4 °C for 6 h, and the immunoprecipitated Myc-tagged Aurora-A proteins were incubated with the indicated amounts of GST-Gadd45a, GST-p21, and GST proteins. Kinase assays were then conducted in the presence of 10 μg of myelin basic protein (MBP; Sigma), 15 mM MgCl₂, 7 mM β-glycerol phosphate, 1.5 mM EDTA, 0.25 mM sodium orthovanadate, 0.25 mM dithiothreitol, and 10 μCi of [γ-32P]ATP in 30-μl total volumes. After 15 min at 30 °C, the reactions were mixed with an equal amount of protein loading buffer, size-separated by 12% SDS-PAGE, and analyzed by autoradiography. In some cases, 1 mg of cellular protein was directly immunoprecipitated by antibody to Aurora-A, and kinase assays using MBP as substrate were then performed.

RESULTS

Centrosome Amplification in gadd45a−/− MEFs—In addition to its role in the control of cell cycle G2-M checkpoint after DNA damage, previous observations have demonstrated that Gadd45a is involved in the maintenance of chromosomal and centrosome stability (11). To further confirm the impact of Gadd45a on genomic stability, the MEFs derived from both wild type or gadd45a-null mice were used for analysis of centrosome abnormalities. MEFs were grown on tissue culture slides and stained with monoclonal antibody against γ-tubulin protein, which is specifically localized at centrosome. After incubation with Cy3-conjugated goat anti-mouse IgG, centrosome numbers were then examined using fluorescence microscopy. It should be noted here that in addition to γ-tubulin antibody, the antibody to pericentrin was also used to further confirm that γ-tubulin staining structures are real centrosomes, and we found that both antibodies presented similar

**FIGURE 1. Detection of centrosome amplifications in mice embryonic fibroblasts.** A, MEFs derived from normal mice (gadd45a+/+) or gadd45a knock-outs (gadd45a−/−) were grown in minimum Eagle’s medium with 10% FBS and placed onto Lab-tek (Nunc) tissue culture slides. After fixation, the slides were stained with monoclonal anti-γ-tubulin antibody and rhodamine-conjugated goat anti-mouse IgG. Cells and nuclei labeled with 4,6-diamidino-2-phenylindole (DAPI) were visualized using an Olympus fluorescence microscope, and photographs were generated using a Kodak digital camera. The quantitative results were obtained from four separate experiments, and in each experiment, more than 400 cells were examined.
Gadd45a Inhibits Aurora-A Activity

FIGURE 2. A, restoration of normal centrosome status by Gadd45a in gadd45a−/− MEFs. pMSCV-GFP, pMSCV-GFP-Gadd45a, pMSCV-GFP-p21, or pMSCV-GFP-p53 expression vectors were transfected into gadd45a−/− MEF cells. 96 h later, cells were stained with anti-γ-tubulin antibody, and centrosomes were examined in 400 GFP-positive cells. The experiment was repeated three times. B, meanwhile cells transfected with different expression vectors were collected for preparation of cell lysates. 100 μg of cellular protein were resolved by 12% SDS-PAGE for measuring the protein expressions of GFP, Gadd45a, p21, and p53. C, effects of Gadd45a and p21 in centrosome reduplication at S phase. CHO cells were transfected with the indicated expression vectors (pEGFP, pEGFP-p21, and pEGFP-Gadd45a). 48 h later, cells were synchronized using hydroxyurea. 96 h post-transfection, cells were stained with anti-γ-tubulin antibody, and centrosomes were examined in 400 GFP-positive cells under a fluorescence microscope. The experiment was repeated more than three times. D, similar to B. Following transfection, cells were collected for preparation of cellular protein 96 h later. 100 μg of whole cell protein were examined for expression of GFP, Gadd45a, and p21.

results (results not shown). As shown in Fig. 1A, MEFs isolated from wild type mice (gadd45a+/+) displayed one or two centrosomes. However, MEFs from gadd45a-null mice (gadd45a−/−) exhibited more than two centrosomes. The quantitative results are summarized in Fig. 1B where ~30% of gadd45a−/− MEF cells display centrosome amplification (defined as centrosome copies equaling three or greater than three per cell) compared with 3.7% of MEFs (gadd45a+/+) showing centrosome amplification. These results indicate that disruption of endogenous gadd45a results in abnormally amplified centrosome.

We next examined whether overexpression of Gadd45a protein in gadd45a-deficient cells restores normal centrosome status. pMSCV-GFP-Gadd45a, a retroviral expression vector that produces GFP-Gadd45a fusion protein, was transfected into gadd45a−/− MEF cells. 96 h later, cells were stained with anti-γ-tubulin antibody, and centrosomes were examined in 400 GFP-positive cells. As shown in Fig. 2A, gadd45a−/− MEFs transfected with GFP empty vector displayed 27% of cells containing three or more than three centrosomes. Following introduction of pMSCV-GFP-Gadd45a expression vector into gadd45a-deficient MEFs, amplified centrosomes were reduced by two-thirds, indicating that expression of exogenous Gadd45a substantially restored the normal centrosome profile in gadd45a−/− MEFs. In the same experiments, p21, a p53-regulated Cdk inhibitor, was also included. Although p21 has been characterized as a major component in regulating centrosome duplication and defect of cellular p21 causes centrosome hyperamplification, overexpression of p21 in gadd45a−/− MEF cells did not restore centrosome abnormality, suggesting that p21 and Gadd45a act on different molecular targets involved in the process of centrosome replication. However, introduction of p53 expression vector into gadd45a−/− MEFs partially rescued centrosome abnormalities. To confirm exogenous expressions of various proteins (Gadd45a, p21, and p53), we examined cellular lysates using immunoblotting analysis and found that those proteins were well expressed in gadd45a−/− MEF cells following transfection (Fig. 2B). Additionally apoptosis was also examined following overexpression of Gadd45a in gadd45a−/− MEF cells, and no evident cell death was observed in the experiments (data not shown).

Gadd45a Expression Does Not Affect Centrosome Duplication in S Phase—Centrosome amplification has been observed to often occur at S and late mitotic phases (8, 9, 16, 24). The former is associated with centrosome over-replication that is carried out by Cdk2-Cyclin E kinase complex, and the latter is associated with aborted cytokinesis that might be due to overactivity of Aurora kinases or polo kinases. A number of investigations have demonstrated that p21 plays a critical role in regulating centrosome replication in S phase through its inhibition of Cdk2-Cyclin E kinase. Although the previous studies have shown that Gadd45a has no inhibitory effect on Cdk2-Cyclin E kinase, we examined whether Gadd45a acts on S phase-related centrosome amplification (Fig. 2C). We transfected pEGFP-Gadd45a or pEGFP-p21 expression vectors into CHO cells and synchronized cells at S phase using hydroxyurea, which is a chemical blocker of DNA synthesis. Following synchronization by hydroxyurea, more than 60% of untransfected cells displayed increased centrosome numbers (three or greater than three copies). Introduction of p21 expression vector substantially inhibited centrosome amplification. In the presence of p21 protein, cells containing amplified centrosome were dropped from 67 to 9%, further confirming that p21 mainly targets Cyclin E-Cdk2 kinase complex and then ensures coordinated initiation of centrosome. In contrast, Gadd45a had no significant effect on centrosome amplification, indicating that Gadd45a does not act on centrosome duplication at S phase. These results are consistent with the findings shown in Fig. 2A that p21 and Gadd45a act on different components involved in centrosome stability.
Gadd45a Physically Interacts with Aurora-A—Previous studies have demonstrated that Gadd45a is neither a kinase nor a phosphatase. Likely the role of Gadd45a in maintenance of centrosome stability might be mediated through its interaction with certain regulatory components involved in centrosome duplication. Using a “yeast two-hybrid” approach, we identified 12 candidates of the Gadd45a-interacting protein, one of which is Aurora-A kinase, which is a key regulator in centrosome separation and maturation. Because deregulated expression of Aurora-A has been shown to result in centrosome instabilities, efforts were made to determine the physical association of Gadd45a with Aurora-A. In Fig. 3A, HeLa cells were transfected with Myc-tagged Gadd45a vector, and cellular lysates were incubated with a group of GST fusion proteins. Clearly Myc-tagged Gadd45a was pulled down by GST-Aurora-A as well as by GST-Cdc2, GST-p21, and GST-B23, which were reported previously to interact with Gadd45a. In contrast, GST-Cyclin B1 and GST alone did not pull down Myc-tagged Gadd45a fusion protein. Next GST-Gadd45a protein was incubated with cell lysates from untreated HeLa cells and followed by pulldown assay. As shown in Fig. 3B, endogenous Aurora-A protein was detected in GST-Gadd45a pulldown complexes. As a positive control, endogenous Cdc2 was pulled down by GST-Gadd45a. An association between cellular Gadd45a and GST-Aurora-A proteins was also demonstrated. GST-Aurora-A, GST-Cdc2, and GST-Cyclin B1 fusion proteins were incubated with cellular lysates isolated from HeLa Gadd45a-inducible cells, which were collected at 24 h after withdrawal of tetracycline and which expressed substantial amounts of Gadd45a protein, and followed by pulldown assays. In Fig. 3C, both GST-Cdc2 and GST-Aurora-A were able to pull down Gadd45a, but GST alone or GST-Cyclin B1 did not associate with cellular Gadd45a. Additionally the physical interactions between endogenous Gadd45a and Aurora-A proteins were further examined. Cellular lysates were prepared from HeLa cells treated with UV radiation (10 J/m²) and incubated with anti-Aurora-A antibody. As shown in Fig. 3D, endogenous Gadd45a protein was highly induced after cell exposure to UV radiation (upper panel). Following immunoprecipitation by the antibody to Aurora-A, endogenous Gadd45a was detected. Interestingly the interactions between Gadd45a and Aurora-A proteins were enhanced by DNA treatment because increased amounts of Gadd45a protein were observed in Aurora-A immunocomplexes following UV radiation. To rule out nonspecific interactions, the antibody to Actin was also included in the immunoprecipitation assays. Both Gadd45a and Aurora-A proteins were not seen in Actin immunocomplexes. Collectively these results indicate a specific physical interaction of Gadd45a with Aurora-A.

Gadd45a Inhibits Aurora-A Kinase Activity—The kinase activity of Aurora-A is closely associated with Aurora-A-induced centrosome abnormalities despite the observation that overexpression of inactive Aurora-A kinase also deregulates the control of centrosome duplication. Interestingly overexpression of wild type Aurora-A or a constitutively active mutant of the kinase in NIH3T3 or Rat1 cells leads to more aggressive properties, but that was not the case when an inactive mutant Aurora-A was overexpressed, suggesting that high activity of Aurora-A is tightly connected with genomic instability and malignant transformation. To determine whether Gadd45a protein inhibits Aurora-A activity, Myc-tagged Aurora-A expression vector was transfected into HeLa cells. 48 h post-transfection, whole cell protein extracts were prepared and pulled down with GST and GST-Cyclin B1, GST-p21, GST-Cdc2, GST-ERK, GST-B23, and GST-Aurora-A. The immunocomplexes were washed three times with lysis buffer and analyzed by SDS-PAGE followed by immunoblotting with anti-Myc antibody. B, GST and GST-Gadd45a were incubated with cell lysates isolated from HeLa cells. The GST pulldown complexes were examined by SDS-PAGE and immunoblotting with anti-Aurora-A and -Cdc2 antibodies. C, GST, GST-Cdc2, GST-Aurora-A, or GST-Cyclin B1 was incubated with cell lysates isolated from HeLa Gadd45a-inducible (Tet-Off) cells, which express high levels of Gadd45a after withdrawal of tetracycline, and followed by immunoblotting assay with antibody to Gadd45a as described in A. D, HeLa cells were treated with UV radiation at a dose of 10 J/m² and collected at the indicated time points for preparation of whole cell lysates. 500 µg of protein were immunoprecipitated with antibody to Aurora-A. The immunocomplexes were analyzed with anti-Gadd45a or -Aurora-A antibodies. To rule out nonspecific interactions, the antibody against Actin was included in the experiments. IP, immunoprecipitate; IB, immunoblot; Ab, antibody.

FIGURE 3. Physical interaction of Gadd45a with Aurora-A. A, Myc-tagged Gadd45a vector was transiently expressed in HeLa cells via Lipofectamine transfection. 48 h post-transfection, whole cell protein extracts were prepared and pulled down with GST and GST-Cyclin B1, GST-p21, GST-Cdc2, GST-ERK, GST-B23, and GST-Aurora-A. The immunocomplexes were washed three times with lysis buffer and analyzed by SDS-PAGE followed by immunoblotting with anti-Myc antibody. B, GST and GST-Gadd45a were incubated with cell lysates isolated from HeLa cells. The GST pulldown complexes were examined by SDS-PAGE and immunoblotting with anti-Aurora-A and -Cdc2 antibodies. C, GST, GST-Cdc2, GST-Aurora-A, or GST-Cyclin B1 was incubated with cell lysates isolated from HeLa Gadd45a-inducible (Tet-Off) cells, which express high levels of Gadd45a after withdrawal of tetracycline, and followed by immunoblotting assay with antibody to Gadd45a as described in A. D, HeLa cells were treated with UV radiation at a dose of 10 J/m² and collected at the indicated time points for preparation of whole cell lysates. 500 µg of protein were immunoprecipitated with antibody to Aurora-A. The immunocomplexes were analyzed with anti-Gadd45a or -Aurora-A antibodies. To rule out nonspecific interactions, the antibody against Actin was included in the experiments. IP, immunoprecipitate; IB, immunoblot; Ab, antibody.
Gadd45a Inhibits Aurora-A Activity

![Graph](image)

**FIGURE 4.** Gadd45a protein inhibits Aurora-A kinase activity. A, HeLa cells were transfected with 5 μg of Myc-tagged Aurora-A expression vector. 48 h later, cells were collected for preparation of cellular protein. 400 μg of whole cell lysates were pulled down with anti-Myc antibody, and the in vitro kinase assays using MBP as the substrate were carried out. The immunoprecipitated Myc-tagged Aurora-A proteins were incubated with the indicated amounts of GST-Gadd45a, GST-p21, and GST proteins. Phosphorylated MBP was detected by autoradiography following size separation by SDS-PAGE. In addition, instead of loading the samples onto the gel for electrophoretic analysis, 20 μl aliquots were spotted on phosphocellulose paper and then washed three times with 0.75% phosphoric acid. After an acetone rinse, radioactivity was measured by scintillation counting. The quantitative data are the average of three separate experiments. B, cellular proteins were prepared from HeLa cells. 1 mg of protein was incubated with the antibodies to Cdc2, Aurora-A, or Cdk2 followed by immunoprecipitation. Immunocomplexes were then used for kinase assays using either histone H1 or MBP as substrates. C, MEFs derived from both wild type and gadd45a knock-outs were treated with UV radiation (10 J/m²) or MMS (50 μg/ml), which are DNA-damaging agents and highly induce cellular Gadd45a protein. Cell lysates were prepared 8 h post-treatment and immunoprecipitated with antibody to Aurora-A. A kinase assay was then carried out in the presence of [γ-32P]ATP and MBP. As shown in Fig. 4C, the levels of Aurora-A kinase activity were similar in untreated MEFs. After UV radiation or MMS treatment, wild type MEFS exhibited a reduction in Aurora-A kinase activity, but MEFS from gadd45a-null mice did not display any significant inhibition on Aurora-A kinase activity. It should be mentioned here that equal amounts of immunoprecipitated Aurora-A were used in the kinase assays as the aliquots of immunoprecipitates were examined with Western analysis. Taken together with the findings in Fig. 4, A and B, these results indicate that Gadd45a inhibits Aurora-A kinase activity.

**FIGURE 5.** Effect of Gadd45a protein on Aurora-A-induced centrosome amplification. 1 × 10^5 CHO cells were placed onto 100-mm dishes and transfected with pEGFP or pEGFP-Aurora-A vectors. In some cases, pEGFP-Aurora-A vector was co-introduced with pCMV-Gadd45a or pCMV-p21. 96 h later, cells were fixed and subjected to centrosome analysis as described in Fig. 2A. Each experiment was performed more than three times. In each experiment, centrosome numbers were examined in 500 GFP-positive cells. Quantitative results represent the average of three individual experiments.

Deregulated expression of Aurora-A is a specific effect. It should be noted here that the inhibition of activities for Cdc2 and Aurora-A kinases but did not affect Cdk2 kinase activity, indicating that Gadd45a inhibition of Aurora-A is a specific effect. It should be noted here that the experiments were conducted to examine whether Aurora-A can directly phosphorylate Gadd45a but failed to get positive results (data not shown), ruling out the possibility that the inhibition of MBP phosphorylation by Aurora-A could be due to competitive inhibition of Gadd45a with Aurora-A.

To explore the effect of Gadd45a on cellular Aurora-A in vivo, Aurora-A kinase activity was examined in MEFS derived from wild type and gadd45a-null mice (gadd45a^−/−). Both types of MEFS were treated with UV radiation (10 J/m²) and MMS (50 μg/ml), which are DNA-damaging agents and highly induce cellular Gadd45a protein. Cell lysates were prepared 8 h post-treatment and immunoprecipitated with antibody to Aurora-A. A kinase assay was then carried out in the presence of [γ-32P]ATP and MBP. As shown in Fig. 4C, the levels of Aurora-A kinase activity were similar in untreated MEFS. After UV radiation or MMS treatment, wild type MEFS exhibited a reduction in Aurora-A kinase activity, but MEFS from gadd45a-null mice did not display any significant inhibition on Aurora-A kinase activity. It should be mentioned here that equal amounts of immunoprecipitated Aurora-A were used in the kinase assays as the aliquots of immunoprecipitates were examined with Western analysis. Taken together with the findings in Fig. 4, A and B, these results indicate that Gadd45a inhibits Aurora-A kinase activity.

Gadd45a Antagonizes Aurora-A-induced Centrosome Amplification—It was thought that deregulated expression of Aurora-A results in centrosome abnormalities. To demonstrate the inhibitory effect of Gadd45a on Aurora-A-induced centrosome amplification, CHO cells were transfected with pEGFP empty vector (control) or pEGFP-Aurora-A expression vector, and centrosome copies were analyzed 96 h post-transfection. As shown in Fig. 5, Aurora-A-transfected CHO cells exhibited evident centrosome amplification as reflected by the fact that 38% of cells overexpressing Aurora-A contained three or greater than three centrosomes. In contrast, only 4% of GFP empty vector-transfected cells revealed increased centrosome numbers (three or greater than three centrosomes per cell).

However, when pEGFP-Aurora-A was co-expressed with a Gadd45a expression vector, the CHO cells containing increased centrosome numbers were reduced from 38 to 11%, but co-expression of p21 was unable to suppress Aurora-A-induced centrosome amplification. These observations demonstrate that Gadd45a antagonizes induction of centrosome amplification by Aurora-A.
DISCUSSION

GADD45a is a downstream target effector gene for both p53 and BRCA1 and is highly induced by a wide spectrum of genotoxic agents, including ionizing radiation, MMS, and UV radiation (25). In addition to its roles in the control of cell cycle G2-M checkpoint and induction of apoptosis, Gadd45a appears to be one of the key components in regulating centrosome stability. MEFs derived from gadd45a-null null mice display aneuploidy, chromosomal aberrations, abnormal cytokinesis, and gene amplification (11). The observations that MEFs with disrupted Gadd45a exhibited increased centrosome copies (Fig. 1, A and B) further confirmed that Gadd45a plays a critical role in the maintenance of centrosome stability. We also conducted cell cycle synchronization using either serum starvation that arrests cell at G0/G1 phase or nocodazole, a microtubule disruptor that arrests cells at late mitotic phase, and examined centrosome numbers but found that centrosome amplification in gadd45a-deficient cells occurred in all different phases and was independent of cell cycle (results not shown). In support of these findings, expression of exogenous Gadd45a in gadd45a-deficient cells was shown to greatly restore normal centrosome states (Fig. 2A). Because centrosome abnormalities are associated with malignant transformation, the finding that disruption of Gadd45a leads to centrosome amplification can at least in part account for susceptibility of the gadd45a−/− knock-out animals to DNA damage-induced carcinogenesis by ionizing radiation, UV radiation, and dimethylbenzanthracene.

Centrosome abnormalities are tightly linked to genetic alterations in the tumor suppressors p53 and BRCA1. p53 directly interacts with Aurora-A kinase and suppresses its kinase activity. In addition, the p53-regulated p21, a potent inhibitor of cell cycle kinases, has been shown to regulate centrosome replication through its inhibition of Cyclin E-Cdk2 activity. In contrast to well characterized mechanism(s) by which p53 plays an important role in maintenance of centrosome stability, the pathway that mediates the role of BRCA1 in regulating normal centrosome states is currently unclear, although BRCA1 has been shown to localize at centrosome via its physical interaction with γ-tubulin, a major component of the centrosome. Several reports by others and by us have demonstrated that GADD45a is transcriptionally up-regulated by BRCA1 in a p53-independent manner, suggesting that Gadd45a possibly might mediate the role of BRCA1 in regulating normal centrosome states is currently unclear, although BRCA1 has been shown to localize at centrosome via its physical interaction with γ-tubulin, a major component of the centrosome. Several reports by others and by us have demonstrated that GADD45a is transcriptionally up-regulated by BRCA1 in a p53-independent manner, suggesting that Gadd45a possibly might mediate the role of BRCA1 in the control of genomic fidelity and cell cycle progression. Recently genetic interactions between BRCA1 and Gadd45a were characterized by crossing genetic mutants of BRCA1 and Gadd45a (31). Because centrosome amplification was seen to dramatically increase in the mice with combined mutations, this synergistic effect suggests that the relationship between BRCA1 and Gadd45a in centrosome duplication is not just epistatic. Although BRCA1 plays a function not only through Gadd45a, this BRCA1 downstream target gene would still be expected to at least in part mediate the role of BRCA1 in regulating centrosome duplication.

Both GADD45a and p21 are transcriptionally activated by p53 and play important roles in the control of cell cycle checkpoints. However, these two proteins appear to act on different phases of the cell cycle. p21 has been demonstrated to guard against premature activation of Cdk2-Cyclin E and function in the initiation of centrosome duplication. In contrast, Gadd45a acts on Aurora-A kinase to ensure centrosome maturation or completion of cytokinesis. Given our previous finding that Gadd45a inhibits Cdc2-Cyclin B1 and blocks cell entry into mitosis, Gadd45a might regulate centrosome stability probably via its inhibitory effect on both Aurora-A and Cdc2 activities. Thus, the observations presented here have broadened the p53-regulated pathways involved in the control of centrosome stability.

Several protein kinases have been implicated in the centrosome cycle, particularly in centrosome amplification. These include Aurora-A kinase, Aurora-B kinase (15–17), polo kinase 1 (18–20), and NIMA-related kinase 2 (21–23). Overexpression of Aurora-A kinase results in centrosome hypertrophy (16). The precise function of Aurora-A is not known, but it may be involved in centrosome separation, spindle assembly, and spindle maintenance. Furthermore Aurora-A is considered as an oncogene and is capable of conferring transformed phenotypes to non-oncogenic cells (16). In the current study, we defined a strong inhibitory effect of Gadd45a on Aurora-A kinase activity (Fig. 4). Addition of GST-Gadd45a protein resulted in suppression of Aurora-A activity (Fig. 4, A and B). Interestingly following UV radiation or MMS treatment, MEFs derived from wild type mice showed a strong reduction of Aurora-A kinase activity, whereas the inhibition was greatly abolished in the cells with disrupted Gadd45a, suggesting that Gadd45a inhibition of Aurora-A is likely enhanced in response to DNA damage.

The Gadd45a inhibition of Aurora-A kinase activity is likely to be mediated through its physical interactions with Aurora-A protein. Although Gadd45a has been shown to physically associate with Cdc2, whose activity is mainly required for G2-M transition, the interactions between Gadd45a and Cdc2 or Aurora-A appear to be different. The Cdc2-binding motif is characterized at the central region of Gadd45a, but Aurora-A-binding domain was found to be located at the N terminus of the Gadd45a protein (results not shown). As discussed earlier, overexpression of Aurora-A leads to centrosome hypertrophy. This was further confirmed by the observations in chinese hamster ovary cells transfected with Aurora-A expression vector (Fig. 5). Consistent with the finding that Gadd45a suppressed Aurora-A kinase activity (Fig. 4), the Aurora-A-induced centrosome amplification was antagonized by co-introduction of Gadd45a, but expression of p21 had no effect on Aurora-A-caused centrosome abnormality. Given the fact that centrosome amplification in cells with disrupted Gadd45a is coupled with aborted cytokinesis, which often occurs due to deregulation of Aurora kinases, our results further support the possibility that the failure to undergo cytokinesis also results in centrosome amplification in addition to failure of inhibition of centrosome reduplication in G/S phases.

Acknowledgments—We thank Dr. Albert J. Fornace and M. Christine Hollander at the National Institutes of Health for providing us with gadd45a knock-out MEFs. In addition, we thank Dr. Sen at the University of Texas M. D. Anderson Cancer Center for providing us with Aurora-A cDNA.
REFERENCES

1. Doxsey, S. J. (2001) Nat. Cell Biol. 3, E105–E108
2. Hinchcliffe, E. H., and Sluder, G. (2001) Genes Dev. 15, 1167–1181
3. Pihan, G. A., Purohit, A., Wallace, J., Malhotra, R., Liotta, L., and Doxsey, S. J. (2001) Cancer Res. 61, 2212–2219
4. Raff, J. W. (2001) Curr. Biol. 11, R159–R161
5. Salisbury, J. L., Whitehead, C. M., Lingle, W. L., and Barrett, S. L. (1999) Biol. Cell 91, 451–460
6. Duensing, S., and Munger, K. (2001) Biochim. Biophys. Acta 2, M81–M88
7. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. (1996) Science 271, 1744–1747
8. Chiba, S., Okuda, M., Musssman, J. G., and Fukasawa, K. (2000) Exp. Cell Res. 258, 310–321
9. Tarapore, P., Horn, H. F., Tokuyama, Y., and Fukasawa, K. (2001) Oncogene 20, 3173–3184
10. Toiyama, Y., Horn, H. F., Kawamura, K., Tarapore, P., and Fukasawa, K. (2001) J. Biol. Chem. 276, 21529–21537
11. Hollander, M. C., Sheikh, M. S., Bulavin, D. V., Lundgren, K., Auger-Hennueller, L., Shehee, R., Molinaro, T. A., Kim, K. E., Tolosa, E., Ashwell, J. D., Rosenberg, M. P., Zhan, Q., Fernandez-Salguero, P. M., Morgan, W. F., Dang, C. X., and Fornace Jr., A. J. (1999) Nat. Genet. 23, 176–184
12. Hsu, L. C., and White, R. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12983–12988
13. Tutt, A., Gabriel, A., Bertwistle, D., Connor, F., Paterson, H., Peacock, J., Ross, G., and Ashworth, A. (1999) Curr. Biol. 9, 1107–1110
14. Smith, L., Liu, S. I., Goodrich, L., Jacobson, D., Deganin, C., Bentley, N., Carr, A., Flagg, G., Keegan, K., Hoekstra, M., and Thayer, M. J. (1998) Nat. Genet. 19, 39–46
15. Giet, R., and Prigent, C. (1999) J. Cell Sci. 112, 3591–3601
16. Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998) Nat. Genet. 20, 189–193
17. Takahashi, T., Futamura, M., Yoshimi, N., Sano, J., Katada, M., Takagi, Y., Kimura, M., Yoshioka, T., Okano, Y., and Saji, S. (2000) Jpn. J. Cancer Res. 91, 1007–1014
18. do Carmo Avides, M., Tavares, A., and Glover, D. M. (2001) Nat. Cell Biol. 3, 421–424
19. Donaldson, M. M., Tavares, A. A., Hagan, I. M., Nigg, E. A., and Glover, D. M. (2001) J. Cell Sci. 114, 2357–2358
20. Goepfert, T. M., and Brinkley, B. R. (2000) Curr. Top. Dev. Biol. 49, 331–342
21. Fry, A. M., Meraldi, P., and Nigg, E. A. (1998) EMBO J. 17, 483–492
22. Fry, A. M., Descombes, P., Twomey, C., Bacchieri, R., and Nigg, E. A. (2000) J. Cell Sci. 113, 1973–1984
23. Ha Kim, Y., Yeol Choi, J., Jeong, Y., Wolgemuth, D. J., and Rhee, K. (2002) Biochem. Biophys. Res. Commun. 290, 730–736
24. Meraldi, P., Honda, R., and Nigg, E. A. (2002) EMBO J. 21, 483–492
25. Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8800–8804
26. Papathanasiou, M. A., Kerr, N. C., Robbins, J. H., McBride, O. W., Alamo, I., Jr., Barnett, S. F., Hickson, I. D., and Fornace, A. J., Jr. (1991) Mol. Cell. Biol. 11, 1009–1016
27. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y. H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. (1999) Cell 97, 575–586
28. Jin, S., Zhao, H., Fan, F., Blanck, P., Fan, W., Colchagie, A. B., Fornace Jr., A. J., and Zhan, Q. (2000) Oncogene 19, 4050–4057
29. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. (1999) Oncogene 18, 2892–2900
30. Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P., Roller, P. P., Fornace, A. J., Jr., and Zhan, Q. (2000) J. Biol. Chem. 275, 16602–16608
31. Wang, X., Wang, R. H., Li, W., Xu, X., Hollander, M. C., Fornace, A. J., Jr., and Dang, C. X. (2004) J. Biol. Chem. 279, 29606–29614