Identification of a Functional Domain in a GADD45-mediated G2/M Checkpoint*

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Cell cycle checkpoints are essential for the maintenance of genomic stability in response to DNA damage. We demonstrated recently that GADD45, a DNA damage-inducible protein, activates a G2/M checkpoint induced by either UV radiation or alkylating agents. GADD45 can interact in vivo with the G2 cell cycle-specific kinase, Cdc2, proliferating cell nuclear antigen (PCNA), and the cell cycle kinase inhibitor p21waf1. The ability of GADD45 to induce a G2/M arrest may be caused in part by the inhibition of Cdc2 kinase activity. Here, we report the identification of a region of GADD45 that is involved in this G2/M checkpoint. Mutants of GADD45 that lacked either the first 35 or the last 80 residues still retained an ability to induce G2/M arrest. A mutant with a deletion of the central region (residues 50–76), which is conserved in the family members GADD45β and GADD45γ, lacked such activity. This mutant also lacked an ability to bind to Cdc2, PCNA, and p21waf1 in vivo. Consistently, either GADD45β or GADD45γ binds to Cdc2. However, unlike GADD45, neither GADD45β nor GADD45γ inhibited the Cdc2 kinase or induced G2/M arrest. The unique effect of GADD45 may be caused by the presence of a region containing DEDDDR residues. Alanine substitutions in the region abolished GADD45 induction of a G2/M arrest and its inactivation of the Cdc2 kinase but not its binding to Cdc2, PCNA, or p21waf1. Therefore, the binding of GADD45 to Cdc2 was insufficient to induce a G2/M arrest, and additional activity contributed by the DEDDDR residues may be necessary to regulate the G2/M checkpoint.

G2/M checkpoints prevent the segregation of damaged chromosomes, which is likely to be important in human tumorigenesis (1, 2). The transition from G2 to M is regulated in part by the G2-specific kinase consisting of Cdc2 and cyclin B1 (3). Many G2/M regulatory genes have been identified recently such as Chk1, Chk2, ATM (MECl and TEL1 in Saccharomyces cerevisiae and RAD3 in Schizosaccharomyces pombe) and the 14-3-3 family (2, 4–7). Their products alter Cdc2 activity by inhibiting diphosphorylation of inhibitory sites on Cdc25C. p53 also has been implicated in an ionizing radiation (IR)−induced G2/M checkpoint (8–12). It may modulate the G2/M transition by up-regulating 14-3-3σ (13) and/or p21waf1 (12, 14, 15). Consequently, cells lacking p53 show chromosome instability (16), a phenotype likely resulting from defects in the G2/M checkpoint. Therefore, a multiplicity of G2/M checkpoints in response to DNA damage may well involve redundant controls involving both p53-independent and -dependent pathways.

We demonstrated recently that GADD45, a 165-amino acid nuclear protein whose expression also is p53-dependent (17), is required for the activation of a G2/M checkpoint induced by either UV radiation or alkylating agents (18). GADD45 was originally identified on the basis of a rapid induction in Chinese hamster ovarian cells after UV irradiation (19). Induction of GADD45 also was observed following treatment with many other types of DNA-damaging agents, including various environmental stresses, hypoxia, IR, genotoxic drugs, and growth factor withdrawal (20). In mammalian cells, two additional family members with extensive sequence homology, GADD45β and GADD45γ, were identified recently (21). Similar to p53-deficient cells, cells from Gadd45-deficient mice also show genomic instability, including chromosome abnormalities and centrosome amplification (22). It is known that GADD45 binds to PCNA, p21waf1 and Cdc2 (23–25). In addition, GADD45 can directly inhibit the kinase activity of the Cdc2-cyclin B1 complex by physically interacting with Cdc2, but not with cyclin B1 (25). This inhibition is specific, because GADD45 has no inhibitory effect on the kinase activity of the G1-specific Cdc2/cyclin E complex (24, 25). Increased expression of GADD45 in primary human fibroblasts arrests cells at the G2/M boundary. This arrest was attenuated by the overexpression of cyclin B1 and Cdc25C. The GADD45-mediated G2/M arrest is dependent on wild-type p53, but does not require p21waf1 (18). When GADD45 is inactivated by expression of antisense GADD45, cells exhibit an attenuated UV-induced inhibition of Cdc2 kinase activity (18). Therefore, a GADD45-induced G2/M checkpoint is modulated through inactivation of the Cdc2-cyclin B1 kinase. To further explore the mechanism(s) and to identify the functional domains of GADD45 that are involved in the G2/M checkpoint, we constructed a series of deletion and point mutants of GADD45. We then tested them for their ability to induce a G2/M arrest; to bind to p21waf1, PCNA, or Cdc2; and to inhibit Cdc2 kinase activity.

EXPERIMENTAL PROCEDURES

Plasmids—The open reading frame of the GADD45 cDNA without its own stop codon was directly amplified by polymerase chain reaction and cloned in-frame into pcDNA3-HA, a derivative of pcDNA3 (Invitrogen) modified to include an HA epitope (24). GADD45 deletion plasmids were generating by polymerase chain reaction and cloned in-frame into pcDNA3-HA.
ated by deleting respective regions from pcDNA3-GADD45-HA. The site-mutagenesis plasmids were constructed by a polymerase chain reaction-based approach, followed by ligation in-frame into pcDNA3-HA. GADD45b-HA and GADD45g-HA vectors were kindly provided by Dr. H. Saito (21).

Cell Culture, Transfections, Microinjection, and Monitors of G2/M Arrest—The colon carcinoma cell line HCT116 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. RKO cells (a colon carcinoma cell line) were maintained in modified Ham's F12 medium containing 10% fetal bovine serum. COS-7 and 293 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Primary human fibroblasts (GM0842) were obtained from Coriell Cell Repositories (Camden, NJ) and grown in Ham's F-10 medium supplemented with 15% fetal bovine serum. Only early passage primary human cells (before passage 9) were used in the experiments. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. Tranfections were performed with LipofectAMINE Plus (Life Technologies, Inc., Manassas, VA) as directed by the manufacturer. The microinjection procedure used was described previously (26). To determine their ability to induce a G2/M arrest, the wild-type and mutant GADD45 expression vectors at a concentration of 100 µg/ml were used for microinjection into normal human fibroblast cells via microinjection to determine their ability to induce a G2/M arrest at 24-h post-microinjection. The data are averages from at least three independent experiments. Error bars represent 1 S.D.

Fig. 1. Mapping of the functional domain of GADD45 required for G2/M arrest. A, sequence comparison of the human, mouse, and rat GADD45 family members was made with the multiple alignment module of the DNASTAR software. The GenBank™ accession numbers of these sequences are as follows: hGADD45, NM001924; hGADD45b, AF078077; hGADD45g, AF078078; mGADD45, B56535; rGADD45, L32591 (h, human; m, mouse; r, rat). Hyphens are used to indicate identical residues to hGADD45. Dotted lines indicate the gaps introduced. Three conserved regions are boxed (Box I, Box II, and Box III). B, the activities of the GADD45 deletion mutants. Different parts of the N terminus, C terminus, or the central region of GADD45 were deleted. The mutants were introduced into normal human fibroblast cells via microinjection to determine their ability to induce a G2/M arrest at 24-h post-microinjection. The data are averages from at least three independent experiments. Error bars represent 1 S.D. C, expression of the GADD45 deletion mutants in COS-7 cells. The deletion mutants were transfected into COS-7 cells. Following a 24-h incubation, cell lysates were prepared, and mutant proteins were detected by immunoprecipitation and Western blot.
primary human fibroblasts, which results in an average of 10 molecules per cell. Morphological determination of the G2/M arrest was described previously (18). GADD45-positive cells display a unique mitotic-like morphology, with characteristics of cells at early prophase, including the expression of a mitotic marker MPMP2, 4N (tetraploidy) DNA contents, and large nonsegregated centrosomes (18). Transfection followed by flow cytometry analysis confirms results obtained by microinjection (18). For microinjection, at least 50 positive cells were analyzed for each experiment. Data were obtained from at least three independent microinjection experiments.

Antibodies—Antibodies against human Cdc2, cyclin B1, p21\textsuperscript{wr} and PCNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA antibody was provided by Roth Molecular Biochemicals.

Immunoprecipitation—Cell extracts were prepared on ice for 30 min in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% Nonidet P-40, 2 μM/ml aprotinin, 2 μM/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride. Two mg of cellular protein was immunoprecipitated by incubating the extracts with the primary antibodies for 2 h at 4 °C, followed by addition of protein A/G-agarose beads and incubation for another 2 h at 4 °C with rotation. After five washes in lysis buffer, bound proteins were released by resuspending the beads in 30 μl of Laemmli sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Novex, San Diego, CA). Membranes were incubated with anti-HA antibody, which was then detected by a chemiluminescence system (Amersham Pharmacia Biotech).

Immunocytochemistry Analysis—Cells were fixed and stained with appropriate antibodies as described previously (26). Anti-GADD45 or anti-HA antibody was used for staining. Secondary antibodies conjugated to fluorescein isothiocyanate or Texas Red (Vector Labs) were used. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

Immunocomplex Protein Kinase Assay—A GADD45-specific kinase assay was done essentially as described previously (26) using cellular protein extracts prepared from RKO cells transiently transfected with various GADD45 constructs. Briefly, the Cdc2-cyclin B1 kinase complex was immunoprecipitated from a total of 100 μg of RKO cellular extracts with an anti-cyclin B1 antibody for 2 h at 4 °C. Immunocomplexes were recovered with the aid of protein A/G-agarose beads (Santa Cruz Biotechnology), washed three times with lysis buffer and twice with kinase buffer (50 mM Tris-HCl, 10 mM MgCl\textsubscript{2}, 2 mM dithiothreitol, 1 mM EGTA and 0.01% Brij35) and resuspended in 20 μl of kinase buffer containing 1 μg of histone H1 (Upstate Biotechnology, Lake Placid, NY). The histone H1 kinase reaction was initiated by the addition of 10 μCi of [γ\textsuperscript{32}P]ATP and 25 μM ATP with incubation for 15 min at 37 °C. Reactions were stopped by addition of an equal volume of 2× Laemmli sample buffer. Histone H1 was separated on a 12% SDS-polyacrylamide gel, which was then dried and visualized by autoradiography.

Cell Cycle Analysis—RKO cells were trypsinized and washed with phosphate-buffered saline, fixed with 70% ethanol overnight at 4 °C, treated with 20 μg/ml RNase A for 30 min and stained with 60 mg/ml of propidium iodide for DNA content. Propidium iodide fluorescence was analyzed with a FACSCaliber cell sorter (Becton-Dickinson). Data for at least 10,000 cells were collected using CellQuest software (Becton-Dickinson). Cell cycle distributions were calculated using ModFit LT software.

RESULTS

Mapping of the Functional Domain of GADD45 Required for G2/M Arrest—Sequence comparison among the members of the GADD45 family (Fig. 1A) indicates that greater than 50% of the residues are conserved. The center region (residues 16–108) shares 55% identical and 81% similar residues. We initially made a series of deletion mutants with deletions at the N terminus, the C terminus, and the central region (Fig. 1B). To determine the expression levels of these constructs, various mutants were transiently transfected into COS-7 cells. Wild-type and truncated GADD45 proteins were detected by Western blot or by immunoprecipitation followed by Western blot analysis. All mutants expressed appreciable amounts of proteins of the appropriate sizes in COS-7 cells as detected by anti-HA antibody (Fig. 1C). Similar results were obtained in 293 and HCT116 cell lines (data not shown). Interestingly, the full-length GADD45/HA fusion gene produced two bands, with the upper band corresponding to the molecular weight of full-length GADD45, and the lower band was identical to the mutant GADD45-(16–165) (Fig. 1C). Both bands were also detected by anti-GADD45 antibody (data not shown). These fusion genes produced identical protein patterns in 293 and HCT116 cells (data not shown). Because codon 16 of GADD45 encodes a methionine and the start codon of the fusion gene is very close to that of the cytomegalovirus promoter, it is likely that the second methionine serves as a secondary transcription start site. This may also account for the second band seen with the GADD45-(1–108) mutant.

The ability of these deletion mutants to induce G2/M arrest in primary human fibroblasts was determined following microinjection (Fig. 1B). G2/M arrest was monitored by a distinct cell
morbidity 24 h following microinjection of the GADD45 constructs. A perfect correlation between the unique cell morphology induced by GADD45 and G2/M arrest was previously confirmed by the expression of a mitotic marker MPM2, 4N DNA contents, and by flow cytometry analysis (18). Consistent with previously published data (18, 25, 27), the HA-tagged full-length GADD45 (1–165) induced both G2/M arrest in normal human fibroblasts (Fig. 1B) and growth suppression in HCT116 cells (data not shown). Deletion of either the N-terminal 16 residues GADD45-(16–165) or the C-terminal 57 residues GADD45-(1–108) did not change the results, suggesting that these domains are dispensable for G2/M arrest. Whereas mutants GADD45-(1–85), GADD45-(16–108), GADD45-(16–85), GADD45-(35–165), and GADD45-(35–108) still partially retained activity on G2/M arrest, mutants del50–95 and del50–76 have no detectable activity. The subcellular localization patterns of wild type as well as every mutants was very similar when overexpressed in normal human fibroblasts following microinjection (data not shown). These data indicate that the central conserved region between residues 50 and 76 may be involved in a G2/M arrest.

The central region (residues 50–76) contains many acidic residues (Fig. 2). To examine if the acidic residues play an important role in GADD45-mediated G2/M arrest, we made three additional mutants, M62–67, M74–79, and M82–87, in which the residues within these regions were changed to alanines by site-directed mutagenesis. The M62–87 mutant is similar to wild-type GADD45 with regard to its ability to induce a G2/M arrest in normal human fibroblasts, whereas M74–79 partially lost activity, and M62–67 was completely deficient (Fig. 2). Again, no noticeable difference of these mutants in subcellular localization was observed (data not shown). These data indicate that the region (residues 62–67) containing DEDDDR residues may be critical for GADD45-mediated G2/M arrest.

Ability of the GADD45 Mutants to Bind to Cdc2, PCNA, or p21waf1 Proteins—To determine whether the GADD45 mutants associate with Cdc2, PCNA, or p21waf1 in vivo, HCT116 cells were transiently transfected with HA-tagged expression vectors. After 24 h, cell lysates were prepared and immunoprecipitated with antibodies to Cdc2, PCNA, or p21waf1. The immunoprecipitated proteins were then analyzed by Western blot with an anti-HA antibody (12CA5).

FIG. 3. In vivo association of GADD45 mutants with Cdc2, PCNA, or p21waf1. To determine whether the GADD45 mutants associate with Cdc2, PCNA, or p21waf1 in vivo, HCT116 cells were transfected with HA-tagged expression vectors. After 24 h, cell lysates were prepared and immunoprecipitated with antibodies to Cdc2, PCNA, or p21waf1. The immunoprecipitated proteins were then analyzed by Western blot with an anti-HA antibody (12CA5).

FIG. 4. Cdc2-cyclin B1 kinase assay in RKO cells treated with ionizing radiation or transfected with GADD45 mutants. A, RKO cells were irradiated with 6.3 gray of IR or left untreated and were harvested at 1, 4, and 24 h. One mg of protein was immunoprecipitated with the anti-cyclin B1 antibody, and histone H1 kinase assays were performed. Anti-mouse IgG was used as a negative control. B, cells in parallel with A were collected and subjected to cell cycle analysis by fluorescence-activated cell sorting. The percent of cells at G1, S, or G2/M of the cell cycle are indicated in A. C and D, the ability of the GADD45 mutants to inhibit Cdc2-cyclin B1 kinase activity in vivo. RKO cells were transfected with wild-type or mutant GADD45 or cotransfected with both wild-type and mutant M62–67. Cells were collected 6 and 24 h later, and the Cdc2-cyclin B1 kinase activity was measured. An empty plasmid vector was used as a control. The amount of phosphorylated histone H1 was quantified with a Fuji Bas2500 phosphorimaging system. The data are normalized to the control lane and expressed as percent of inhibition.
50–76 of GADD45. The 3 site-mutation mutants (M62–67, M74–79, M82–87) can still bind to Cdc2, PCNA, and p21waf1/z, although M62–67 binding is slightly reduced, implying that residues 62–67 may be a part of the actual interaction domain with Cdc2, PCNA, and p21waf1/z.

**Ability of GADD45 Mutants to Inhibit Cdc2 Kinase**—To establish conditions for measuring the in vivo Cdc2 kinase activity and to confirm previous findings that radiation alters the Cdc2 kinase activity, RKO cells were irradiated with 6.5 gray IR and incubated for 1, 4, or 24 h. Cell lysates were prepared and the Cdc2-cyclin B1 complex was immunoprecipitated with cyclin B1 antibody. Cdc2 kinase activity was measured using the substrate histone H1. Anti-mouse IgG was used as a control (Fig. 4A). Levels of the Cdc2-cyclin B1 kinase activity were similar in the nonirradiated cells during the incubation period (Fig. 4A). RKO cells showed a strong reduction in Cdc2 kinase activity after a 1-h incubation following irradiation, but activity returned to normal levels by 4 h and was further increased at 24 h (Fig. 4A). Cell cycle analysis using cell sorting in parallel indicated that IR-induced a G2/M arrest as early as 4 h after treatment, and this significantly increased through 24 h (Fig. 4B). These data indicate that whereas immediate G2/M arrest may involve inactivation of Cdc2 kinase activity, the prolonged arrest may require additional processes.

To test whether GADD45 mutants inhibited Cdc2 kinase activity in vivo, RKO cells were transfected with wild-type and mutant GADD45. The Cdc2 kinase activity was then determined as described above. GADD45 inhibited the Cdc2 kinase activity within 6 h of transfection (Fig. 4C, upper), consistent with previously published data (25). Interestingly, the inhibition was reversed within 24 h (Fig. 4C, lower). In contrast, mutants M62–67 and del50–76 had no inhibitory effect on the Cdc2 kinase following a 6-h transfection (Fig. 4C). Because M62–67 is still able to bind to Cdc2, suggesting that this mutant may act in a dominant-negative fashion. Consistently, M62–67 mutant was able to block wild-type GADD45 to inhibit the Cdc2 kinase activity (Fig. 4D).

We have shown previously that GADD45-mediated inhibition of Cdc2 kinase activity may be due to the dissociation of the cyclin B1 and Cdc2 complex (25). Consistently, transient transfection of wild-type GADD45 resulted in a dose-dependent decrease in the binding between cyclin B1 and Cdc2 (Fig. 5, A and B). In contrast, mutants M62–67 and del50–76 did not reduce cyclin B1-Cdc2 binding (Fig. 5C).

**GADD45β and GADD45γ Do Not Induce a G2/M Arrest**—To examine whether other GADD45 family members are involved in the G2 checkpoint, normal primary human fibroblasts were microinjected with GADD45, GADD45β, or GADD45γ expression vector. Whereas GADD45 predominantly induced a G2/M arrest, no arrest was observed with the expression of either GADD45β or GADD45γ (Fig. 6A). All the GADD45 family members displayed a similar subcellular distribution pattern when transiently overexpressed (Fig. 6B), suggesting that lack of a G2/M arrest by GADD45β or GADD45γ was not caused by differences in subcellular localization. To determine whether the GADD45 family members associate with Cdc2 in vivo, HCT116 cells were transfected with HA-tagged expression vectors and immunoprecipitated with anti-Cdc2 antibody. Following immunoblot analysis, the HA-tagged GADD45 family proteins were detected with the anti-HA antibody. These results indicate that GADD45β and GADD45γ were able to bind to Cdc2 in vivo (Fig. 6D) but were unable to inhibit its kinase activity (Fig. 6E).

**Discussion**

We have demonstrated recently that GADD45 activates a G2/M checkpoint after damage induced by either UV radiation or alkylating agents. Increased expression of GADD45 in normal human fibroblasts arrests the cells in G2/M. The data presented in this study demonstrate that the N and C termini of GADD45 are dispensable for G2/M arrest but the central region (residues 50–76) may be required. It appears that this region contains a unique acidic motif DEDDDR that may play a key role in the inhibition of Cdc2-cyclin B1 kinase activity and in the induction of a G2/M arrest because mutating the acidic residues to alanines abolished these activities. Interestingly, Ran, a small nuclear GTPase implicated in both cell cycle progression and nuclear export (28, 29), also contains this unique acidic motif with a similar amino acid composition in its C-terminal domain (DEDDDL) (30). Overexpression of Ran also predominantly induces a G2/M arrest, whereas deletion of the DEDDDL motif abolishes this activity (28). However, this motif does not appear to be required for Ran-mediated nuclear transport activity. These data suggest that GADD45 and Ran may utilize a similar pathway to regulate cell cycle progression
from the G2 phase to mitosis, and this unique motif may serve as a common structural entity that activates the G2/M checkpoint. Additional experiments including investigating the ability of the DEDDDR peptide to inhibit the Cdc2 kinase activity will be needed to prove this hypothesis. It will be interesting to examine whether one can exploit this acidic motif therapeutically to inactivate the G2/M checkpoint mediated by GADD45. Such a strategy would potentially enhance cancer chemotherapy because inactivation of a G2/M checkpoint can sensitize cancer cells for DNA damage-induced apoptosis (31).

One of the key steps in regulating the progression of mammalian cells from G2 into mitosis is the activation of the G2-specific Cdc2-cyclin B1 kinase (3, 32). p53 plays an essential role in G2/M checkpoints. Interestingly, the expression products of three p53-regulated genes, p21\textsuperscript{waf1}, 14-3-3\textsuperscript{s}, and GADD45, are involved in G2/M checkpoints (12, 18, 33). Both p21\textsuperscript{waf1} and 14-3-3\textsuperscript{s} may initiate a G2/M arrest through direct binding to and sequestering of Cdc2 in the cytoplasm, thereby preventing Cdc2-cyclin B1 from becoming activated and initiating mitosis (33). We reported recently that GADD45 physically interacts with Cdc2 and directly inhibits Cdc2-cyclin B1 kinase through its disruption of the Cdc2-cyclin B1 complex (25). Our data indicate that GADD45 may be involved in a G2/M checkpoint at least partially through direct inactivation of the Cdc2-cyclin B1 kinase activity. These findings suggest that the G2/M checkpoint may utilize a redundant system. It has been reported that GADD45 interacts with the nuclear proteins p21\textsuperscript{waf1/2} and PCNA (23, 24). GADD45 can disrupt the ability of p21\textsuperscript{waf1/2} to bind to PCNA, and, conversely, p21\textsuperscript{waf1/2} blocks the ability of GADD45 to bind to PCNA (34). Although the central region (residues 50–76) of GADD45 may be required for interaction with both Cdc2 and p21\textsuperscript{waf1/2}, the mutant M62–67 that lost its inhibitory effect on the Cdc2-cyclin B1 kinase can still bind to Cdc2 and p21\textsuperscript{waf1/2}. Moreover, p21\textsuperscript{waf1/2} is not required for GADD45-induced G2/M arrest (18). Therefore, the binding of GADD45 to Cdc2 and p21\textsuperscript{waf1/2} is insufficient to define the mechanism of a GADD45-mediated G2/M checkpoint. The additional activity of the DEDDDR motif may be needed to inactivate the Cdc2-cyclin B1 kinase and to induce a G2/M arrest. In addition, the GADD45-mediated G2/M checkpoint is independent of 14-3-3\textsuperscript{s}, because overexpression of GADD45 is still able to induce a G2/M arrest in 14-3-3\textsuperscript{s}-deficient HCT116 cells (data not shown). These data indicate that three p53 downstream genes may utilize different mechanisms to activate the G2/M checkpoints.

While we were preparing this manuscript, Zhan and coworkers (35) showed that the central region of GADD45 is necessary for GADD45 inhibition of Cdc2 kinase and G2/M arrest. Our data are in agreement with their findings and support the hypothesis that the central domain of GADD45 may represent a unique mechanism for induction of a G2/M checkpoint.

In mammalian cells, at least two additional GADD45 family members, GADD45\textbeta and GADD45\textgamma, have been identified based on the extensive region of conserved sequence and the induction following DNA damage and/or other environmental stresses (21). The central regions of these proteins share more than 80% sequence homology with GADD45. Similar to GADD45, both proteins also bind to Cdc2 in vivo. Surprisingly, we found that only GADD45 is able to induce a G2/M arrest and...
to inhibit the Cdc2-cyclin B1 kinase, whereas neither GADD45β nor GADD45γ have such activity. These data indicate that the binding of these proteins to Cdc2 is insufficient to regulate the transition from G₂ to mitosis. One possibility for the lack of G₂/M arrest by either GADD45β or GADD45γ is that GADD45 is a nuclear protein (23, 24) whereas GADD45β and GADD45γ have been shown to be localized in cytoplasm and to activate the MAPK (mitogen-activated protein kinase) cascade (21). However, we did not observe any significant difference in subcellular localization of these proteins when they were transiently overexpressed following microinjection. Alternatively, the DEDDDR residues only present in GADD45 provides a unique charged pattern, and this composition may provide a unique mechanism to induce a G₂/M arrest. Our data also suggest that the lack of G₂/M arrest by either GADD45 and GADD45γ plasmid constructs and Dr. Bert Vogelstein for providing us with the 14-3-3-deficient HCT116 cells. We are grateful to Dr. Curtis Harris and Elisa Spillare for their invaluable advice and continued support. We also thank Dorothea Dudek for her excellent editorial assistance.

Acknowledgments—We thank Drs. Albert Fornace and Haruo Saito for their generosity in providing the GADD45, GADD45β, and GADD45γ plasmid constructs and Dr. Bert Vogelstein for providing us with the 14-3-3-deficient HCT116 cells. We are grateful to Dr. Curtis Harris and Elisa Spillare for their invaluable advice and continued support. We also thank Dorothea Dudek for her excellent editorial assistance.

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