Gadd45 Proteins Induce G2/M Arrest and Modulate Apoptosis in Kidney Cells Exposed to Hyperosmotic Stress*

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Gadd45 proteins are induced by hyperosmolality in renal inner medullary (IM) cells, but their role for cell adaptation to osmotic stress is not known. We show that a cell line derived from murine renal IM cells responds to moderate hyperosmotic stress (540 mosmol/kg) by activation of G2/M arrest without significant apoptosis. If the severity of hyperosmotic stress exceeds the tolerance limit of this cell line (620 mosmol/kg) apoptosis is strongly induced. Using transient overexpression of ectopic Gadd45 proteins and simultaneous analysis of transfected versus non-transfected cells by laser-scanning cytometry, we were able to measure the effects of Gadd45 super-induction during hyperosmolality on G2/M arrest and apoptosis. Our results demonstrate that induction of all three Gadd45 isoforms inhibits mitosis and promotes G2/M arrest during moderate hyperosmotic stress but not in isosmotic controls. Furthermore, all three Gadd45 proteins are also involved in control of apoptosis during severe hyperosmotic stress. Under these conditions Gadd45 induction strongly potentiates apoptosis. In contrast, Gadd45α/β induction transiently increases caspase 3/7 and annexin V binding before 12 h but inhibits later stages of apoptosis during severe hyperosmolality. These results show that Gadd45 isoforms function in common but also in distinct pathways during hyperosmolality and that their increased abundance contributes to the low mitotic index and protection of genomic integrity in cells of the mammalian renal inner medulla.

Cells of the mammalian renal inner medulla (IM) are characterized by a stress-tolerant phenotype that enables them to resist extremes in multiple environmental variables, including osmolality. We have shown that these cells induce all three Gadd45 isoforms during hyperosmotic stress via posttranscriptional mechanisms (1, 2). Gadd45α was initially discovered based on its induction in hamster cells exposed to ionizing radiation stress (3). Gadd45α based on its induction in hamster cells exposed to ionizing radiation stress (3). Gadd45α initially described as myeloid differentiation immediate early gene MyD118 (4). More recently, GADD45α was described under different names, including GADD45γ (5, 6), GRP17 (7), CR6 (8), and OIG37 (9).

Despite their hyperosmotic induction, functional consequences of Gadd45 up-regulation in cells exposed to osmotic stress are unknown. Potential Gadd45 functions during environmental stress include promotion of cell cycle arrest, apoptosis, chromatin remodeling, and DNA repair (10). All of these cell functions are altered during hyperosmolality (1, 11–15). To assess the mechanistic relevance of hyperosmotic Gadd45 induction, it is necessary to determine which of these cell functions are altered as a result of increased levels of each of the three mammalian Gadd45 isoforms. The present study addresses the question of how super-induction of Gadd45α, Gadd45β, and Gadd45γ during hyperosmotic stress affects cell proliferation and apoptosis.

Renal IM cells exposed to hyperosmolality undergo growth arrest in G2 phase of the cell cycle (1, 16). This hyperosmotic cell cycle delay is likely a consequence of activation of DNA damage checkpoints because hyperosmolality causes multiple forms of DNA damage (13, 15, 17). However, whether any of the three Gadd45 isoforms plays a role in mediating hyperosmotic G2/M arrest remains unclear. A direct role of Gadd45 in cell cycle checkpoint pathways responsible for G2/M arrest has been demonstrated in human keratinocytes exposed to UV radiation and alkylating agents, where it inhibits the activity of the Cdc2-cyclin B1 complex (18, 19). Moreover, Gadd45β and Gadd45γ are also Cdc2/cyclin B1 inhibitors that are involved in controlling the G2/M checkpoint (20). Therefore, one goal of the present study was to investigate whether increases of any Gadd45 isoform during hyperosmotic stress inhibit mitosis and promote G2 arrest.

The role of Gadd45 proteins for the regulation of apoptosis is controversial and may depend on cell type and the nature of the environmental stimuli that triggers apoptosis. For instance, in medulloblastoma cells Gadd45α induces apoptosis in response to nerve growth factor stimulation independent of the p38 and c-Jun NH2-terminal kinase pathways (21), whereas in UV-irradiated skin tumors its stimulation of apoptosis depends on p38 and c-Jun NH2-terminal kinase activity (22). Overexpression of Gadd45α in human fibroblasts does not induce apoptosis nor does it alter p38 and c-Jun NH2-terminal kinase activity (10). Gadd45γ has similarly wide-ranging effects on apoptosis as Gadd45α. For instance, Gadd45β inhibits apoptosis caused by tumor necrosis factor-α by mediating inhibition of the c-Jun NH2-terminal kinase pathway via the NF-kB pathway (23, 24), but it promotes apoptosis caused by TGFβ via activation of the p38 pathway (25). Gadd45γ also activates the p38 pathway in some...
Hyperosmolality diminishes proliferation and plasma membrane integrity of mIMCD3 cells. Cells were exposed to medium of 300, 540, and 620 mosmol/kg of H\(_2\)O for the times indicated. A, cell proliferation as indicated by absorbance of WST reagent is delayed at 540 and even more so at 620 mosmol/kg of H\(_2\)O. B, plasma membrane damage as indicated by lactate dehydrogenase (LDH) leakage into the medium is not affected at 540 mosmol/kg of H\(_2\)O but increases steeply at 620 mosmol/kg H\(_2\)O. Error bars are shorter than symbols when they are not visible.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Proliferation, and Cell Integrity Assays—Murine inner medullary collecting duct (mIMCD3) cells of passage 18 were used for all experiments (27). Cell culture medium consisted of 45% Ham F-12, 5% fetal bovine serum, and 5% charcoal-treated calf serum. Cells were grown on 8-chamber slides (Nalge-Nunc) and staining with propidium iodide (PI) as previously described (28). In addition, mitotic cells were identified and quantified by bivariate LSC analysis of the chromatin content. Apoptotic nuclei were counterstained with Hoechst 33342.

Cell cycle stages were analyzed by laser-scan cytometry (LSC, CompuCyte, Cambridge, MA) after fixing cells grown on 8-chamber slides (Nalge-Nunc) and staining with propidium iodide (PI) as previously described (28). In addition, mitotic cells were identified and quantified by bivariate LSC analysis of the chromatin content. Apoptotic nuclei were counterstained with Hoechst 33342.

Cell Cycle Analysis—Cell cycle stages were analyzed by laser-scanning cytometry (LSC, CompuCyte, Cambridge, MA) after fixing cells grown on 8-chamber slides (Nalge-Nunc) and staining with propidium iodide (PI) as previously described (28). In addition, mitotic cells were identified and quantified by bivariate LSC analysis of the chromatin content. Apoptotic nuclei were counterstained with Hoechst 33342.

Cell-specific caspase 3/7 activity was determined by LSC analysis after growing cells in 8-chamber slides. After exposure to different osmolalities, cells were treated with 10 \(\mu\)M carboxyfluorescein-labeled caspase 3/7-specific inhibitor (FAM-DEVD-FMK) obtained from Immunohistochemistry Technologies (Bloomington, IN). Cells were rinsed briefly with PBS, fixed for 2 h with 1% formaldehyde in PBS, and rinsed again briefly with PBS. After counterstaining slides with PI, caspase 3/7 activity in each cell was quantified by scanning slides with the LSC using an argon laser (488 nm excitation) and standard settings of the green and orange/red channel emission filter.

Cell-specific annexin V binding to the outer cell membrane was also determined by LSC analysis after growing cells in 8-chamber slides. After exposure to different osmolalities, cells were washed twice in PBS and stained with annexin V conjugated to either fluorescein or Alexa 647 (Alexis Biochemicals, San Diego, CA) as previously described (29). Cells were then fixed for 15 min in 1% methanol-free formaldehyde, and annexin V binding to the outer cell membrane was quantified by scanning slides with the LSC using an argon laser (488 nm excitation) and green and red emission channel or a helium-neon laser (633 nm excitation) and far-red LSC emission channel. When using Alexa 488, conjugate cells were counterstained with PI. For Alexa 647 conjugate, Hoechst 33342 (Molecular Probes, Eugene, OR) was used for nuclear counterstaining. This approach was validated by co-localization of annexin V fluorescence and PI fluorescence in unfixed cells that are only permeated by PI when they are dead (apoptotic).

Late apoptosis was quantified by DNA strand break labeling (TUNEL) assay as previously described (30). Briefly, cells were fixed in 1% methanol-free formaldehyde for 15 min, incubated in 70% ethanol at \(-20^\circ\)C for at least 1 h, and rinsed twice with 50% PBS. DNA strand breaks were then labeled with APO-Direct reagents, including fluorescein-dUTP with the enzyme terminal deoxynucleotidyltransferase, as instructed by the manufacturer (Phoenix Flow Systems, San Diego, CA). Green fluorescence per cell was measured by LSC using an argon laser (488 nm excitation) and green LSC emission channel. Cell nuclei were counterstained with Hoechst 33342.

Cloning and Expression of Erythrotype-tagged Gadd45 Genes in Renal Cells—Total RNA from mIMCD3 cells was extracted as previously described (2) and reverse-transcribed using oligo(dT) primers and SuperScript III first strand synthesis reagents (Invitrogen). PCR primer pairs were performed using Vector NTI 9 software (Informax, Frederick, MD) and GenBank numbers NM_008655 and NM_011817 as templates: Gadd45\(\alpha\)-1-CTGTGGAGTGTGACTGCATCAT, 2-GCGTTCCTCTAGAGAGTCACGTC; Gadd45\(\beta\)-1-GAGGCAGCATGGTGATGG, 2-CGCCCATCCTTCCCACTA, 2-CTCGGGAGATTTAACGCGGC; Gadd45\(\gamma\)-1-CTGGTGAGTTGCTGACCGCTA, 2-CCGGATCCTCAGAGAGTATA, 2-CCTGCCCATGTTGAGTGAGTCTGCTG. PCR products were directly cloned into pEF6/V5-His-TOPO vector (Invitrogen) and propagated in Escherichia coli strain DH5\(\alpha\) (Invitrogen). Endotoxin-free plasmid Mega-preps were performed using a kit as described by the manufacturer (Qiagen GmbH, Hilden, Germany). Transient transfections were carried out by mixing 2 \(\mu\)g of plasmid DNA with 4 \(\mu\)l of LipofectAMINE 2000 reagent (Invitrogen) in 8-chamber slides, and cells were allowed to express recombinant cell types, indicating that this third Gadd45 isoform plays an important role in the regulation of apoptosis during cellular stress (26). Thus, a second goal of the present study was to determine how increased levels of each of the three Gadd45 proteins modulate apoptosis during exposure of renal IM cells to hyperosmolality.

Gadd45 Modulates Apoptosis during Hyperosmolality
binant protein during a 24-h incubation period before dosing at different osmolalities. Transfection efficiency was similar in all samples (~5%) as determined by LSC analysis of V5-positive cells versus total the number of cells (see “Discussion”).

Compartmentation of Epitope-tagged Gadd45 in Transfected Cells—Recombinant Gadd45 proteins were detected using an antibody against the V5 epitope tag (Invitrogen; catalog #R960-25, 1:250 in PBS) and Alexa 488 conjugated secondary anti-IgG antibody (Molecular Probes; catalog #A11029, 1:100 in PBS). After immunocytochemistry, nuclei were counterstained with PI. Bivariate LSC analysis was carried out using excitation with argon ion laser at 400 nm in combination with green and orange emission filter photomultiplier tubes. For some experiments Hoechst 33342 DNA stain was used for nuclear counterstaining instead of PI. In these cases, bivariate LSC analysis was performed using argon ion laser excitation at 488 nm and violet diode laser excitation at 400 nm in combination with green and violet emission filter photomultiplier tubes.

Differential Laser-scanning Cytometry of Transfected Cells—LSC technology is ideal for analyzing mixed populations of cells. We applied this feature of LSC for differential analysis of transfected and non-transfected cells in the same sample. Trivariate LSC analysis was performed as follows. First, cells were probed with FAM-SEVD-FMK (caspase 3/7 activity), annexin V, or fluorescein-dUTP. All three probes contained a green fluorescent label, either fluorescein or Alexa 488 as described above. Then slides were kept in 70% ethanol at ~20 °C for at least 1 h, washed briefly with PBS, and processed for immunocytochemistry with anti-V5 epitope tag antibody (Invitrogen, catalog #R960-25, 1:250 in PBS) and Alexa 647 conjugated secondary anti-IgG antibody.
Finally, nuclei were counterstained with Hoechst 33342 DNA stain. The three fluorophores (fluorescein/Alexa 488, Alexa 647, and Hoechst 33342) were chosen to yield minimally overlapping emission spectra. Trivariate LSC analysis was performed using an argon ion laser (488 nm) for exciting fluorescein/Alexa 488, helium neon laser (633 nm) for exciting Alexa 647, and violet diode laser (400 nm) for exciting Hoechst 33342 during slide scanning. Fluorescence was recorded using violet, green, and far-red emission filter photomultiplier tubes of the LSC.

**RESULTS**

Hyperosmolality Inhibits Cell Proliferation by Growth Arrest and Apoptosis—Exposure of mIMCD3 cells to hyperosmotic medium causes growth arrest that lasts for about 1 day. After a day cell number increase resumes (Fig. 1A). The rate of cell number increase depends on the severity of hyperosmolality, fast at 540 mosmol/kg and slow at 620 mosmol/kg (Fig. 1A). The slow rate of cell doubling in 620 mosmol/kg of medium can at least in part be attributed to accelerated cell death (Fig. 1B). To assess whether cell proliferation rates are also affected by hyperosmolality, we analyzed individual cell cycle stages. Moderate hyperosmolality (540 mosmol/kg) causes an increase of

![Fig. 3. Gadd45 proteins are localized in nuclear and cytosolic compartments.](image)

LSC images collected from slides stained with anti-V5 primary and Alexa 488-conjugated secondary antibodies illustrate that all three Gadd45 isoforms are preferentially located in the nucleus of mIMCD3 cells and, less abundantly, also in the cytosol. A, Gadd45α (green) counterstained with Hoechst 33342 (blue). B, Gadd45β green counterstained with propidium iodide (red). C, Gadd45γ (green) counterstained with propidium iodide (red). Counterstaining illustrates the relatively low transfection efficiency.

![Fig. 4. Differential laser-scanning cytometry enables direct comparison of transfected versus non-transfected cells in the same sample.](image)

Cells grown in eight-chamber slides were evaluated by trivariate LSC analysis of Hoechst 33342 (violet), FAM-DEVD-FMK (green), and Gadd45-V5 (far-red) fluorescence. A, each chamber on the slide contained cells grown in either iso- or hyperosmotic medium and was analyzed separately. B, scattergram displaying the area and Hoechst 33342 content of cells. C, histogram displaying numbers of cells in different cell cycle stages. These cells representing ~5% of the total cell population are labeled blue in all panels. Untransfected cells in the same sample are labeled orange in all panels E, scattergram showing FAM-DEVDFMK fluorescence in non-transfected cells. FLICA-positive cells were defined as cells exceeding maximum pixel fluorescence of 4000 units. F, scattergram showing FAM-DEVD-FMK fluorescence in transfected cells. In this example 8.9% of transfected cells are apoptotic (compared with 4.9% of non-transfected cells (E)) based on positive FAM-DEVD-FMK staining.
cells in G2 and a decrease in S-phase cells relative to isosmotic controls (Fig. 2, A and B). In contrast, severe hyperosmolarity induces G1 arrest and apoptotic DNA fragmentation (Fig. 2C). The induction of G2 arrest by moderate hyperosmolarity is significant at 6 and 24 h, but the decrease in S phase cells becomes significant only at 24 h (Fig. 2, D and E). During severe hyperosmolarity, apoptotic cell numbers increase, and S phase cell numbers decrease significantly at 6 and 24 h, but significant G1 arrest is only apparent at 24 h. These results demonstrate that moderate hyperosmolarity (540 mosmol/kg) only causes G1 arrest but no apoptosis, whereas severe hyperosmolarity approaching the osmotic tolerance limit of mIMCD3 cells causes some G1 arrest but mostly apoptosis.

**Differential Laser-scanning Cytometry Localizes Gadd45 in Transiently Transfected Cells**—To investigate how Gadd45 induction affects growth arrest and apoptosis during hyperosmolarity, we transfected epitope-tagged Gadd45 transiently into mIMCD3 cells. Expressed Gadd45 proteins were detected by immunocytochemistry and LSC. All three isoforms of Gadd45 proteins are distributed evenly in the cytosol, but their concentration is highest in the nucleus (Fig. 3). This compartmentalization profile resembles wild-type Gadd45 and confirms that the level of recombinant Gadd45 expression is consistent with normal processing of the protein. Nuclear co-localization of Gadd45 proteins with PI or Hoechst 33342 DNA stain enabled us to quantify the percentage of transfected cells using the LSC (Fig. 4). Transfection efficiency was ~5% in all experiments and not influenced by subsequent treatment of cells in hyperosmotic media (data not shown). Triple staining of mIMCD3 cells with nuclear DNA counterstain, Gadd45-V5 antibody, and fluorophores that are markers for mitosis and apoptosis made it possible to quantify hyperosmotic effects on mitosis and apoptosis in transfected cells that overexpress Gadd45 (Fig. 4). In addition, we were able to compare these data directly with hyperosmotic effects on mitosis and apoptosis in non-transfected cells in the same sample treated under identical conditions (Fig. 4). This method represents a powerful new way of analyzing properties of transiently transfected cells.

**Gadd45 Inhibits Mitosis during Hyperosmolality**—Cells in prophase, metaphase, and telophase of mitosis can be identified with antibodies against the V5 epitope of Gadd45 and against phospho-histone H3 (Ser-10) and counterstained with propidium iodide. Mitotic cells in transfected and non-transfected cells were analyzed by LSC. Hyperosmolality consistently decreases the number of mitotic cells by 5–10% after 6 h and 10–30% after 24 h in all experiments (Fig. 5). Overexpression of different Gadd45 isoforms has similar effects on mitosis during hyperosmolality. All three Gadd45 isoforms significantly potentiate the inhibitory effect of hyperosmolality on mitosis (Fig. 5). Gadd45-induced inhibition of mitosis was observed at moderate (540 mosmol/kg) and severe (620 mosmol/kg) hyperosmolality but not in isosmotic medium. In contrast, overexpression of a control (LacZ) from the same plasmid has no significant effect on the number of mitotic cells (Fig. 5). These results indicate that Gadd45 induction contributes to growth arrest during hyperosmotic stress.

**Gadd45 Promotes Early Apoptotic Events during Hyperosmolality**—Because severe hyperosmolality also causes apoptosis, we investigated whether Gadd45 induction at 620 mosmol/kg modulates apoptosis. Early apoptosis was monitored by measuring caspase 3/7 activity. The time course of caspase 3/7 activity measured with a fluorescence-based microplate assay shows that significant hyperosmotic caspase activation takes place within 6 h (Fig. 6A). In addition, caspase 3/7 activity was also measured by LSC (Fig. 6B). The time course of hyperosmotic caspase 3/7 activation as measured by LSC confirms that a significant activity increase takes place within 6 h (Fig. 6C). Of interest, despite the low number of apoptotic cells after 24 h at 540 mosmol/kg (Figs. 1, 2, and 6), we observed a significant increase in demonstrable caspase 3/7 activity.

**Fig. 5. Overexpression of all three Gadd45 isoforms inhibits mitosis during hyperosmolality.** Cells were stained with PI (red) and anti-phospho-histone H3 (Ser-10) antibody (green). A, mitotic cells in prophase (P), metaphase (M), and telophase (T) are yellow due to nuclear colocalization of phospho-histone H3 and PI fluorescence. Overexpression of Gadd45 significantly inhibits mitosis in hyperosmotic but not isosmotic medium. B, Gadd45α; C, Gadd45β; D, Gadd45γ; E, LacZ (control). An asterisk symbolizes statistical significant difference to non-transfected cells at the same osmolality.
increase in caspase 3/7 activity for up to 12 h at this osmolality. Effects of Gadd45 overexpression on caspase 3/7 activity were evaluated by trivariate LSC analysis. The results show a slight but insignificant increase of caspase 3/7 activity in response to overexpression of each Gadd45 isoform in isosmotic medium (Fig. 6D). However, when cells are exposed to hyperosmotic medium (620 mosmol/kg) for 4 h, Gadd45 overexpression significantly potentiates caspase 3/7 induction. This potentiation is 1.9-fold for Gadd45α, 1.5-fold for Gadd45β, and 3-fold for Gadd45γ (Fig. 6E). Overexpression of a LacZ control plasmid has no effect on caspase 3/7 activity.

Next, we analyzed hyperosmotic and Gadd45 effects on another early indicator of apoptosis, the binding of annexin V to outer cell membranes. To verify that Annexin V binds only to dead mIMCD3 cells, we initially performed experiments in which live cells were stained with PI and fluorescein-labeled annexin V (Fig. 7A). Then additional experiments were done for trivariate LSC analysis of fixed cells. These experiments show a significant increase of annexin V binding as early as 2 h after hyperosmolality ranging from 540 to 625 mosmol/kg (Fig. 7B). Similar to caspase 3/7 activity, Gadd45 overexpression increases annexin V binding slightly but insignificantly in isosmotic medium (Fig. 7C). Moreover, when cells were treated in hyperosmotic medium (620 mosmol/kg) for 4 h Gadd45 overexpression also significantly potentiates annexin V binding. The degree of potentiation is similar to that observed for caspase 3/7, 1.9-fold for Gadd45α, 1.8-fold for Gadd45β, and 3-fold for Gadd45γ (Fig. 7D). Overexpression of a LacZ control plasmid has no effect on annexin V binding.

Gadd45αββ and Gadd45γ Have Opposite Effects on Late DNA Breaks—A late stage of apoptosis consists in DNA degradation by caspase-activated DNase-mediated cleavage of intranucleosomal linker regions. We measured the amount of such cleavage by TUNEL assay in combination with trivariate LSC analysis. Apoptotic nuclei containing fragmented DNA were detected with fluorescein-dUTP incorporated by terminal deoxynucleotidyltransferase (Fig. 8A). Apoptotic DNA fragmentation does not increase in mIMCD3 cells exposed to moderate hyperosmolality (540 mosmol/kg) compared with isosmotic controls (Fig. 8B). This result is very interesting because two early apoptosis markers (caspase 3/7 and annexin V) are clearly increased under these conditions (Figs. 6, 7). Severe hyperosmolality (600–625 mosmol/kg) leads to a significant increase in DNA breaks (TUNEL-positive cells) after 12 h and an even higher increase after 24 h (Fig. 8B). Thus, we measured the effect of Gadd45 overexpression on late apoptotic DNA breaks after 24 h of incubation of cells in isosmotic and severely hyperosmotic medium (300 and 620 mosmol/kg). Gadd45 overexpression has no significant effect on the number of TUNEL-positive cells in isosmotic medium (Fig. 8C). However, Gadd45α and Gadd45β overexpression significantly reduces and Gadd45γ overexpression significantly increases the number of TUNEL-positive cells in severely hyperosmotic medium (Fig. 8D). This result indicates that Gadd45γ is functionally
different from Gadd45α/β with regard to its involvement in late-stage apoptotic events during hyperosmolality.

**DISCUSSION**

This study demonstrates that induction of all three Gadd45 isoforms in renal IM cells exposed to hyperosmolality inhibits mitosis and promotes early stages of apoptosis. Gadd45α also promotes late apoptosis as evidenced by increased nuclear DNA fragmentation. In contrast, Gadd45α and Gadd45β inhibit late apoptotic events and decrease the number of apoptotic DNA breaks resulting from hyperosmolality. These data indicate functional differences between Gadd45 isoforms during hyperosmotic stress.

Hyperosmotic stress (hypertonicity) causes rapid cell shrinkage as a result of passive water loss due to osmosis. Cell shrinkage plays an important role in the regulation of apoptosis, and apoptotic volume decrease represents a key element for cells to decide whether to die by activation of programmed cell death (31). Thus, volume regulatory mechanisms of renal IMCD cells are central to their hyperosmotic stress tolerance and are probably involved in monitoring severity of hypertonicity. IMCD cells exposed to anisotonic media swell and shrink within seconds (32). In hypotonic media they display virtually complete regulatory volume decrease within 15 min, which is mediated by release of organic osmolytes via ion channels (32, 33). In contrast, after exposure to hypertonic media rapid volume regulatory increase of IMCD cells is only partial (34), and this property of IMCD cells may be important for tuning the regulation of apoptosis during hyperosmotic stress. Antidiuretic hormone promotes rapid volume regulatory increase, probably by increasing sodium and water permeability of IMCD cells. IMCD cells complete rapid volume regulatory increase in the long term by uptake and synthesis of compatible organic osmolytes, which takes much longer (12–36 h) than their rapid release during regulatory volume decrease (35).

For the experiments reported in this study we have used osmolalities just below and slightly above the tolerance limit of 600 mosmol/kg for mIMCD3 cells in culture to differentiate between Gadd45 effects on the cell cycle versus effects on apoptosis, which increases greatly above 600 mosmol/kg (1). Our data show that just below this tolerance limit (at 540 mosmol/kg) mIMCD3 cells stop proliferation and accumulate in the G2 phase of the cell cycle without becoming apoptotic. Interest-
ingly, when this tolerance limit is exceeded (at 620 mosmol/kg), the number of cells in G2 phase decreases below control levels, and apoptotic cell number increases dramatically. This interesting result raises the possibility that Gadd45 isoforms contribute to the hyperosmotic activation of a G2/M cell cycle checkpoint at moderate hyperosmolality and to the onset of apoptosis at severe hyperosmolality. Indeed, all three isoforms of Gadd45 are induced by moderate and severe hyperosmolality (2).

Gadd45 proteins play an important role in the activation of the G2/M checkpoint in cells exposed to UV radiation and alkylating agents by inhibiting the Cdc2-cyclin B1 complex (18, 20). All Gadd45 isoforms physically associate with Cdc2, but only Gadd45α and Gadd45β disrupt Cdc2/cyclin B1 complexes (18, 20, 36). In addition to their interaction with Cdc2, Gadd45α and Gadd45β also interact with other proteins involved in cell cycle checkpoint control, including proliferating cell nuclear antigen and p21 (37–39). A central, highly conserved region (amino acids 50–76 in Gadd45α) is required for interaction with Cdc2, proliferating cell nuclear antigen, and p21 in vivo (36).

Our observation that during hyperosmolality all three Gadd45 isoforms localize mainly to the nucleus of mIMCD3 cells is in agreement with potential interactions between Gadd45 and Cdc2, proliferating cell nuclear antigen, and p21, which are also predominantly nuclear proteins. These results are consistent with the hypothesis that Gadd45 proteins bind Cdc2 and other cell cycle regulators in the nucleus, leading to inhibition of G2/M progression during hyperosmotic stress. Additional support for this hypothesis is provided by data on human keratinocytes exposed to UV radiation stress. In these cells Gadd45 promotes G2 arrest by nuclear export and cytoplasmic sequestration of Cdc2 (19) and cyclin B1 (40). Indeed, we also found significant amounts of each Gadd45 isoform in the cytosol of mIMCD3 cells, supporting a similar nuclear export function during hyperosmotic stress.

Although no studies have investigated Gadd45 function during hyperosmotic stress, several reverse genetics approaches...
have been applied to study Gadd45 function in mammalian cells in other contexts. These approaches include utilization of knockout mice and cells derived from such mice, (41, 42), antisense RNA approaches (43), and stable overexpression of ectopic Gadd45 (44). Here we report a novel approach that is based on transient expression of ectopic Gadd45 and simultaneous analysis of transfected versus non-transfected cells in the same sample using laser-scanning cytometry. This approach has two major advantages. First, it provides internal controls within each sample that are treated under identical conditions, thus eliminating potential side effects due to antibiotic selection or developmental/adaptive compensation of Gadd45 function. Second, super-induction of Gadd45 can be initiated just before dividing cells in hyperosmotic media without the need to add potentially interfering inducing agents for conditional gene expression.

Using this approach we show that super-induction of each Gadd45 isoform during hyperosmotic stress significantly reduces the number of mitotic cells by about 20–40%. These data indicate that Gadd45 is directly involved in the induction of G2 arrest at moderate hyperosmolality (540 mosmol/kg) and of apoptosis at severe hyperosmolality (620 mosmol/kg). Of interest, significant Gadd45-mediated induction of G2 arrest and apoptosis was only observed during hyperosmotic stress and not in isosmotic controls, indicating that Gadd45 super-induction alone is not sufficient to cause either G2 arrest or apoptosis in mIMCD3 cells. Because compartmentation of Gadd45 proteins and their potentiation of hyperosmotic induction of G2 arrest are not isoform-specific, we conclude that all Gadd45 proteins have similar effects on the cell cycle during hyperosmolality.

We have previously shown that hyperosmotic induction of G2 arrest in mIMCD3 cells proceeds in parallel with Gadd45α induction that partially depends on p38 kinase (1). This study and another study published independently (5) were the first to report demonstrating a functional link between stress-activated mitogen-activated protein kinase pathways and Gadd45. The present study, viewed in the context of our previously reported data, suggests that the p38 pathway promotes G2 arrest via Gadd45α induction in response to hyperosmolality. Gadd45α induction also depends on p38 activity in human neuroblastoma cells exposed to oxidative stress (45), human epithelial cells exposed to flavonoids (46), and esophageal cancer cells exposed to peripheral benzodiazepine receptor-specific ligands (47). Conversely, p38 activity depends on MEK44 activation mediated by each of the three Gadd45 proteins in response to a diverse array of stimuli (5, 26, 48, 49). Other mitogen-activated protein pathways such as c-Jun NH2-terminal kinase and extracellular signal-regulated kinase are also known regulators and effectors of Gadd45 proteins (1, 22, 50, 51). However, the feedback loop between Gadd45 and mitogen-activated proteins is not always stimulatory but sometimes absent or inhibitory. A recent study demonstrates that Gadd45β enhances NF-κB-mediated suppression of c-Jun NH2-terminal kinase signaling and inhibits apoptosis by targeting MKK7 in mouse fibroblasts exposed to tumor necrosis factor-α (24). Therefore, an autoregulatory loop consisting of mitogen-activated protein pathways, NF-κB pathway, Gadd45 proteins, and p35 may coordinate induction of G2 arrest versus apoptosis in dependence of the nature and amplitude of cellular or environmental signals.

Because of the different and cell-stimulus-specific effects of Gadd45 proteins on apoptosis, we investigated how super-induction of each Gadd45 isoform influences early and late hallmarks of apoptosis. Interestingly, we observed that even moderate hyperosmotic stress (540 mosmol/kg) increases caspase 3/7 activation and annexin V binding up to 12 h after the onset of hyperosmolality. Nevertheless, these effects were transient, and no late apoptosis (>12 h) or apoptotic DNA breaks were observed, suggesting that irreversible commitment to apoptosis occurs only after 12 h. An alternative explanation would be that cells committed to apoptosis before 12 h detach or die off rapidly and are, therefore, not detected at later time points. This scenario unlikely because at severe hyperosmolality (620 mosmol/kg) cells that are in late apoptotic stages are readily detected at 24 h. It is not unusual that irreversible commitment to apoptosis can take many hours or even days. For instance, irreversible commitment to apoptosis in a B cell lymphoma cell line exposed to oxidative stress occurs between 48 and 72 h (54). In addition, escape of cells from apoptosis has been documented before, and emerging evidence demonstrates that caspase activation can also be indicative of other, non-lethal processes (55).

The balance between survival and pro-apoptotic signals and the persistence of such signals clearly favors cell survival at moderate, and apoptosis at severe hyperosmolality. Gadd45 proteins may play an important role in coordinating this process. Our results suggest that individual Gadd45 isoforms have different survival functions during hyperosmolality. Gadd45α is strongly pro-apoptotic at all times. However, Gadd45α and Gadd45β prevent progression of apoptosis and reduce the number of late DNA breaks in cells exposed to severe hyperosmolality. They stimulate early caspase 3/7 activation and annexin V binding but to a lesser degree (<2-fold) than Gadd45γ (3-fold). Therefore, the time course and persistence of Gadd45 α induction may be critical for survival and pro-apoptotic signaling. In this context it is interesting that hyperosmotic activation of Gadd45α is slower than in response to other stimuli (1). Gadd45β also promotes cell survival in response to stimuli other than hyperosmolality. Gadd45γ is induced by CD40 in B cells and halts the apoptotic cascade in these cells (52). In contrast, all three Gadd45 proteins are pro-apoptotic in human cancer cell lines exposed to high doses of ionizing radiation and genotoxic chemicals (39). These data collectively suggest that Gadd45 proteins are part of the cellular machinery that regulates the balance between cell survival and apoptosis during stress and, perhaps, of a mechanism that monitors severity of cellular stress effects.

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