Oligomerization of Human Gadd45a Protein*

Gadd45a is an 18-kDa acidic protein that is induced by genotoxic and certain other cellular stresses. The exact function of this protein is not known. However, there is evidence for its involvement in growth control, maintenance of genomic stability, DNA repair, cell cycle control, and apoptosis. Consistently, Gadd45a has previously been shown to interact in vitro and/or in vivo with a number of proteins playing central roles in these cellular processes: proliferating cell nuclear antigen, p21\(^{cip1/waf1}\), Cdc2-CyclinB complex, MTK1, and histones. Adding to this complexity, we have found that Gadd45a self-associates in solution, both in vitro and when expressed in the cell. Moreover, Gadd45a can complex with the two other members of the Gadd45 family of stress-induced proteins, human Gadd45b (MyD118) and Gadd45g (CR6). Gel-exclusion chromatography, native gel electrophoretic analysis, enzyme-linked immunosorbent assay, and chemical cross-linking showed that recombinant Gadd45a forms dimeric, trimeric, and tetrameric species in vitro, the dimers being the predominant form. Deletion mutant and peptide scanning analyses suggest that Gadd45a has two self-association sites: within N-terminal amino acids 33–61 and within 40 C-terminal amino acids. Despite the low abundance of Gadd45a in the cell, oligomer-forming concentrations can probably be achieved in the foci-like nuclear structures formed by the protein upon overexpression. Evidence for a potential role of Gadd45a self-association in altering DNA accessibility on damaged nucleosomes is presented.

Gadd45a is a member of a mammalian protein family that includes two other homologous proteins, Gadd45b/MyD118 and Gadd45g/CR6 (1–4). Transcription of the genes encoding these proteins is induced by various kinds of DNA-damaging agents and/or a number of stresses associated with growth arrest. Gadd45a is the only member of the family whose up-regulation has a p53 component. Its gene has a very strong p53-binding site in the third intron (5).

Gadd45a is a relatively small, 18.4 kDa, and highly acidic protein. It is of low abundance in the cell and is thought to localize primarily in the nucleus (6). Gadd45a shares with Gadd45b and Gadd45g this extreme charge characteristic and may act synergistically with them in promoting cell growth arrest upon overexpression in the cell (7). Besides this originally discovered role in suppression of cell growth (1), recent findings suggest specific function(s) for Gadd45a and/or the two other Gadd45 proteins in DNA repair (8–10), apoptosis (11, 12), maintenance of genomic stability (13), and regulation of signaling pathways (4).

The role(s) of Gadd45 proteins in these fundamental cellular processes are realized through interactions made both in vitro and in a mouse model. Studies of the Gadd45a/mouse show genomic instability (13), reduced nucleotide excision repair as well as increased levels of mutations and chemically induced tumorigenesis (14). Gadd45a and Gadd45b interact with PCNA,\(^*\) which is an indispensable component of the DNA repair mechanism (8, 11, 12). This interaction is also suggested to impede UV-induced cellular apoptosis (12). The role of Gadd45a in repair may also be realized through its ability to bind histones and modify accessibility of DNA on damaged chromatin (15). Gadd45a interacts with p21 (Cip1, Wip1, Cdkn1A), a particularly important cell cycle regulator protein (16–18). Gadd45a forms a specific complex with Cdc2-CyclinB mitosis promoting complex and inhibits its kinase activity, which is crucial for G\(_2\)-M progression (19–21). In addition Gadd45 proteins seem to directly bind and activate stress-responsive MTK1/MEKK4 kinase (4).

Even though these findings provide important clues to the cellular role(s) of Gadd45a protein, its function(s) remains poorly understood. In characterization of the function of any protein, especially of a protein participating in as multiple interactions as Gadd45a, it is important to establish a potential for self-association. In many cases formation of higher order oligomeric structures contributes crucially to protein functionality and its regulation and extends its ability to interact with other cellular targets (22–23). Hence, understanding of potential homo- and hetero-interactions among Gadd45 proteins can aid in our understanding of the mechanism(s) by which these proteins carry out their roles in multiple cellular processes.

Here we present evidence that Gadd45a protein can self-associate both in vitro and in the cell. It was found that two distinct regions of Gadd45a are involved in self-association. These regions at least partially overlap with PCNA binding domains, thus implying functional roles for self-association. As another clue for its functional relevance, it was found that the oligomeric state of Gadd45a may regulate its effect on accessibility of DNA on damaged chromatin. Moreover, Gadd45a is capable of forming hetero-complexes with two other Gadd45 proteins. These observations suggest a potentially complex network of interactions underlying the functions of Gadd45 proteins.

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\(\dagger\) To whom correspondence should be addressed: NCI, NIH, Bldg. 37, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: 301-402-0744; Fax: 301-480-2514; E-mail: fornace@pop.nic.nih.gov.

\(\ddagger\) The abbreviations used are: PCNA, proliferating cell nuclear antigen; Ab, antibody; DMS, dimethylsulfoximide; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; T4 UV endo, bacteriophage T4 UV endonuclease V; UV, ultraviolet radiation (254 nm); PCR, polymerase chain reaction; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors—**E. coli and human constructs expressing Gadd45 proteins with or without N-terminal tag were obtained by inserting PCR amplified fragment containing Gadd45 open reading frames into suitable expression vectors. Primers for PCR amplification of inserts consisted of 15 or 18 nucleotides corresponding to open reading frame sequence flanked with restriction endonuclease sites that were used for cloning. The following combinations of templates containing Gadd45 open reading frames, restriction endonuclease sites, and endonuclease-digested expression vectors were employed. Plasmid pET21-Gadd45a, expressing untagged Gadd45a in *E. coli*: pET14b-Gadd45a and Gadd45b plasmids expressing N-terminally HA-tagged Gadd45a, pcDNA3.1/H11011 Gadd45a-(48-132) deletion mutant was constructed by introducing I-flanked primers, EcoRI- and XhoI-treated pET21a vector, containing FLAG sequence; pHA-Gadd45a and pHA-Gadd45b plasmids expressing N-terminally HA-tagged Gadd45a, Gadd45b, respectively, in human cells: pET14b-Gadd45a, Mun I- and XhoI-flanked primers, EcoRI- and XhoI-treated plasmid pDNA3.1 plus, containing FLAG sequence; pH-A-Gadd45a and pH-A-Gadd45b plasmids expressing HA sequence. Plasmids pHA-Gadd45g and pHA-Gadd45h (24), containing HA sequence. Plasmids pHA-Gadd45a and pDNA3.1-HA were kindly provided by Drs. K. Smith (Cornell University) and Y. Gao (NIH, NCI), respectively. Expand High Fidelity PCR System (Roche Molecular Biochemicals) was used for PCR amplification. Cloning was carried out according to manufacturer’s recommendations. As a DNA cross-linker, a stock solution of reagent (25 mg/ml) was prepared in 20 mM HEPES, pH 7.9. Reactions were conducted for 30 min at 25 °C in 20 μl volume of 20 mM HEPES, pH 7.9, at 10 μM protein, and 5 mg/ml DMS and varying KCl and DTT concentrations (Fig. 3). Reactions were stopped by adding 2 μl of 1 M Tris-Cl, pH 6.8. Reactions were adjusted to 1× SDS loading buffer, boiled, and resolved by 12% SDS-PAGE. Protein products were detected by Coomassie Blue G-250 staining. Unstained protein molecular mass markers were calculated according to Refs. 26 and 27 and Sigma protocol (technical bulletin No. MKR-137).

**Chemical Cross-linking in Solution—**Dimethylsuberimidate (DMS) and disuccinimidyl glutarate chemical cross-linkers were from Pierce. Solutions of these cross-linkers were prepared and reactions carried out according to manufacturer’s recommendations. For DMS, a 10-fold solution of reagent (25 mg/ml) was prepared in 20 mM HEPES, pH 7.9. Reactions were conducted for 30 min at 25 °C in 20 μl volume of 20 mM HEPES, pH 7.9, at 10 μM protein, and 5 mg/ml DMS and varying KCl and DTT concentrations (Fig. 3). Reactions were stopped by adding 2 μl of 1 M Tris-Cl, pH 6.8. Reactions were adjusted to 1× SDS loading buffer, boiled, and resolved by 12% SDS-PAGE. Protein products were detected by Coomassie Blue G-250 staining. Unstained protein molecular mass markers for denaturing gels (broad range, Bio-Rad) were run along the samples on the same gels.

**ELISA Assay—**Microtiter plates (Immuno II, Dynatech) were coated with highly purified Gadd45a protein by incubating wells with 50 μl of protein diluted in PBS (2 μg/ml) for 1 h at room temperature. After washing with PBS (PBS with 0.05% Tween 20) and blocking with 4% BSA, purified GST-Gadd45a (or GST in control) protein 2-fold serially diluted in 20 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1 M EDTA was added to the wells. Following 2-h incubation at 25 °C and extensive washing with PBS, anti-GST monoclonal Ab (Covance Research Products) diluted 1:1000 in 1% BSA/PBS was applied to the wells for 1 h. Following reactions, plates were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse Ab (Amersham Pharmacia Biotech) diluted 1:10000 in 1% BSA/PBS. Following 1-h incubation and washing, 0.4 mg/ml solution of 0-phenylenediamine (Sigma) in citric-phosphate buffer, pH 5.0, containing 0.02% hydrogen peroxide was added for color development at 37 °C. Reactions were stopped in 30 min with 2 mM sulfuric acid, and optical density at 490 nm was measured using a plate reader ( Molecular Dynamics). In the case of peptide analysis, the same serial steps were followed except that different Gadd45a-derived peptides were immobilized on microtiter plates. The synthesis and sequence of peptides as well as details of ELISA procedure were described previously (21).  

**Transfections, Immunoblot, and Immunoprecipitation Analyses—**Transfections were carried out with Effectene reagent according to manufacturer’s protocol (Qiagen). For immunoanalyses, cell cultures were washed twice in ice-cold PBS, lysed on ice in ice-cold RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 20 mM β-glycerophosphate; 0.6 ml per P100 plate) supplemented with protease inhibitor mixture (for mammalian cell and tissue extracts, Sigma). Lysates were cleared by centrifugation, and total protein concentration was determined by Bradford assay kit (Bio-Rad) using BSA as a standard. For immunoblot, an aliquot of lysate was supplemented with SDS loading buffer to 1× final concentration, and denatured by boiling. Twenty-five to 100 μg of total protein were typically loaded onto pre-made 12% SDS-PAGE (Novex) and electrophoresed. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% dry milk in PBST and proteins analyzed with specific primary Ab diluted in 5% milk or 1% BSA, and then with HRP-conjugated anti-mouse Ab (Amersham Pharmacia Biotech) conjugated Ab diluted 1:5000. Following extensive washing in PBS, blots were developed with ECL reagents (PerkinElmer Life Sciences).

The following primary Abs were used for immunoblot analysis: anti-FLAG monoclonal Ab M2 (Sigma) or anti-FLAO monoclonal antibody M2 conjugated with HRP (Sigma), anti-HA polyclonal Ab ( Covance...
Gel-filtration Chromatography—Gel-filtration chromatography was used as a final step in the purification of Gadd45 (see "Experimental Procedures"). Protein was already about 90% pure after the two preceding chromatographic steps. Chromatography on Sephacryl S200 clearly separates most of remaining higher molecular mass contaminants. The resulting protein was estimated to be more than 99% pure. Gadd45a samples with preferentially monomeric species (Gadd45m) were treated in the same way except that NiCl₂ exposure was omitted.

RESULTS

Analysis of Gadd45a Electrophoretic Mobility in Native Polyacrylamide Gels (Ferguson’s Analysis)—Analysis of the rela-

2 It is assumed that Gadd45 is roughly globular, which is strongly suggested by secondary structure predictions (EXPASY proteomics tools server).
tive electrophoretic mobilities ($R_f$) of the globular proteins in nondenaturing gels provides a sensitive technique to determine the aggregation state of the protein and estimate molecular masses of the oligomeric species (26, 27). At least four bands can be resolved when Gadd45a at 1 mg/ml (starting concentration) is electrophoresed through such gels (Fig. 2A). In contrast, only one band can be detected when the same amount of the protein is run under denaturing conditions (Fig. 2B). Thus, the additional bands in native conditions may represent oligomers of Gadd45a containing from two to four subunits. To estimate molecular masses of these presumed oligomers, molecular mass protein standards for native gel (Sigma) were electrophoresed along with the Gadd45a in duplicate through native gels of differing acrylamide percentage (%$T$). E. coli UvrB protein (28) was also used in some experiments. The log($R_f$) values were plotted against %$T$. In all cases the plots were found to be linear ($r^2 > 0.97$, data not shown). As a part of analysis, the linear plots for all four Gadd45a species would intercept log($R_f$)-axis in the same point (data not shown) if extrapolated to %$T$ equal 0. This means that all Gadd45a species have the same charge per subunit, thus confirming that they are oligomers and not charge isomers (26, 27). In further analysis, the logs of the retardation coefficients, represented by the slopes of $R_f$ versus %$T$ graphs, for molecular mass standards were plotted against logs of their molecular masses, thus producing the molecular mass calibration plot (26, 27). Comparison of the log(retardation coefficient) for Gadd45a species I–IV with this calibration plot (Fig. 2C) allowed us to estimate their molecular masses (Table I). Comparison of thus obtained molecular masses with those calculated from amino acid composition for oligomers of Gadd45a suggests that I and II are most probably monomer and dimer, while III and IV contain three and four subunits, respectively, although additional higher subunit contents cannot be ruled out.

Chemical Cross-linking of Gadd45a Protein in Solution—
The hydrodynamic methods (gel filtration and gel electrophoresis) strongly suggest a potential for Gadd45a self-association. These methods utilize movement of the protein through porous medium. Some artifacts can be generated due to the interaction with this medium. To rule out such a possibility and to further study Gadd45a self-association, chemical cross-linking of the protein in solution was examined with denaturing (Fig. 3) gel electrophoresis. Such cross-linking was, indeed, achieved with a number of bifunctional reagents that react primarily with lysines, DMS and disuccinimidyl glutarate (Pierce). These reagents yielded a similar pattern of cross-linking. The results

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3 It should be noted, however, that although this analysis accounts for the different charges of the proteins, it is assumed that the shapes of the proteins of interest are roughly the same as that of standards.
TABLE I

| Proteins   | Molecular weight | Predicted |
|------------|------------------|-----------|
| Gadd45a, I | 19 ± 2           | 18.4, monomer |
| Gadd45a, II| 36 ± 2           | 36.8, dimer |
| Gadd45a, III| 64 ± 8          | 55.2, trimer |
| Gadd45a, IV| 83 ± 3           | 73.6, tetramer |

FIG. 3. Chemical cross-linking of Gadd45a protein in solution. Gadd45a (10 μM) was cross-linked by DMS under varying composition of reaction mixture and analyzed by 12% SDS-PAGE followed by Coomassie staining. Positions of molecular mass markers (lane M) are shown by arrows.

obtained with DMS are presented in Fig. 3. Incubation of Gadd45a with DMS resulted in the appearance of covalently linked oligomers of the protein, which were resolved by SDS-PAGE under denaturing conditions (Fig. 3). Comparison of the mobilities of the cross-linked species with those of molecular mass standards for denaturing gels (Bio-Rad) reveals them to be dimers and trimers, the former being the major species at this protein:cross-linker ratio. Increase in salt (KCl) concentration from 25 to 300 mM did not alter the yield of cross-linked species, implying the hydrophobic nature of oligomerization. Likewise, a 10-fold increase in DTT concentration had no effect on the extent and pattern of cross-linking (Fig. 3).

Estimation of the Overall Affinity of Gadd45a Self-association—An ELISA-based immunoassay (29) was used to roughly estimate the overall affinity of Gadd45a self-association. This estimation was obtained to determine whether association occurred at physiologically relevant concentrations. Highly purified Gadd45a protein coated onto a 96-well microtiter plate was incubated with glutathione-agarose purified GST-Gadd45a at different concentrations. After washing, the amount of bound GST-Gadd45a was assessed by sequential incubations of the wells with anti-GST monoclonal Ab and HRP-conjugated secondary Ab intermittent with extensive washing. Following another washing and incubation with HRP cologenic substrate o-phenylenediamine, plates were read and a binding curve generated (Fig. 4). GST protein was used to monitor for possible nonspecific binding in this assay. It was found to be insignificant even at high concentrations (data not shown). The concentration, ~2.5 μM, of GST-Gadd45a resulting in 50% of maximal binding was taken as an estimate of apparent Kd of self-association. It should be emphasized that this value can constitute only a rough estimate, because self-association of GST-Gadd45a in solution as well as potential masking of binding sites by immobilization in the well were not taken into account. In addition, some oligomerization might be expected for the protein bound to the plate. All these effects probably contributed to an underestimate of the actual affinity for self-association.

FIG. 4. Binding curves of Gadd45a self-association. A binding curve was obtained by ELISA assay as described under “Experimental Procedures.” Binding of GST protein alone was insignificant in the range of concentrations used. The concentration of GST-Gadd45a resulting in a 50% of maximum binding is ~2.5 μM. It was taken as an estimate of the dissociation constant of self-association.
used in co-precipitation experiments (Fig. 7A). These results provide evidence that Gadd45a can self-associate in mammalian cells.

Using a similar approach, the interaction of Gadd45 with the two other human Gadd45-related proteins, Gadd45b and Gadd45g, was investigated in RKO cells. These cells were co-transfected with plasmids expressing myc-tagged Gadd45a and either HA-tagged Gadd45b or HA-tagged Gadd45g. Tag-specific immunoprecipitation showed that Gadd45a co-precipitates with both Gadd45b and Gadd45g (Fig. 6B, upper panels). It was also found by the same approach that FLAG-tagged Gadd45b and HA-tagged Gadd45g co-precipitate when co-expressed in RKO cells (data not shown). These findings indicate that all three members of the Gadd45 protein family can form homo- and/or hetero-complexes in the cell.

Immunoblotting analysis of tagged-protein levels in total lysates, used for immunoprecipitation, was employed as a control for their comparable expression (Fig. 6, A and B, lower panels).

Identification of the Self-Association Domain(s) of Gadd45a Protein.—To map the Gadd45a domain(s) involved in self-association, a series of myc-tagged Gadd45a deletion mutants was employed. RKO cells were co-transfected with plasmids expressing myc-tagged Gadd45a deletion mutants and FLAG-tagged full-length Gadd45a. The lysates from transfected cells were immunoprecipitated with anti-FLAG Abs and analyzed by immunoblotting with anti-myc Ab (Fig. 7A). The expression levels of myc-tagged Gadd45a proteins were analyzed after immunoprecipitation (upper panel) or in lysates (lower panel). The lysate levels were found to be abundant and comparable in all samples (Fig. 7A, lower panel). Full-length myc-Gadd45a and myc-tagged C-terminal deletion mutant Gadd45a-(1–71) were able to interact with full-length FLAG-tagged Gadd45a. N-terminal deletion Gadd45a-(74–165) was also able to efficiently interact with FLAG-tagged Gadd45a. In contrast, the deletion mutant Gadd45a-(48–132), lacking both N-terminal and C-terminal portions of the protein, was significantly deficient in the binding (Fig. 7A, upper panel). This suggests the existence of two self-association domains of Gadd45a, one within the N-terminal region and another near the C-terminal region.

A series of overlapping peptides spanning through the entire sequence of Gadd45a (21) were used to further delineate interacting domains. Gadd45a peptides were immobilized onto microtiter plates and incubated with purified GST-Gadd45a protein. The amount of bound GST-Gadd45a was assessed with anti-GST Abs as described above (see “ELISA Assays”). Incubation of peptides with GST protein alone was used as the control for specificity and background. It was found that N-terminal peptides 33–52 and 42–61 as well as C-terminal peptides 113–132, 129–148, and 145–165 bound GST-Gadd45a two to three times stronger than neighboring peptides or peptides in the central region (Fig. 7B). The absence of a correlation between binding and the presence of cysteines in peptide provides another argument against nonspecific disulfide formation-driven interaction.

Oligomerization State of Gadd45a Protein Influences Its Ability to Protect Cyclobutane Pyrimidine Dimers in in Vitro UV-irradiated Nucleosomes from T4 UV Endonuclease Cleavage.—It was previously found that Gadd45a can bind nucleosomes in vitro and protect cyclobutane pyrimidine dimers in nucleosomes from T4 UV endo digestion (15). These effects, however, were observed only with Gadd45a purified without usage of nickel-chelating chromatography, although nickel column-purified protein was similarly active in other nucleosome related assays (Ref. 15; data not shown). Consequently, it was found that small amounts of nickel result in a significantly increased level of oligomerization of Gadd45a, which is persistent even after nickel removal4; these oligomers appear indistinguishable from spontaneous oligomers. Total nucleosomes obtained from chicken erythrocytes were UV-irradiated and incubated with Gadd45a preparations having different levels of oligomerization followed by incubation with T4 UV endo. The unirradiated nucleosomes were used as a control. The T4 UV endo digestion of total UV-irradiated nucleosomes resulted in a decrease of full-length nucleosomal DNA and the appearance of a diffuse smear of lower molecular mass DNA fragments (Fig. 8). Total nucleosomes contain DNA of the same length but different sequence, and digestion of these DNAs produces DNA fragments of many possible lengths. The electrophoretic mobility of these fragments can additionally be influenced by their sequence, thus resulting in a smear-like pattern instead of distinct bands. It was found that an increased level of Gadd45a oligomerization significantly reduced the protection of nucleosomes (Fig. 8). Consistent with previous observations, there was no protective effect in the case of DNA devoid of nucleosomes regardless of the Gadd45a oligomerization level. Thus it appears that oligomerization of Gadd45a protein abolishes its nucleosomal damage protective effect on T4 UV endo digestion. The effect of nickel on Gadd45a other than increase of its nucleosomal damage protective effect, appears that oligomerization followed by incubation with T4 UV endo. The unirradiated nucleosomes were used as a control. The T4 UV endo digestion of total UV-irradiated nucleosomes resulted in a decrease of full-length nucleosomal DNA and the appearance of a diffuse smear of lower molecular mass DNA fragments (Fig. 8). Total nucleosomes contain DNA of the same length but different sequence, and digestion of these DNAs produces DNA fragments of many possible lengths. The electrophoretic mobility of these fragments can additionally be influenced by their sequence, thus resulting in a smear-like pattern instead of distinct bands. It was found that an increased level of Gadd45a oligomerization significantly reduced the protection of nucleosomes (Fig. 8). Consistent with previous observations, there was no protective effect in the case of DNA devoid of nucleosomes regardless of the Gadd45a oligomerization level. Thus it appears that oligomerization of Gadd45a protein abolishes its nucleosomal damage protective effect on T4 UV endo digestion. The effect of nickel on Gadd45a other than increase of its oligomerization cannot be ruled out at present. However, the ability of nickel-exposed protein to efficiently promote chromatin relaxation by eukaryotic topoisomerase I (Ref. 15; data not shown) lends support to the above suggestion.

4 This effect was found to be nickel-specific and is currently under study (O. Kovalsky, Y. Higashimoto, E. Appella, and A. Fornace, unpublished observation).
FIG. 6. Association of Gadd45a protein with itself and the other two members of the Gadd45 protein family, Gadd45b and Gadd45g, in mammalian cells. RKO cells were transiently co-transfected with: FLAG-Gadd45a and HA-Gadd45a constructs (A). Either FLAG-Gadd45a (left panels) or HA-Gadd45a (right panels) was immunoprecipitated from total lysates of transfected cells with anti-tag immunomatrix (IP), and the presence of HA-Gadd45a or FLAG-Gadd45a was probed by immunoblotting (IB) with the respective anti-tag Ab. The amounts of HA- (left) and FLAG-Gadd45a (right) proteins in total lysates detected by immunoblotting are shown in the lower panels. B, myc-Gadd45a and either HA-Gadd45b or HA-Gadd45g constructs. Samples were immunoprecipitated and immunoblotted as described above using anti-myc and anti-HA immunomatrix and Abs. The amounts of myc- and HA-tagged Gadd45 proteins in total lysates are shown in the lower panels. For controls, co-expression with FLAG, HA, and myc vectors was employed.

**DISCUSSION**

Determining the oligomeric state of Gadd45a protein is important for understanding the molecular mechanism(s) by which this protein carries out its multiple functional interactions. Using a variety of approaches, it was shown that Gadd45a is capable of self-association forming stable and reproducible oligomeric structures both in vitro and when expressed in the cell. Gel filtration, electrophoresis, and chemical cross-linking data strongly suggest that under in vitro physiological solution conditions dimers are the predominant oligomeric form of Gadd45a protein, although oligomers of higher order can also be detected by these methods.

The self-association seems to be driven largely by hydrophobic interactions, because neither the extent nor the pattern of cross-linking are changed by a 10-fold increase in salt concentration. Consistently, it was found that Gadd45a is strongly retained by hydrophobic matrix, phenyl-Sepharose. This property provided a very efficient step in chromatographic purification of Gadd45a protein. It also indicates the presence of hydrophobic patch(es) on the surface of the protein, which is accessible for interaction with other proteins and cellular components. Gadd45a is overall negatively charged under physiological conditions (pH 4.2) (7), which hints of involvement of the protein in ionic interactions. The presence of surface-accessible hydrophobic domain(s) extends the spectrum of potential interactions of Gadd45a protein. As a potential reflection of its hydrophobicity, it was found that Gadd45a is only partially extractable from the cell by non-ionic detergent-based RIPA-like lysis buffers, even after increase of ionic strength (data not shown). A significant part of cellular Gadd45a remained bound to the insoluble nuclear matrix/chromatin fraction. This strong binding can be mediated in vivo by hydrophobic interactions.

It has been shown that a number of cellular proteins involved in DNA processing form foci-like structures in the nucleus, particularly in response to cell stress associated with DNA damage. These structures may constitute repair sites at which specialized proteins assembled on chromatin/nuclear matrix carry out functional activities (30–33). Gadd45a was found to also form foci-like structures in the nucleus (Fig. 5). This observation together with the abovementioned low extractability of Gadd45a suggests that Gadd45a may be a component of such repair assemblies. These foci are found in cells stably transfected with a Gadd45a-expressing construct even in the absence of exogenous stress. However, the increased level of Gadd45a protein in these cells seems to be itself “stressful” for the cells judging from their slow growth (data not shown). Moreover, this level, estimated to be about 10-fold higher than in control cells, is comparable with the levels of the protein induced in this type of cells by methylmethane sulfonate, x-ray, and UV radiation treatment (6, 9). Therefore, these cells may to some extent mimic the situation in normal cells upon exogenous stress. In agreement with the latter suggestion, the foci-like pattern of Gadd45a localization was not qualitatively changed after genotoxic stress, although the number of foci appeared to be increased (preliminary experiments with UV radiation and methylmethane sulfonate, data not shown). Compartmentalization of Gadd45a molecules in foci structures would be expected to further drive oligomerization through the increase of effective concentration.

An important issue is whether Gadd45a can oligomerize at physiologically relevant concentrations. For example, a $K_d$ of association in the millimolar range would be unlikely to be achieved in vivo. To address this issue, an ELISA approach (29) gave an estimated $K_d$ of $\sim 2.5 \mu M$, although the actual value is probably less for the reasons already discussed. However, even this approximate result increases the confidence that self-association occurs in vivo. Indeed, we estimate that there are $\sim 40,000$ copies of Gadd45a per RKO cell, which translates into a $\sim 0.1 \mu M$ concentration assuming a $10-\mu M$ diameter

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6 F. Carrier and A. Fornace, unpublished observation.
6 F. Carrier, O. Kovalsky, and A. Fornace, unpublished observations.
spheroid-shaped cell. Taking into account that Gadd45a is predominantly a nuclear protein, and that the RKO nucleus is estimated to be at least 5-fold smaller in volume, the nuclear concentration of Gadd45a falls into the hundreds of nanomolar to micromolar range. Further compartmentalization in Gadd45a foci would easily bring its concentration into the micromolar range.

Two regions of Gadd45a protein were found in this study to be implicated in self-association. One is localized within N-terminal amino acids 33–61 and another within the C-terminal amino acids 129–165. The N-terminal binding site is enriched in hydrophobic and non-charged amino acids and, hence, may contribute crucially to the observed hydrophobic nature of oligomerization. It is not clear whether these two sites form a single binding surface in the protein. However, removal of one of the sites in deletion mutants does not abolish the capability of the other site to mediate self-association in vitro and in the cell.

It was previously shown that Gadd45a protein can interact in vitro and/or in vivo with a number of other cellular proteins: p21, PCNA, cdc2-cyclinB complex, histones, and MTK1/MEKK4 kinase. Delineation of Gadd45a regions, which are involved in binding of these proteins and self-association domains, shows that the latter overlaps with PCNA binding sites (Fig. 9). The physiological significance of the overlap remains to be determined. However, it may imply a potentially regulatory role for Gadd45a oligomerization. In an attractive model, the formation of oligomers would render Gadd45a incapable of interacting with one of these proteins while promoting interaction with another. Stress to the cell induces Gadd45a levels and, hence, should influence the oligomeric state of the protein. Consequently, it may result in altering the pattern of Gadd45a interactions in the cell. Because different stresses up-regulate Gadd45a to different extents (34), the oligomeric status of the protein may be stress-dependent. Hence, cellular interactions of Gadd45a, which are necessary to adequately respond to
specific stresses, can be regulated by adjusting its oligomerization status. Taking into account structural and functional similarities between all three Gadd45 proteins (24), it is highly probable that Gadd45b and Gadd45g are also capable of efficient oligomerization. Together with the evidence of hetero-complexing of these proteins (Fig. 6B), this property of Gadd45 proteins significantly extends the spectrum of possible responses to a multiplicity of stresses and stimuli to the cell. As an example of potential cooperation of Gadd45a and Gadd45b proteins, it was found that they synergistically suppressed cell growth after overexpression (7). Also, tissue and stress/stimulus specificity of Gadd45 proteins distribution and induction (35) may further contribute to the specificity of cellular response.

As evidence of oligomerization dependence of Gadd45a interactions, it was found that self-association of the protein influences protection of DNA on damaged nucleosomes (Fig. 8). It was shown previously that Gadd45a protects cyclobutane pyrimidine dimers induced by UV irradiation of isolated nucleosomes from digestion by T4 UV endo (15). This effect suggested that Gadd45a may preferentially recognize and bind DNA-damaged sites in chromatin, thus influencing their accessibility to DNA processing proteins, e.g., DNA repair machinery. The protective effect was significantly reduced in a Gadd45a preparation enriched in oligomers, so that cyclobutane pyrimidine dimers are no longer efficiently protected from digestion (Fig. 8). Hence, the mode of interaction of Gadd45a with damaged chromatin is dependent on its oligomeric status.

In summary we show here that Gadd45a protein is capable of self-assembly and interaction with two other Gadd45 proteins both in vitro and in the cell. These interactions may constitute a basis for regulation and tuning of the functional activities of Gadd45 proteins in growth arrest, DNA repair, and other stress-induced processes.

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