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Solution Structure of Human Growth Arrest and DNA Damage 45α (Gadd45α) and Its Interactions with Proliferating Cell Nuclear Antigen (PCNA) and Aurora A Kinase*[S]

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Gadd45α is a nuclear protein encoded by a DNA damage-inducible gene. Through its interactions with other proteins, Gadd45α participates in the regulation of DNA repair, cell cycle, cell proliferation, and apoptosis. The NMR structure of human Gadd45α has been determined and shows an α/β fold with two long disordered and flexible regions at the N terminus and one of the loops. Human Gadd45α is predominantly monomeric in solution but exists in equilibrium with dimers and other oligomers whose population increases with protein concentration. NMR analysis shows that Aurora A interacts through its N-terminal domain with a region of human Gadd45α encompassing the site of dimerization, suggesting that the oligomerization of Gadd45α could be a regulatory mechanism to modulate its interactions with Aurora A, and possibly with other proteins too. However, Gadd45α appears to interact only weakly with PCNA through its flexible loop, in contrast with previous and contradictory reports.

The Gadd45[4] family of proteins consists of isoforms α, β, and γ, with sequence identities of ~56% (1). Transcription of the *Gadd45* genes is induced by DNA-damaging agents and other cellular stresses and is associated with growth arrest.

Human Gadd45α (hGadd45α) is an acidic protein of low abundance in the cell, localized mainly in the nucleus (2). It participates in cell growth and cell cycle control, DNA repair, apoptosis, maintenance of genomic stability, and the regulation of signaling pathways (3, 4). Gadd45 proteins exert these functions by their interactions with other proteins. hGadd45α interacts with PCNA (5), an essential factor for DNA replication and repair, the cyclin-dependent kinase inhibitor $p21^{WAF1}$ (6), and with the protein kinases cyclin-dependent kinase 1, mitogen-activated protein kinase kinase kinase 4 (MAPKKK4), and Aurora A (7, 8). hGadd45α maintains centrosome stability by down-regulation of Aurora A kinase activity (7). The pleiotropic effects of hGadd45α have partly been explained by its interaction with the xeroderma pigmentosum complementing group G endonuclease and the promotion of DNA repair, which erases methylation marks relieving epigenetic gene silencing (9). However, the role of Gadd45α in active DNA demethylation is still controversial (10, 11). Gadd45α interacts with the four core histones of the nucleosome and modifies the access to DNA on damaged chromatin (12). Gadd45 proteins can form oligomers, which could have a role in regulating their function (13).

We describe the NMR structure of the full-length monomeric hGadd45α protein, which adopts a globular α/β fold with two long disordered and flexible regions at the N terminus and one of the loops. The folded region is similar to the recent crystal structure of a construct of the murine Gadd45γ isoform truncated at the N terminus, which forms dimers (14). We show that hGadd45α interacts with Aurora A through a region opposite to the location of the flexible protein chain termini and encompassing a putative dimerization site, whereas it interacts with PCNA through the flexible loop albeit very weakly.

EXPERIMENTAL PROCEDURES

Protein Production and Purification—All the human proteins were produced in *Escherichia coli* and purified from the soluble fractions of the lysed cells by liquid chromatography. Two different wild type hGadd45α protein samples were prepared, one of them without any tag, the other fused to an N-terminal His-tag, which was removed by proteolysis (15). PCNA was also purified with an N-terminal His-tag cleaved in the same way (16). Aurora A full-length and the kinase domain (residues 122–403) were produced fused to an N-terminal His-tag.

Analytical Ultracentrifugation and Gel Filtration—Sedimentation velocities were measured at 25 °C at a speed of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental methods, Table S1, Figs. S1–S11, and additional references.

The atomic coordinates and structure factors (code 2KG4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[4] The abbreviations used are: Gadd45α, growth arrest and DNA damage 45 α; PCNA, proliferating cellular nuclear antigen; PIP, PCNA-interacting protein; HSQC, heteronuclear single quantum coherence spectroscopy.

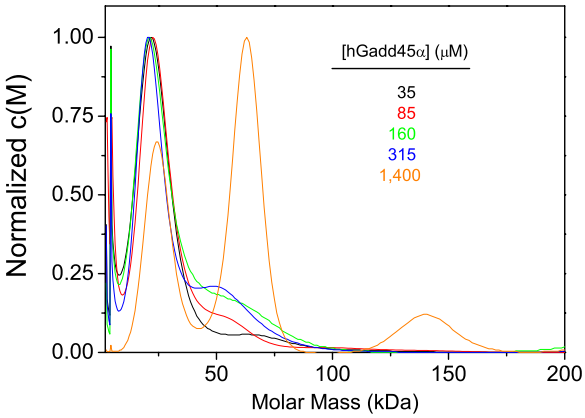

FIGURE 1. **hGadd45α is predominantly monomeric in solution.** The distributions of molar masses measured in sedimentation velocity experiments at 42,000 rpm of hGadd45α are shown at different protein concentrations. The values of the distributions of molar masses have been normalized so that the maximum value is set to 1 for every one of the samples. The measurement at 1,400 μM was done with the protein expressed with a hexahistidine tag that, after cleavage, results in an extra GlyProHis amino acid sequence preceding the native M1 residue. Except for this high concentration sample, the measured molecular mass of the major peaks ranges from 17,500 to 19,500 Da, which correspond to the monomeric species.

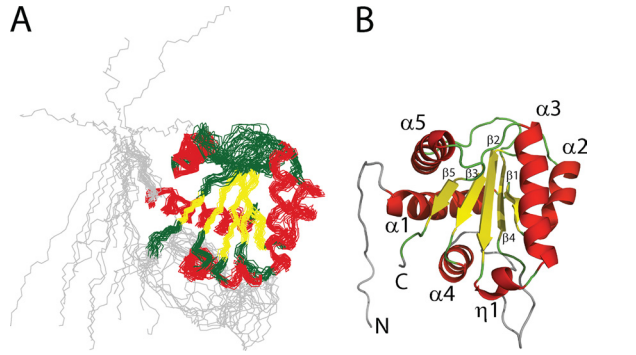

FIGURE 2. **Solution structure of hGadd45α.** The mixed five-stranded β-sheet is shown in *yellow*, the α-helices are colored *red*, flexible regions are colored *gray* (see below), and coil regions are shown in *green*. *A*, superposition of the backbone heavy atoms (N, C^{α}, C′) of the 20 NMR conformers. *B*, *ribbon representation* of hGadd45α. Labels indicate the location of the N and C termini and the individual secondary structure elements. *η1* indicates the 3_{10} helix.

42,000 rpm in buffer: 20 mM sodium phosphate, pH 7.4, 100 mM KCl, 2 mM EDTA, 2 mM dithiothreitol. Analytical size-exclusion chromatography of hGadd45α proteins was done at room temperature on a calibrated Superdex-75 10/300 column using the same buffer.

NMR Spectroscopy, Structure Calculations, and Measurement of Interactions—NMR measurements were performed at 25 °C on Gadd45α samples with protein concentrations in the range 160–450 μM. Distance, dihedral angle, and residual dipolar coupling restraints were measured and used to calculate an ensemble of 20 models that were refined by restrained energy minimization in explicit water. ^{1}H-^{15}N HSQC spectra of U-^{15}N Gadd45α in the absence and presence of Aurora A or PCNA were recorded sequentially and under identical conditions on samples dialyzed simultaneously against the same buffer. The same procedure was used to prepare the samples and record the spectra of U-^{2}H-^{15}N PCNA (with protonated amides) in the absence and in the presence of Gadd45α, except that the measurement temperature was 35 °C. Detailed experimental methods are provided in the supplemental information methods section.

RESULTS AND DISCUSSION

Human Gadd45α Is Predominantly Monomeric in Solution—Sedimentation velocity measurements show that hGadd45α is predominantly monomeric up to concentrations of a few hundred μM, in equilibrium with dimers and other oligomeric species (Fig. 1). Although the theoretical molecular mass of the monomer is 18.3 kDa, analytical gel filtration yields an apparent value of 29 kDa (supplemental Fig. S1). This is probably due to the long flexible segments of the molecule (see below), which accelerate the advance of the molecule through the column with respect to a compact and globular one. The line widths of most of the NMR signals of hGadd45α are also consistent with a protein of the size of the monomer (15). The average R_2 relaxation rates measured for the backbone ^{15}N nuclei in the ordered regions of the protein is 20 ± 0.1 s^{-1}, which is consistent with monomeric hGadd45α. Two-dimensional diffusion ordered spectroscopy yielded a diffusion coefficient slightly larger than the value measured for lysozyme (14.6 kDa) and smaller than the value measured for maltose binding protein (42 kDa; supplemental Fig. S2). These results are in agreement with published gel filtration data (providing that a smaller elution volume is not interpreted as a size larger than the monomer) and with chemical cross-linking data (13), but not with the measured electrophoretic mobility which, in addition to monomers, trimers, and tetramers, suggest a predominantly dimeric species (13). At concentrations in the low mM range, the NMR signals of hGadd45α broaden, and their intensity decreases with time, eventually becoming invisible except for a set of non-dispersed signals from the flexible regions of the molecule. These observations can be explained by the irreversible self-association of hGadd45α into large soluble aggregates, which sequester the monomeric species and render them invisible by NMR. The tendency to aggregate could be related to the low global and local structural stability of the protein. Thus, the thermal denaturation monitored by circular dichroism is less cooperative than expected for a protein of its size (data not shown), and the solvent exchange of the amide protons is very fast (supplemental Fig. S3). These observations indicate a complex dynamic behavior of hGadd45α, which could be of functional relevance.

Solution Structure of Human Gadd45α—The solution structure of hGadd45α is represented by an ensemble of 20 refined NMR models whose superposition (Fig. 2*A*) shows three disordered regions: residues 1–16, 105–118, and 164–165. The high root mean square deviations in these regions are a consequence of their high flexibility, as confirmed by small local heteronuclear {^{1}H}-^{15}N Overhauser effects (supplemental Fig. S4). The R_2 relaxation rates of the backbone ^{15}N nuclei in these mobile regions also are smaller than those of the ordered regions; however, the low signal to noise ratio in these experiments resulted in large errors in the estimation of the values of R_2 preventing a reliable full quantitative relaxation study of the protein.

The ordered regions of the chain are defined with an root mean square deviation of 0.95 and 1.50 Å for the backbone and all the heavy atoms, respectively (supplemental Table S1 and supplemental Fig. S5), and the root mean square deviation is even smaller for the secondary structure elements (0.75 Å for the backbone heavy atoms). The structure shows a segregated α/β fold with a five-stranded mixed β-sheet at the core and five surrounding helices (Fig. 2*B*). Helices α2 and α3 pack on one side of the β-sheet, whereas helices α1, α4, and α5 pack on the opposite side. The β2–α3 connecting loop contains one turn of 3_{10} helix. The two long flexible regions at (the N terminus and the loop connecting α4 with β4), are not completely random, but show a propensity to form local helical structures in fast exchange with other conformations. These nonrandom structures can be detected by the deviations of the corresponding backbone chemical shifts from tabulated random coil values (Supplemental Fig. S6), which are small but larger than the range observed in denatured polypeptide chains. These conformational propensities could be relevant for the interactions of hGadd45α with other proteins (see below) as well as for its monomeric state. In this regard we note that the published (17) analytical gel filtration chromatography on the human Gadd45β isoform (performed on the full length construct) is consistent with a monomeric molecule providing that the elution volume, larger than expected for a globular protein of its size, is due to the N-terminal and α4-β4 loops being flexible as are the homologous regions of mGadd45α. Therefore, it may be that the dimerization equilibrium of hGadd45γ is shifted toward the dimer when the N-terminal segment is eliminated. Indeed, it is tempting to speculate that, in solution, Gadd45 proteins in general exist in dynamic equilibria with oligomeric populations, which can be modulated by the proteins concentration, mutations, deletions, and ligand binding.

The structure of the ordered regions of hGadd45α is similar to the reported crystal structure of a deletion mutant of mGadd45γ, superimposing with a root mean square deviation of 1.7 Å for the C^{α} atoms (Fig. 3*A* and supplemental Fig. S7). This construct of the murine γ-isoform was found to form dimers in solution, with the dimerization interface formed by helices α2 and α3. The possibility that other Gadd45 proteins dimerize in the same way is suggested by the high conservation of residues located in helices α2 and α3 (supplemental Fig. S8). However, earlier studies with deletion mutants and peptide binding suggested a different oligomerization site for hGadd45α (13).

Our hydrodynamic results on the monomeric nature of hGadd45α are confirmed by the amide residual dipolar couplings, an independent and purely orientational measurement that constrains the arrangement of the possible oligomers (18, 19). For several residues of the well defined regions of the protein, the residual dipolar couplings are incompatible with a dimer analogous to the reported mGadd45γ ($r = 0.88$ and 0.82, each for one of the two chains in the dimer), but compatible with the set of 20 solution structures of monomeric hGadd45α ($r = 0.94 \pm 0.01$; see also supplemental Fig. S9). Still, a small population of dimeric hGadd45α exists in equilibrium with the monomers (Fig. 1 and supplemental Fig. S1). The dimer population is much smaller or even undetectable (depending on the technique used) when the mutation L77E is introduced in helix α3. This mutation is analogous to the mutation L80E in mGadd45γ, which strongly destabilizes the dimer and confirms that the dimerization interface is formed by helices α2 and α3 rather than the other three possible interfaces observed in the mGadd45γ crystal structure. These results suggest that a similar dimerization surface may exist for both proteins, although the oligomerization equilibrium is shifted toward monomeric hGadd45α and to dimeric mGadd45γ. The different populations of dimeric and monomeric species may be explained by two facts. First, residues differ at three positions within the α2 and α3 helices in the two isoforms (Fig. 3*B* and supplemental Fig. S8). Second, interhelical packing differs, with an angle (between the longitudinal axes of both helices) of $12 \pm 4°$ and $29 \pm 2°$ for the hGadd45α NMR structures and for the mGadd45γ crystal monomers, respectively (Fig. 3*B*). This different orientation results from the reorganization of the packing at the hydrophobic core, due to the different size of the Ala^{43} and Leu^{46} side chains in hGadd45α compared with the corresponding residues in mGadd45γ (Ser^{46} and Val^{49}, respectively), and to the burial of the Ser^{46} hydroxyl group of mGadd45γ. This polar group would destabilize the structure

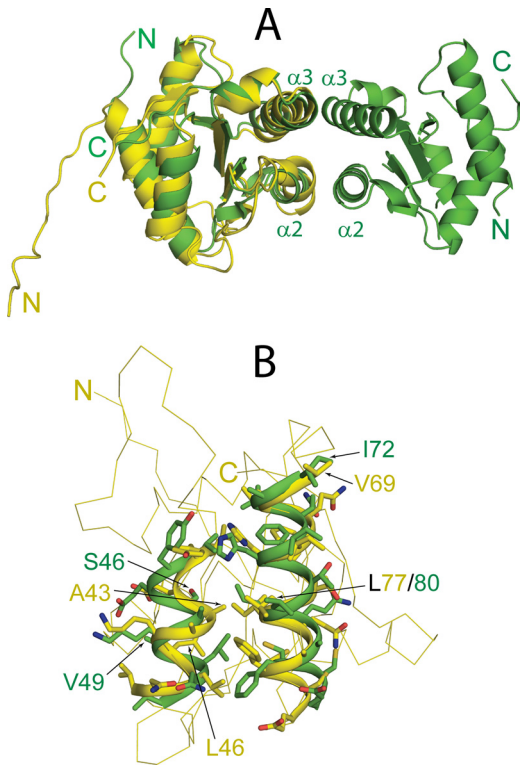

FIGURE 3. **Structure comparison between hGadd45α and mGadd45γ.** *A*, *ribbon diagram* of hGadd45α (*yellow*) superposed onto one of the monomers of mGadd45γ (*green*). Chain termini and helices α2 and α3 are labeled in the corresponding color. The crystallized construct of mGadd45γ lacks the N-terminal 15 residues, and loops α4–β4 and β4–α5 are not seen in the crystal. *B*, superposition of helices α2 and α3 of hGadd45α (*yellow*) and mGadd45γ (*green*) illustrating the different relative orientation of the helix pair in each protein. The side chains of the helical residues are shown in *sticks*, with carbon atoms colored as the corresponding backbone. The C^{α} trace of hGadd45α is shown as a *thin yellow line*. Labels indicate the N and C termini of hGadd45α as well as the nonconserved residues in helices α2 and α3 (with the corresponding residue number and color). Residue Leu^{77} in Gadd45α (which corresponds to Leu^{80} in mGadd45γ) is labeled in *black*.

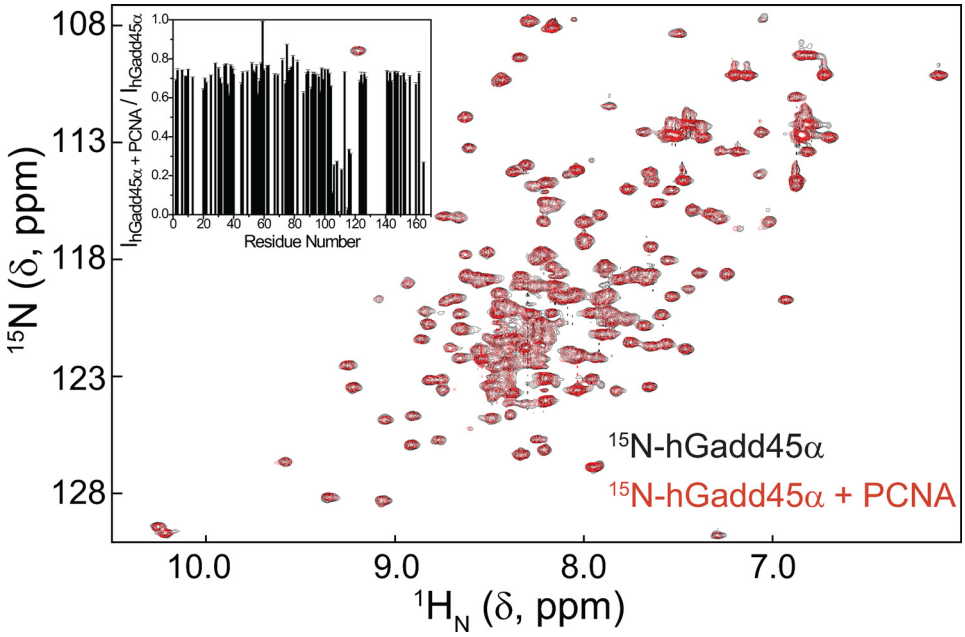

FIGURE 4. **Interaction of hGadd45α with PCNA.** Overlay of the ^{1}H-^{15}N HSQC spectra of 450 μM hGadd45α in the absence (*black*) and in the presence (*red*) of PCNA (1:2 monomer molar ratio). The spectra were acquired in 20 mM sodium phosphate, pH 7.4, 100 mM KCl, 2 mM dithiothreitol, 2 mM EDTA at 25 °C. *Inset*, relative hGadd45α peak intensities with *versus* without PCNA plotted against the residue number; the experimental error is indicated by the *error bars*. An overall reduction of ~25% in the cross-peak intensities in the sample with PCNA is caused by the dilution of the mixed sample during dialysis with respect to the sample with only hGadd45α (because of the higher osmotic pressure). For some residues, a quantitative measurement was not possible, and the corresponding position is left empty in the plot.

were it not hydrogen bonded with the carbonyl of G[42]. A third factor that may affect the association properties of the Gadd45 proteins is the flexibility of the N-terminal region, which is absent in the reported mGadd45γ construct. This segment of the chain would lie at the opposite side of the proposed dimerization interface, but considering its length and its high mobility, it cannot be excluded that it influences the tendency of two molecules to associate.

Interaction of hGadd45α with PCNA and Aurora A Kinase—Among the few proteins reported to interact directly with hGadd45α, we have tried to structurally characterize the binding of human PCNA and Aurora A kinase because both of them can be expressed in *E. coli* and can be purified in the amount and with the purity necessary for NMR studies (20, 21). In the case of PCNA, its backbone NMR spectrum has been assigned previously (16), making it possible to map the interaction site on both hGadd45α and PCNA.

The interaction with PCNA is the earliest and most documented interaction of Gadd45 proteins. It has been reported that hGadd45α binds to human PCNA (6), as do mGadd45β and mGadd45γ (22), though none of them contain the typical PCNA interacting protein (PIP) box motif (23). The PIP box is a conserved sequence that binds to three equivalent sites on the PCNA homotrimeric ring, with one PIP sequence for each PCNA monomer (24, 25). Thus, binding of Gadd45 proteins to a site on PCNA different from the PIP binding site might be compatible with the binding of PIP-containing proteins to PCNA, whereas these other proteins compete among them (26). However, the experimental evidence for the identification of the hGadd45α/PCNA interaction sites is contradictory (27). Yeast two-hybrid data reported by two independent groups map the interaction site to different non-overlapping Gadd45 regions, and inconsistent results also were obtained for the regions of PCNA binding to hGadd45α (6, 22). We have analyzed in detail this interaction by NMR observing the changes in the signals of hGadd45α in the presence of the homotrimeric 87-kDa PCNA ring. A reduction in the signal intensities would be expected as a result of the large size of the complex, with a larger decrease for those residues at the interface. We could observe significant changes only for a few residues in the flexible α4–β4 loop and at the C terminus (Fig. 4). Binding through these regions are consistent with one set of reported yeast two-hybrid data (27), but not with the other set (6) because opposite results were reported for hGadd45α-(1–136) binding to PCNA. The available assignment of the NMR spectrum of PCNA (16) allowed us to examine the interaction from the other side, observing the changes in the spectrum of PCNA in the presence of hGadd45α. Because of the large size of PCNA, it must be perdeuterated to observe the NMR signals of its protonated amide groups. The size increase on binding to Gadd45α (18 kDa) should not reduce much their intensities, but should cause chemical shift perturbations on those PCNA residues at the binding site. However, the values that could be measured were all small and below the experimental error (supplemental Fig. S10). Based on the absence of significant chemical shift perturbations, we estimate that the dissociation constant of the possible complex would be larger than 2.2 mM (assuming a 1:1 PCNA:hGadd45α monomer ratio) or larger than 0.64 mM (if we assume a 3:1 ratio, one molecule of hGadd54α per PCNA ring). It has been proposed that the hGadd45α/PCNA interaction coordinate the cell cycle and the DNA damage response (6), reducing the negative control of cell growth and reducing apoptosis (27). We have measured here that a direct physical interaction between the two pure proteins in solution is, at best, transitory and very weak, although it could be enhanced by other factors present in the cell nucleus.

The interaction between hGadd45α and the mitotic kinase Aurora A has been observed by yeast two-hybrid, immunoprecipitation, and pulldown assays (7, 11). Mouse embryonic fibroblast cells deficient in mGadd45α exhibit genome instability, centrosome amplification, and aberrant mitosis, with multiple spindles and failed cytokinesis (3). Since ectopic expression of hGadd45α in Chinese hamster ovary cells antagonizes the centrosome amplification induced by Aurora A (7),

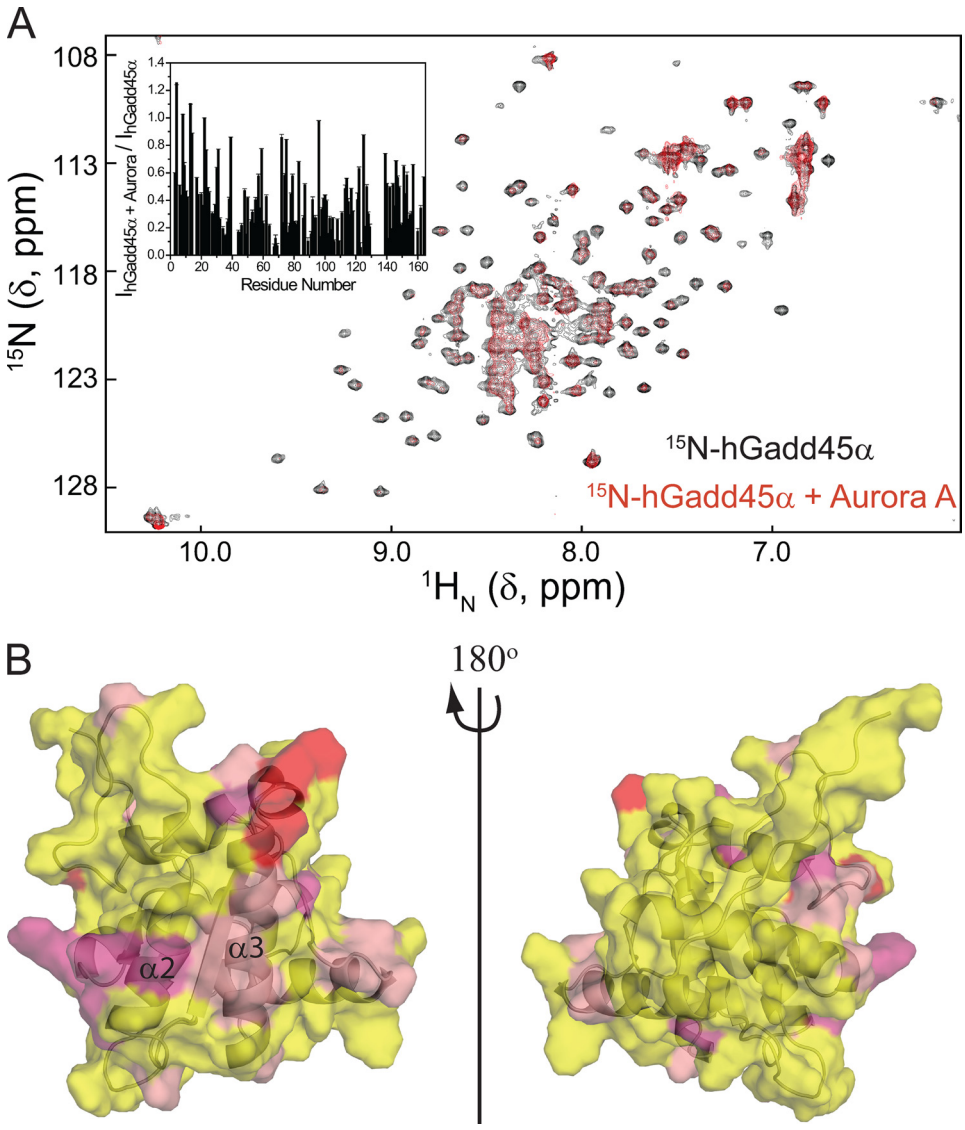

FIGURE 5. **Interaction of Gadd45α with Aurora A.** *A*, overlay of the ^{1}H-^{15}N HSQC spectra of ~65 μM Gadd45α in the absence (*black*) and in the presence (*red*) of Aurora A (1:1 molar ratio). The spectra were acquired in 20 mM Tris, pH 8.0, 200 mM NaCl, 2 mM dithiothreitol, 0.5 mM EDTA at 25 °C. *Inset*, relative hGadd45α peak intensities with *versus* without Aurora A, plotted against the residue number. *Error bars* indicate the estimated experimental error. *B*, surface representation of hGadd45α with residues colored according to relative intensity (bound *versus* free hGadd45α): larger than 0.3 (or not possible to analyze) in *yellow*, between 0.3 and 0.2 in *pink*, between 0.2 and 0.1 in *magenta*, and smaller than 0.1 in *red*. The left-hand view has the same orientation as Fig. 3*B*, and the right-hand view resulted from a 180° rotation along the indicated axis. Helices α2 and α3 are indicated on the left-hand view.

and hGadd45α inhibits Aurora A in HeLa cells, it can be hypothesized that hGadd45α maintains centrosome stability by down-regulation of Aurora A kinase activity. Direct interaction with Aurora A in solution is confirmed by the analysis of the hGadd45α ^{1}H,^{15}N HSQC spectra (Fig. 5*A*), with a large reduction in its signal intensities as a result of the large molecular mass of the complex formed (67 kDa or larger). The intensity reduction is not uniform along the sequence, with the residues changing the most located in a region of the molecule opposite to the location of the flexible chain termini and encompassing helices α2 and α3 (Fig. 5*B*). The location of the binding site is confirmed by the same measurements on hGadd45α mutant L77E (in helix α3), whose NMR signals show smaller changes (and mostly uniform along the sequence) in the presence of Aurora A (supplemental Fig. S11). A truncated mutant of Aurora A containing only the kinase domain (residues 122–403) causes also a smaller and uniform reduction in the hGadd45α signal intensities, indicating that hGadd45α interacts principally with the N-terminal domain of Aurora A (supplemental Fig. S11). This domain is a nonconserved region that could be the binding site to regulatory proteins. Because the Aurora A binding site of hGadd45α overlaps with the Gadd45α dimerization site, it is likely that the two interactions compete with each other. Indeed, the dimerization interface, mapped by an analogous procedure (differential decrease in the intensity of hGadd45α signals in spectra at 200 and 1,300 μM protein concentration), overlaps with the Aurora A binding site (data not shown). The NMR data do not rule out a possible interaction of Aurora A with hGadd45α dimers, binding on an accessible surface in the proximity of the dimer interface. However, this alternative mode of binding is less probable because a larger number of perturbed residues on the surface of hGadd45α than those shown in Fig. 5 would be expected if both dimerization and binding to the much bigger Aurora A molecule were happening. Dynamic light scattering measurements on a sample identical to that used for the NMR measurements did not clarify this issue as the estimated diameters of the species present in the sample were quite variable over consecutive measurements (data not shown).

In any case, the hGadd45α/Aurora A interaction that can be measured by NMR should not be interpreted in terms of a single species. In solution, the complex coexists with different populations of free hGadd45α and free Aurora A protein, in addition to a certain amount of dimeric hGadd45, all of them in dynamic equilibrium.

CONCLUSIONS

Self-association of hGadd45α has been detected in mammalian cells by co-immunoprecipitation of the protein expressed with different tags, as has formation of hetero-oligomers of Gadd45 isoforms (13). However, it is unclear whether this association is direct or mediated by other cellular components. The relevance of hetero-oligomerization is also unclear, as the

expression of each gene is induced by different signals, human *Gadd45α* being the only one up-regulated by p53 (28). Oligomeric hGadd45α does not protect UV-irradiated nucleosomes from nuclease digestion, indicating that it loses the ability to modify DNA accessibility on damaged chromatin (13); thus, the oligomeric state of hGadd45α may affect its function. Our results show that hGadd45α is primarily monomeric, in dynamic equilibrium with minor populations of oligomeric forms. Competition and shifting of this equilibrium could be the basis for a temporal/spatial regulatory mechanism of the interactions with different effectors in the nucleus. The direct interaction with Aurora A through an overlapping site is consistent with a regulatory role of hGadd45α self-association, which could release active Aurora A.

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