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Gadd45β forms a Homodimeric Complex that Binds Tightly to MKK7

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Edited by M. Yaniv

Gadd45α, β, and γ proteins, also known as growth arrest and DNA damage-inducible factors, have a number of cellular functions, including cell-cycle regulation and propagation of signals produced by a variety of cellular stimuli, maintaining genomic stability and apoptosis. Furthermore, Gadd45β has been indicated as a major player in the endogenous NF-κB-mediated resistance to apoptosis in a variety of cell lines. In fibroblasts this mechanism involves the inactivation of MKK7, the upstream activator of JNK, by direct binding within the kinase ATP pocket. On the basis of a number of experimental data, the structures of Gadd45β and the Gadd45β-MKK7 complex have been predicted recently and data show that interactions are mediated by acidic loops 1 and 2, and helices 3 and 4 of Gadd45β. Here, we provide further evidence that Gadd45β is a prevailingly α-helical protein and that in solution it is able to form non covalent dimers but not higher-order oligomers, in contrast to what has been reported for the homologous Gadd45α. We show that the contact region between the two monomers is comprised of the predicted helix 1 (residues Q17–Q33) and helix 5 (residues K131–R146) of the protein, which appear to be antiparallel and to form a large dimerisation surface not involved in MKK7 recognition. The results suggest the occurrence of a large complex containing at least an MKK7-Gadd45β:Gadd45β-MKK7 tetrameric unit whose complexity could be further increased by the dimeric nature of the isolated MKK7.

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Introduction

The *gadd45* growth arrest and DNA damage-inducible family of genes, *gadd45a*, *gadd45b* and *gadd45g*, encode for the corresponding Gadd45α, Gadd45β and Gadd45γ acidic proteins of about 18 kDa. They are ubiquitously expressed and exert

the primary function of growth arrest and apoptosis induction in response to several genotoxic stresses thus contributing to cellular homeostasis.[1–6] They have been implicated in a variety of other cell functions, such as DNA replication and repair,[7] cell-cycle regulation,[8] and, depending on cell type and cell metabolic state, also in cell survival.[4,9–16] This last property is seemingly exhibited mostly by Gadd45β, which has been described as an NF-kB-inducible gene and as a prominent mediator of the NF-kB protective response to TNFα- and UV-induced apoptosis.[4,11,17] However, this aspect is still controversial and several reports indicate Gadd45β as an effective pro-apoptotic factor.[5,6,17] The mechanisms by which Gadd45β can promote cell survival have been investigated extensively and it has been found

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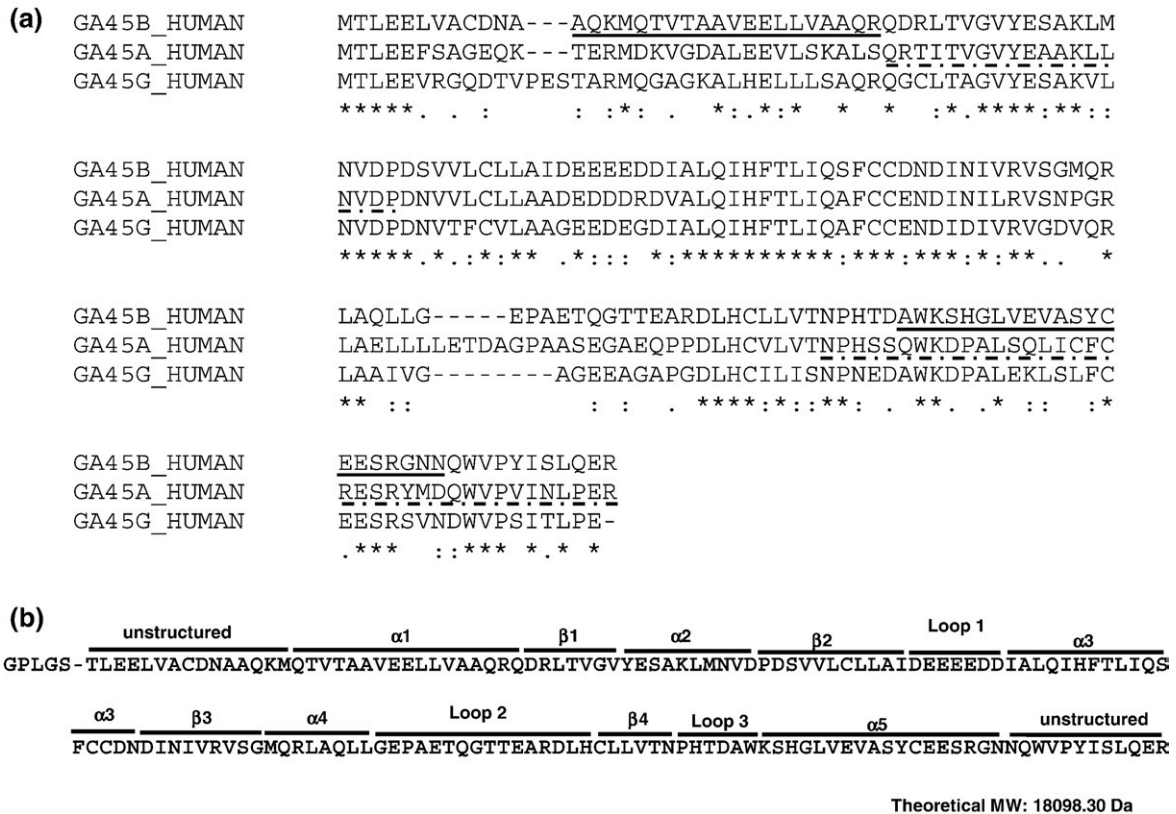menotti.ruvo@unina.it; marmonti@unina.it.

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Abbreviations used: GST, glutathione-*S*-transferase; TFE, trifluoroethanol; MD, molecular dynamics; GdnHCl, guanidinium hydrochloride; TFA, trifluoroacetic acid.

that in MEFs and other cells, upon NF-kB induction, it provides selective JNK inactivation by inhibition of the upstream MKK7.[1,10,18] In hematopoietic cells, instead, it blocks JNK activation by binding to MKK4,[4,19] and in B cells it is a critical mediator of the pro-survival activity of CD40 elicited in response to Fas stimulation.[9]

Since there is no reported enzymatic activity for the Gadd45 proteins, it is believed that they exert their functions by interacting with protein partners.
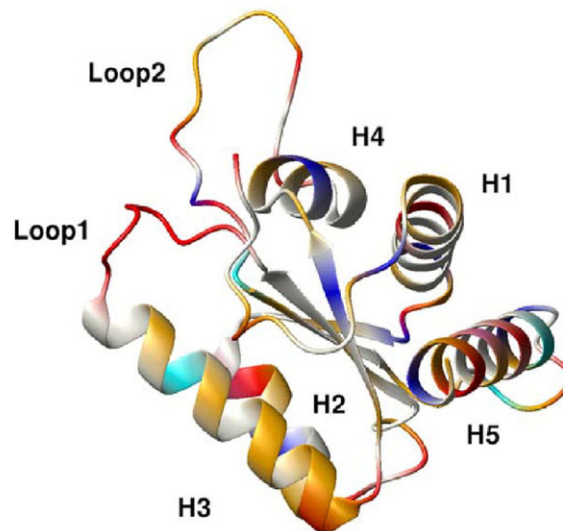
(a)

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GA45B_HUMAN    MTLEELVACDNA---AQKMQTVTAAVEELLVAAQRQDRLTVGVYESAKLM
GA45A_HUMAN    MTLEEFSAGEQK---TERMDKVGDALEEVLSKALSQRTITVGVYEAAKLL
GA45G_HUMAN    MTLEEVRGQDTVPESTARMQGAGKALHELLLSAQRQGCLTAGVYESAKVL
               *****. . :        : :*: .  *:.*:*   *   *   :*.****:**::

GA45B_HUMAN    NVDPDSVVLCLLAIDEEEEDDIALQIHFTLIQSFCCDNDINIVRVSGMQR
GA45A_HUMAN    NVDPDNVVLCLLAADEDDDRDVALQIHFTLIQAFCCENDINILRVSNPGR
GA45G_HUMAN    NVDPDNVTFCVLAAGEEDEGDIALQIHFTLIQAFCCENDIDIVRVGDVQR
               *****.*.:*:**  .*.:: *:**********:***:***:*.:**.. *

GA45B_HUMAN    LAQLLG-----EPAETQGTTEARDLHCLLVTNPHTDAWKSHGLVEVASYC
GA45A_HUMAN    LAELLLLETDAGPAASEGAEQPPDLHCVLVTNPHSSQWKDPALSQLICFC
GA45G_HUMAN    LAAIVG--------AGEEAGAPGDLHCILISNPNEDAWKDPALEKLSLFC
               ** ::           :  :  .  ****:*::**: . **. .* ::   :*

GA45B_HUMAN    EESRGNNQWVPYISLQER
GA45A_HUMAN    RESRYMDQWVPVINLPER
GA45G_HUMAN    EESRSVNDWVPSITLPE-
               .***   ::*** *.* *
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(b)

(c)

Fig. 1. (a) Alignment of Gadd45α, β and γ sequences. An identity of about 60% is observed. Most differences are present in the region corresponding to the second acidic loop, with a Gadd45γ pentapeptide stretch remaining unpaired with the other two sequences. To align the Gadd45α variant correctly, an eight-residue long stretch is required. Within the acidic loop 1, residue Asp67 of Gadd45β is mutated to Arg in the α variant and to Gly in Gadd45γ. Regions involved in self-association are underlined. (b) A representation of the protein secondary structure is schematised based on the model described in Ref. 1. (c) A ribbon representation of Gadd45β three-dimensional model, coloured by residue type according to the following scheme: hydrophobic residues, white; polar residues, yellow; acidic residues, red; basic residues, blue; histidine residues, cyan; tyrosine residues, in pink.

Indeed, other than MKK7 and MKK4, highly specific interactions with PCNA,[6,20–22] cdc2,[23] waf/p21,[24] cdk1/cyclinB1,[25] MEKK4[26] and CRIF1[27] are involved in Gadd45 regulation of the cell cycle and the response to external cell stimuli. A further interaction with the protein nucleophosmin has been described for Gadd45α,[28] and it has been shown that this protein can work as a vehicle for nuclear import. However, it is not known whether Gadd45β and Gadd45γ share a similar mechanism of nuclear translocation. Importantly, it has been reported that Gadd45 proteins are also able to homo- and heterodimerise or oligomerise, and regions involved in self-association of Gadd45α have been investigated using overlapping synthetic peptides spanning the entire protein sequence.[29]

The primary sequences of Gadd45 proteins share an overall 70% homology (about 60% identity, see Fig. 1a) and all contain six cysteines, five of which (from the second to the sixth) are located in highly conserved positions.

An unusually long stretch of glutamic and aspartic acid residues, only partially conserved within the Gadd45γ variant, can be found starting from position 60 of Gadd45α and Gadd45β. These residues have been described as having a key role in inhibition of both cdc2[23] and MKK7.[1,10] However, the 3D structure of this important class of proteins is not known, and so far a predicted model has been reported[1] for Gadd45β (Fig. 1b and c) and its complex with MKK7. These structures, supported by various experimental evidences, present a central four-stranded β-sheet surrounded by five α-helices and, as expected for a nuclear protein, all cysteine residues are predicted to be far from each other and thus in the reduced state.[1] The acidic stretch appears to be in a large loop interacting with several basic and polar residues within the kinase active site, and it is part of the minimum region of Gadd45β needed to bind and block MKK7 activity (A60-D86 fragment). Other regions involving the putative helix 3 (H3), a second acidic loop and part of helix 4 (H4) harbour other key residues contacting MKK7.

Following an approach of protein enzymatic fragmentation and HPLC fractionation, here we identify the regions of Gadd45β implicated in self-association and confirm those involved in binding with MKK7. We show also that Gadd45β is unable to form higher-order oligomers, as only dimers are detected using different methods. These studies extend our knowledge of Gadd45β properties and suggest that protein self-association can have a primary role in regulating its biological activity.

Results

Gadd45β purification

Recombinant construct pGEX6P-GADD45β allowed expression of the protein as a glutathione-*S*-transferase (GST)-fusion product containing a highly specific cleavage site for PreScission Protease upstream of the Gadd45β protein. The applied overexpression system was quite efficient, producing more than 6 mg of highly purified protein from 1 L of induced culture under the reported conditions.[1] The recombinant protein obtained after removal of GST had the sequence reported in Fig. 1b and was used only for oligomerisation studies. The protein was characterised by SDS-PAGE and LC-MS analysis determining
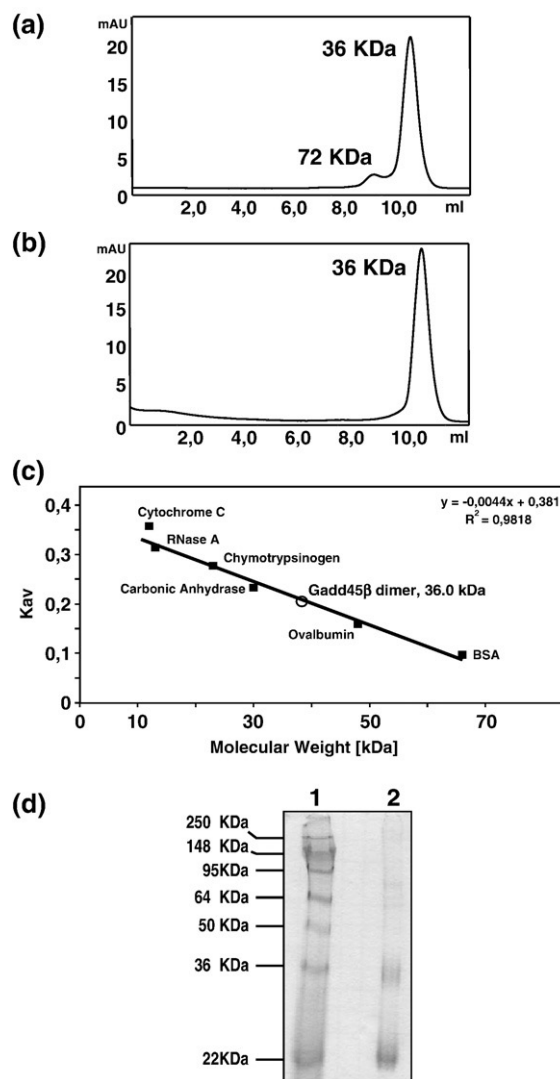

Fig. 2. Characterisation of the oligomeric state of Gadd45β. (a) Gel-filtration analysis of the recombinant protein on Superdex 75 10/30 under non reducing conditions. Two peaks at elution volumes compatible with dimeric and tetrameric forms are detected. (b) The same analysis carried out after inclusion of 1 mM DTT in the running buffer. The peak at lower elution volumes disappears, suggesting that it is due to covalent disulfide bridges. (c) The calibration curve used to determine M_r is reported. Cytochrome *c*, 12.4 kDa; RNase A, 14.7 kDa; chymotrypsinogen, 25.0 kDa; carbonic anhydrase, 29.0 kDa; ovalbumin, 44.0 kDa; BSA, 66.0 kDa. All measurements were done at least twice. (d) Non-denaturing polyacrylamide gel analysis of Gadd45β at a concentration of 1.8 mg/mL (100 μM).

the exact MW ($MW_{Exp/Theor}$, 18096.6±1.0 Da/ 18096.1 Da). His$_6$-Gadd45β used in ELISA assays was obtained similarly with high yields and purity. The value of 19196.5 amu as determined by LC-MS was consistent with the protein sequence (MW_{Theor} 19196.52 amu). The protein was derivatised efficiently with biotin as described in Experimental Procedures. Protein derivatisation was assessed by mass spectrometry showing that about 90% of the protein harboured one biotin molecule, while the remaining appeared underivatised (data not shown).

Investigation of Gadd45β oligomerisation by gel filtration and CD studies

Since previous studies reported on the capacity of Gadd45 proteins to oligomerise,[29] we investigated this point by carrying out a gel-filtration analysis in the presence and/or in the absence of DTT and by native electrophoresis experiments. Gel-filtration analysis of protein aliquots at a concentration of 5.0 μM showed that, in the absence of DTT, two

peaks were eluted at column volumes corresponding to a dimeric and tetrameric protein, i.e., 36 kDa and 72 kDa, respectively (Fig. 2a and c). Conversely, under reducing conditions, the tetramer peak disappeared (Fig. 2b), indicating that it was held together by disulfide bridges, whereas the dimer was associated through strong non-covalent interactions. The presence of protein dimers or oligomers was investigated by native gel electrophoresis, observing in this case the dimer and the monomer (Fig. 2d). According to these data, Gadd45β seemingly exists in solution prevalently as a dimeric protein, in partial equilibrium – under certain conditions (the non-denaturing gel) – with the monomer. Indeed, no higher-order oligomer has been detected by either techniques.

The far-UV CD spectrum of Gadd45β (11×10^{-6} M) in aqueous solution showed negative bands at 209 nm and 222 nm, and a positive band at 195 nm indicative of a high content of α-helical conformations (Fig. 3a), as reported.[1] Importantly, the His$_6$-Gadd45β, used in different assays exhibited a very
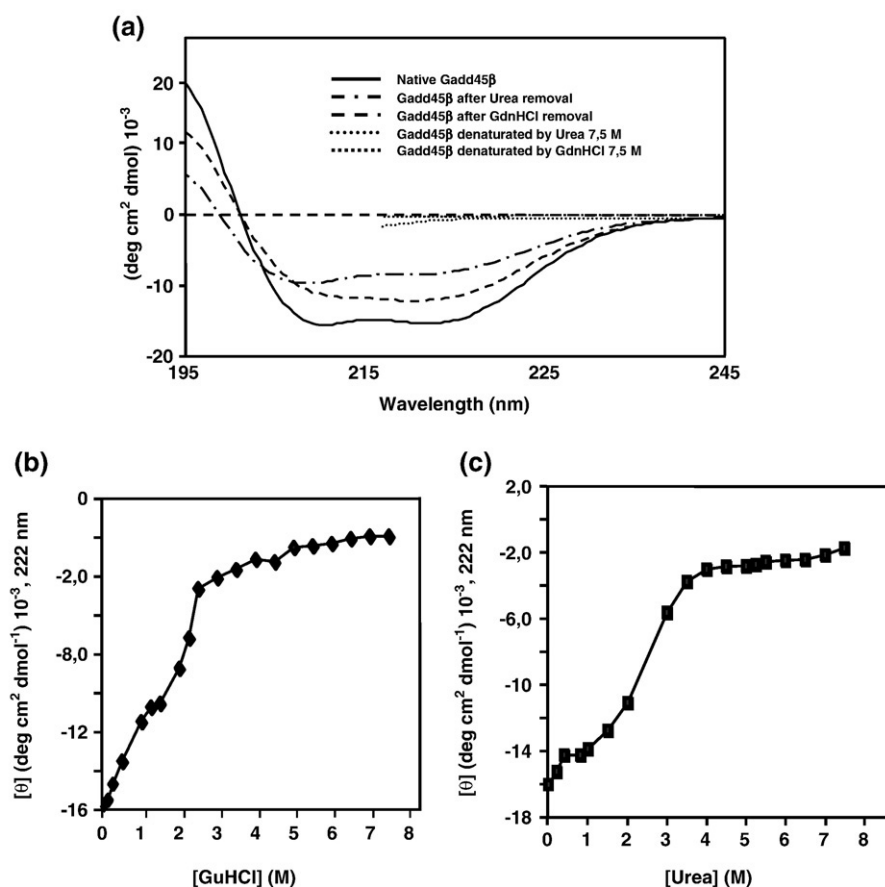

Fig. 3. Circular dichroism characterisation of the recombinant Gadd45β. (a) CD spectra of the recombinant protein before and after denaturation with guanidinium and urea. The protein appears to refold properly after the chemical treatment. Spectra obtained in the presence of 7.5 M urea and guanidinium (dotted lines) are also reported. Curves of chemical denaturation are representative of at least two independent experiments. CD curves have been obtained by averaging at least three consecutive acquisitions. CD curves of denatured Gadd45B are missing below 215 nm since CD signals are suppressed by the high concentrations of urea and guanidinium. (b) Chemical denaturation by GdnHCl. The protein unfolds in a cooperative way at concentrations of guanidinium higher than ~2.5 M (and see (a)). (c) Chemical denaturation by urea is reported. In this case also, the protein unfolds cooperatively. Complete denaturation occurs at concentrations of urea higher than ~4.0 M.

similar CD spectrum (not shown). The occurrence of oligomers was also investigated by this technique by comparative analysis of protein solutions at different concentrations. No difference was detectable between CD curves recorded for protein solutions at concentrations ranging between 5.5×10^{-5} M and 5.5×10^{-6} M (not shown) suggesting that dilution, at least in this range of concentrations, did not affect the protein global folding or the quaternary structure. Therefore, no information was obtained about the monomer–dimer status of the protein by this experiment. A CD analysis of the dependence on denaturant concentration was performed to further assess the protein structure stability or the capacity to eventually dissociate into monomers. Chemical denaturation data (Fig. 3b and c) showed that Gadd45β unfolds cooperatively at about 2.0 M GdnHCl and 3.0 M urea. This may indicate that electrostatic interactions, which are weakened more efficiently by GdnHCl than urea,[30] have an important role in the stabilisation of Gadd45β.

The protein secondary structure was recovered following removal of the denaturant (Fig. 3a), suggesting that denaturation, as well as the eventual dimer dissociation (assuming it occurs at high concentrations of denaturant), is a reversible event. Thermal denaturation experiments were carried out to confirm these observations; a single transition was observed at about 46.5 °C but, most importantly, after slow cooling to 20 °C the protein did not recover the original structure because of disulfide cross-linking (detected by SDS-PAGE under reducing and non-reducing conditions; not shown). The melting temperature is quite low In comparison to those of other globular proteins,[31] indicating that Gadd45β is not a thermodynamically stable protein, in agreement with the predicted structure that exhibits large and flexible loops.[1]

Expression and purification of MKK7

MKK7 was produced in bacteria as a soluble GST-MKK7 fusion product. About 7 mg/L of the fusion protein were obtained in typical fermentations. The protein was initially purified by standard GSTrap and on-column PreScission protease cleavage. The material recovered was further purified by gel-filtration chromatography, obtaining a product more than 95% pure. The protein was characterised by SDS-PAGE and liquid chromatography-mass spectrometry (LC-MS) analysis and the experimental molecular mass was consistent with that expected (not shown). Protein identity was further confirmed by trypsin digestion and LC-MS/MS analysis of the resulting fragments (not shown). The folding of MKK7 was assessed by CD analysis observing, as expected,[1] a spectrum with a mixed α-β content (Fig. 4a). In Fig. 4b the determination of the oligomeric state of the protein by gel-filtration analysis is reported: the protein appears as a dimer, exhibiting an apparent mass of ~90 kDa. Importantly, the fusion protein GST-MKK7, used in several
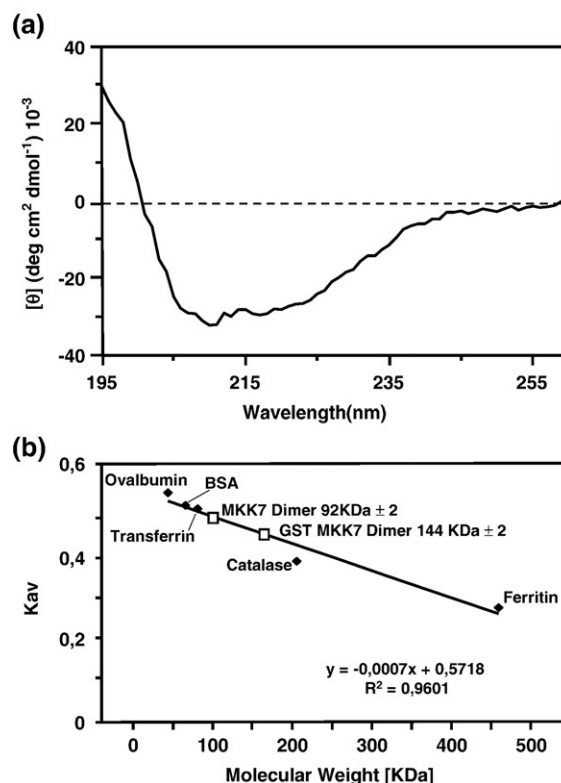

Fig. 4. Recombinant human MKK7 is folded properly and appears to be dimeric. (a) CD spectrum in the near UV of the protein showing that it adopts a prevailingly α-helical conformation, as expected on the basis of the model proposed in Ref. 7. (b) The calibration curve used to determine the apparent mass of the recombinant protein by gel-filtration analysis. Ovalbumin, 44 kDa; BSA, 66 kDa; transferrin, 81 kDa; catalase, 206 kDa; ferritin, 460 kDa. The protein appears to be dimeric, as reported.[35,36] Consistently, GST-MKK7, used in several experiments in this work, is also dimeric. All determinations were carried out at least twice under the same conditions.

assays in this study, eluted from the gel-filtration column as a dimer.

Identification of regions of Gadd45β involved in auto-association

In an attempt to determine the protein self-association surface, we used Gadd45β fragments as competitors of the protein self-association in an ELISA-like assay (see below). For this purpose, protein digestion by several enzymes was considered, comparing enzyme efficacy and specificity other than the length and complexity of the obtainable peptides. Following this analysis, an extensive digestion of the protein with trypsin resulted in complete digestion and formation of fragments of suitable length to ensure a complete coverage of the protein primary structure (Table 1). Upon reverse phase (RP)-HPLC fractionation (13 fractions), Gadd45β peptides, identified by molecular mass and MS/MS sequencing, were essentially

Table 1. Tryptic Gadd45β peptidic fragments as identified by LC-MS/MS analysis

Fraction	Gadd45β tryptic peptides	Relative distribution of fragments within fractions (%)	Relative composition of the fraction (%)	Predicted secondary structure[a]
6	G147-R160	98	100	Unstructured
7	L36-K45	98	50	Part of β1, part of α2
	L98-R115	99	50	Part of α4; most of loop 2
8	S132-R146	87	85	α5
	D116-K131	99	15	β4
9	-13-15	100	75	Tag
	S132-R146	5	25	α5
10	M16-R32	92	95	α1
12	L46-R91	10	100	Part of α2, β2, loop1, α3, part β3
13	L46-R91	87	100	Part of α2, β2, loop1, α3, part β3

Fragments less than 5% were not considered. Relative distributions of fragments in column 3 were calculated by comparing area integration of extracted ion peaks from a given fragment taken from the different fractions. The relative composition within each fraction was derived by comparing area integrations of extracted ion peaks of all the fraction components.
  [a] As reported in Ref. 1.

distributed along seven main fractions, since fractions 1–5 contained no material and fraction 11 contained only very small amounts (less than 4%) of fragment L46-R91. All identified trypsin fragments are reported in Table 1 along with a correspondence with the predicted Gadd45β secondary structure.[1] Fractions 6, 7, 8 ,9, 10, 12 and 13 contained relevant amounts of the protein fragments. Notably, fraction 6 contained only the C-terminal G147-R160 peptide (unstructured); fraction 7 contained, in a 1:1 ratio, the fragments L36-K45 (part of β1 and part of α2) and L98-R115 (part of α4; most of loop 2); fraction 8 contained (in an 85:15 ratio), a major fraction of fragments S132-R146 (87%, the central region of α5, hereinafter short H5) and the peptide D116-K131 (β4); fraction 9 contained, in a 75:25 ratio, the N-terminal polyhistidine tag and a minor fraction of the peptide S132-R146 (α5); fraction 10 contained only the fragment M16-R32 (92%, the remaining being distributed along the contiguous fractions), corresponding to most of helix 1; fraction 12 and 13 contained the fragment L46-R91, corresponding to part of α2, β2, loop1, α3, and part of β3. The ELISA assay to monitor Gadd45β self-association was carried out by coating the His$_6$-protein on the surface of microtiter wells. Protein association was followed by adding increasing amounts of biotinylated His$_6$-Gadd45β and detecting the bound protein by using horseradish peroxidase-conjugated streptavidin. As shown in Fig. 5a, the protein associated efficiently in a dose-dependent way, reaching signal saturation at a nearly 1:1 (mol/mol) ratio, as expected for a dimeric complex. The concentration of His$_6$-Gadd45β resulting in 50% of maximum binding was ~100 nM and it was taken as an estimation of the dissociation constant of self-association. To identify the Gadd45β regions involved in protein dimerisation, binding competition assays were then carried out using peptide fragments derived from Gadd45β digestion. The assays were performed using a constant 1:0.5 (mol/mol) ratio of coated/soluble His$_6$-Gadd45β and a 2:1 (mol/mol) ratio of peptide competitor/soluble protein. The results are summarised as a

plot of representative data in Fig. 5b. Gadd45β trypsin fractions 1–5 and fraction 11 were not used. Fractions 6, 7, 12 and 13, which contained consistent amounts of protein fragments (see Table 1), were essentially ineffective. In contrast, fractions 8–10 interfered with the association. In particular, fraction 8 exhibited a nearly 30% binding reduction and fraction 10, which virtually contained only fragment M16-R32 (see Table 1) including most residues from helix 1, decreased protein association to about 50%. Fraction 9, containing most residues of the tag and a minor part of fragment S132-R146, disrupted Gadd45β self-association by 25%. Interestingly, the synthetic peptides Gadd45β(A60-D86) and MKK7(G132-N156), identified as forming the binding interface between Gadd45β and MKK7,[1,10] appeared totally ineffective in this assay (also discussed later). To refine the ELISA data, fragments unable to disrupt Gadd45β-Gadd45β interaction were not considered; furthermore, on examination of the predicted Gadd45β 3D model, it was decided not to further investigate fragment D116-K131, present in fraction 8 at 15%, since it corresponds to the fourth β-strand, which should be buried within the protein core and therefore virtually inaccessible to external interactions (Fig. 1c). Hence, we decided to investigate regions M16-R32 and S132-R146 corresponding to the regions within the putative H1 and H5, respectively. Examining the protein model (Fig. 1c), the peptides were opportunely designed by adding N- and C-terminal amino acids in order to complete the helices, hence the corresponding synthetic peptides, A12-R35 (hereinafter extended helix 1, eH1) and A129-N148 (hereinafter extended helix 5, eH5) were prepared by chemical synthesis and purified to homogeneity by RP-HPLC. The peptide R91-E104, corresponding to the extended predicted eH4, was similarly prepared and used as a negative control. These peptides were then tested in the Gadd45β-Gadd45β competition assay, using the protein–protein and protein–competitors ratios described above. As shown in Fig. 5b, eH1 and eH5 blocked the Gadd45β self-association by 82% and 78%,
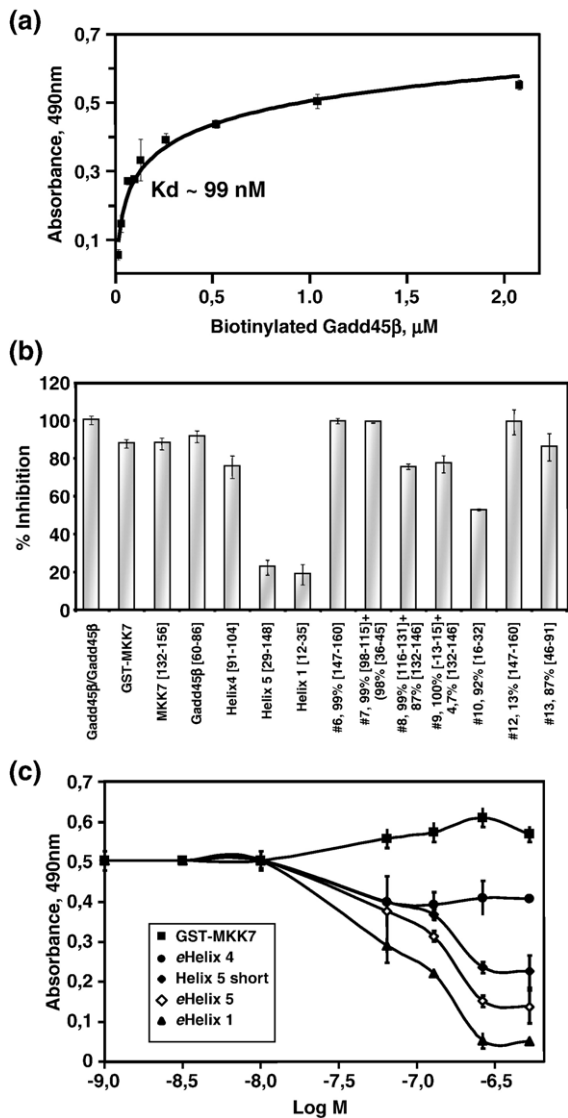
(a)

(b)

(c)

Fig. 5. Gadd45β is able to self-associate, the interaction being disrupted by Gadd45β trypsin fragments. (a) Dose-dependent binding of biotinylated Gadd45β to the plate-adsorbed protein: Gadd45β self associates with an estimated K_D of ~100 nM, assumed as the concentration of Gadd45β resulting in 50% maximum binding.[29] Notably, the signal reaches saturation at a 1:1 protein ratio, suggesting the formation of dimers only. (b) Competition assay of the Gadd45β self-association by the trypsin-generated protein fragments and by synthetic peptides designed to reproduce helices as predicted in the protein model.[1] (c) Dose-dependent inhibition of Gadd45β self-association by full-length GST-MKK7, eH4 (residues 91–104), eH5 (residues 129–148), eH1 (residues 12–35) and the short helix 5 (residues 132–146). While the kinase and eH4 are not able to block the Gadd45β self-association, peptides corresponding to eH1 and eH5, reduce the association markedly. Short helix 5, derived by trypsin cleavage, is less effective than the entire eH5. Data are representative of at least three independent experiments.

respectively, supporting the hypothesis that these regions are strongly involved in the interaction. In contrast, peptide R91-E104 (eH4), lying in the close proximity of H1 in the model (Fig. 1c), as well as peptides Gadd45β(A60-D86) and MKK7(G132-N156), and the full-length kinase did not interfere with the protein homodimerisation. A dose-dependent competition assay carried out with eH1, eH5, the short H5, eH4 and full-length MKK7, further confirmed the properties of H1 and H5 and the inefficacy of H4 to abrogate dimerisation (See Fig. 5c). As expected, the full-length kinase (fused to GST) was unable to block the Gadd45β self-association even at higher concentrations. Remarkably, the IC_{50} for these competitors was 100 nM for H1, 180 nM for the eH5 and only 600 nM for the short H5, indicating that the full H5 was more than three times more efficient in disrupting the protein self-association. These results might account for the relatively weak inhibition exhibited by fractions 8 (30%) and 9 (25%), which contained only short H5. To further investigate this point, the synthetic peptides reproducing eH1, eH5, short H5 and eH4 were analyzed by CD. The analyses were carried out both in phosphate buffer and in the presence of 20% (v/v) trifluoroethanol (TFE) to measure the relative propensity of peptides to adopt α-helical conformations. As reported in Fig. 6a–d, whereas eH1, eH4 and eH5 adopted a partially folded structure in buffer and readily folded into α-helices upon the addition of TFE, the short H5 remained in the unfolded state even after addition of the structuring solvent, suggesting an intrinsic incapacity to form organised structures. Thus, increasing the length at the N and C termini of the putative H5 provided a fivefold increase in potency that can be ascribed, in part, to the lack of any structure of shorter variant.

To further extend our knowledge of the interaction between the two monomers, we developed an assay to monitor the binding between MKK7 and Gadd45β. This interaction has been thoroughly investigated in previous work by pull-down assays using several point-mutated variants of both proteins.[1,10] As shown in Fig. 7a, the association between the two purified protein was very strong: a rough estimation of the K_D, deduced by the Gadd45β concentration at half of the saturation signal, was about 13 nM. Noticeably, signal saturation was reached at a molar ratio of ~1:1, suggesting that one MKK7 molecule should be sufficient to bind to one molecule of Gadd45β. The competition experiment carried out using 42 nM MKK7, 21 nM Gadd45β and a twofold excess of competitors over the soluble protein, is reported in Fig. 7b. As shown, peptides corresponding to the putative eH1, eH4 and eH5, MKK7(G132-N156) and Gadd45β(A60-D86) were used. Consistent with the notion that MKK7 proximity does not interfere with the Gadd45β dimerisation, eH1 and eH5 do not affect the binding, whereas peptides Gadd45β(A60-D86) and MKK7(G132-N156), believed to form part of the interface between the two proteins, completely

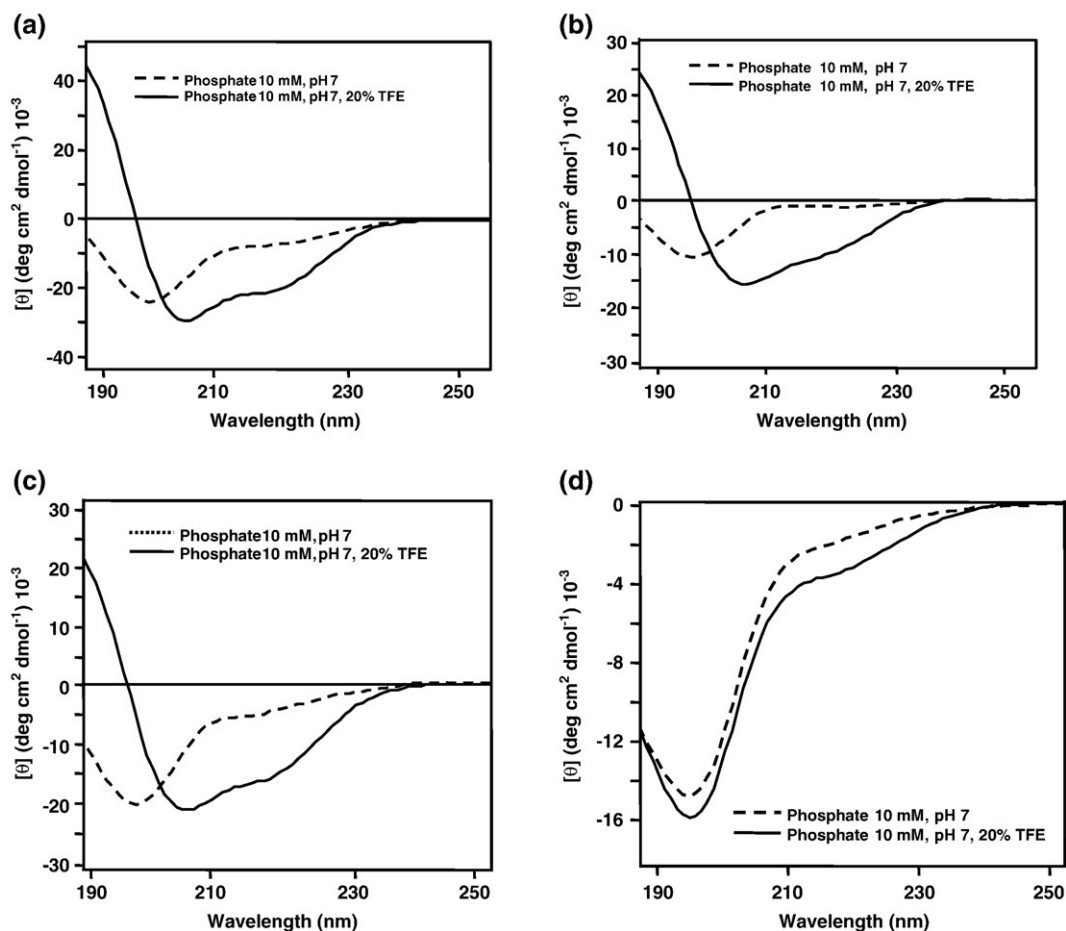**Fig. 6.** CD spectra of the synthetic peptides in neutral buffers and in the presence of 20% TFE. a–d, CD spectra of the synthetic eH1 (a), eH4 (b), eH5 (c) and short helix 5 (d) in phosphate buffer and in the presence of 20% TFE. As shown, the complete predicted helices have a good propensity to adopt an α-helical conformation, whereas the short helix 5 persists in a random conformation even after addition of the structuring agent. This different property is likely contributing to the reduced peptide capacity to block the MKK7-Gadd45β interaction. CD curves were obtained by averaging at least three consecutive acquisitions.

abolish the interaction. The peptide R91-E104 corresponding to the putative eH4 and containing two key residues (M95/Q96) involved in kinase recognition,[1] also proved unable to antagonise the Gadd45β-kinase binding, at the concentration used. Instead, soluble GST-MKK7, as expected, totally abolished the binding. Thus, these results corroborate the view that the Gadd45β-MKK7 interaction is essentially mediated by residues comprised within loop 1 and H3 (region A60–D86),[1] while other regions, such as H4, contribute only partially or may have only a structural role.

Modelling of the Gadd45β homodimer

To have a more detailed depiction of interactions occurring at the interface between the two monomers, we performed manual docking of the two units. Examining the Gadd45β model, H1 and H5 helices form a continuous surface and constitute a putative half of a four-helix bundle motif. On this basis, a number of Gadd45β homodimer models were constructed. Several relative orientations, both parallel and antiparallel, of the two monomers were tested in order to optimise both steric and electrostatic complementariness. An accurate analysis was then carried out to assess the effective stability and consistence of the resulting complexes, therefore each starting model underwent energy minimisation and then 200 ps of molecular dynamics (MD) simulations in solution. The best model in terms of specific side chain interactions is reported in Fig. 8a and b. The model suggests that the dimerisation region can form a four-helix bundle in which H1 and H5 of one monomer interact in an antiparallel fashion with the corresponding helices of the other monomer. This representation shows also how the proposed complex is stabilised by a network of intermolecular polar interactions involving Gln13, Thr14 and Glu21 on H1 and Glu140, Tyr137, Glu133 and His 129 on H5. The presence of Glu residues unequivocally determines the antiparallel orientation of the subunits, because in the parallel orientation these residues face each other, thus
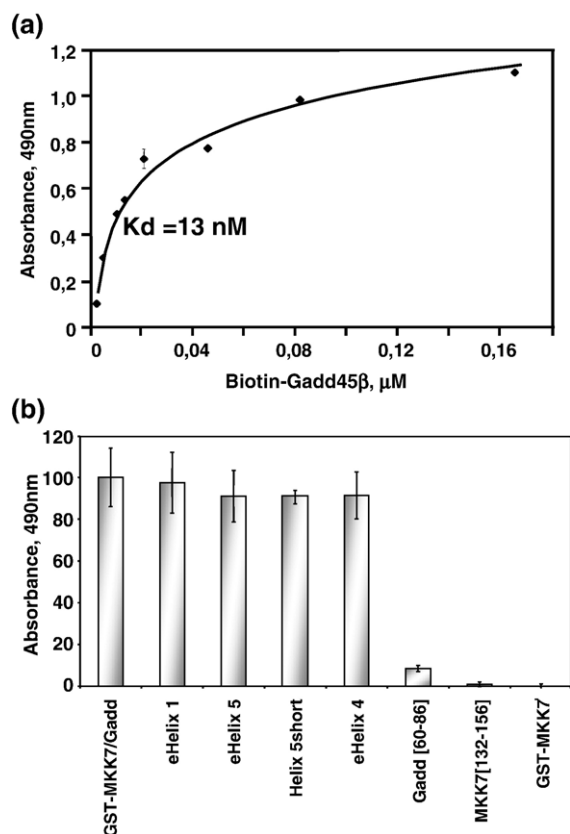
(a)

(b)

Fig. 7. Gadd45β and MKK7 interact strongly and their association is not influenced by helix 1 or helix 5. (a) Binding curve of the association between GST-MKK7 and Gadd45β. The estimated K_D is 13 nM. (b) Binding competition of the MKK7–Gadd45β association by Gadd45β and MKK peptides. eH1, eH5, short helix 5 and eH4 cannot disrupt the binding, whereas Gadd45β [A60-D86] and MKK7 [G132-N156], corresponding to the interface between the two proteins, abolish the interaction completely. Also, the GST-fused full-length kinase abolishes the interaction, as expected. Data are representative of at least three independent experiments.

destabilising the complex. Interestingly, a four-helix bundle search in the Protein Data Bank reveals the occurrence of a homodimer stabilised by intermolecular polar interactions in which Glu residues interact with Tyr and His (PDB entry 1U7M), similar to what was observed in our Gadd45β dimer model.

Discussion

Protein dimerisation and oligomerisation are often associated with protein function. Indeed, auto-association can be seen as a way to mask or unmask functional sites or, for example, to regulate protein degradation by making inaccessible regions otherwise marked for degradation.[32,33] Protein self-association is therefore believed to be a regulatory mechanism under both physiological and pathological conditions.

This mechanism has been speculated for Gadd45 proteins, a family of intracellular and intranuclear acidic proteins, as, reportedly, they homo- and heterodimerise and oligomerise under a variety of conditions;[29] the oligomerisation properties of Gadd45α have been investigated and it was reported that the protein can form dimers and higher-order oligomers also with Gadd45β and Gadd45γ.[29] Given the high number of molecular interactions they establish, a deeper knowledge of regions involved in self-association is of pre-eminent importance, as the occurrence of homo-oligomerisation can impair Gadd45 functions as well as strongly influencing hetero-interactions.

To extend our knowledge of the properties of Gadd45 proteins, we have investigated whether Gadd45β can dimerise–oligomerise and whether this ability can influence its interaction with MKK7, an important component of the MAP kinase cascade whose function in cellular apoptosis is regulated by Gadd45β itself.[10] As a first step, oligomerisation was investigated by gel-filtration chromatography, CD and native gel analyses. Gel-filtration experiments showed unequivocally that Gadd45β exists in solution as a non-covalent dimer at a concentration of about 5 μM, a 50-fold higher concentration than the estimated self-association K_D (~100 nM). Indeed, under reducing conditions, only a single sharp peak at the elution volume of the dimer (36 kDa) was detected. Consistently, by CD analysis, no structural change was observed for concentrations of protein between 5.5×10^{-6} M and 5.5×10^{-5}; i.e., 55-fold and 550-fold excess over the estimated K_D, respectively. The ELISA-like assay used to determine the K_D, showed that the protein self-binding is saturated for protein to protein molar ratios higher than 1:1, suggesting, likewise, the occurrence of dimers only. In agreement with all these findings, the analysis on native gels revealed the presence of the dimeric protein and some monomer, whereas no higher-order oligomer was detected. Therefore, in contrast to previous data on Gadd45α, Gadd45β seems able to form only dimers, which, under some conditions appear to be in equilibrium with monomers. Importantly, the self-association constant, estimated to be about 100 nM, is much lower than that estimated for Gadd45α (about 2.5 μM)[29] and close to the cellular concentration estimated for proteins of this family (~100 nM).[29] This discrepancy, partly due to the differences in primary structure, can also be explained by the large differences of protein concentrations used in the two studies. Indeed, as the self-association is concentration-dependent, we can reasonably assume that higher-order oligomers occur only at very high concentrations of protein, a condition that is not actually reflected within the cytoplasm and nucleus. Furthermore, the high level of similarity between the values of K_D and cell concentration suggest that an equilibrium between monomers and dimers can occur in the cytoplasm, where up- and down-regulation of the protein could be a way to finely modulate self-association as well as other external interactions.
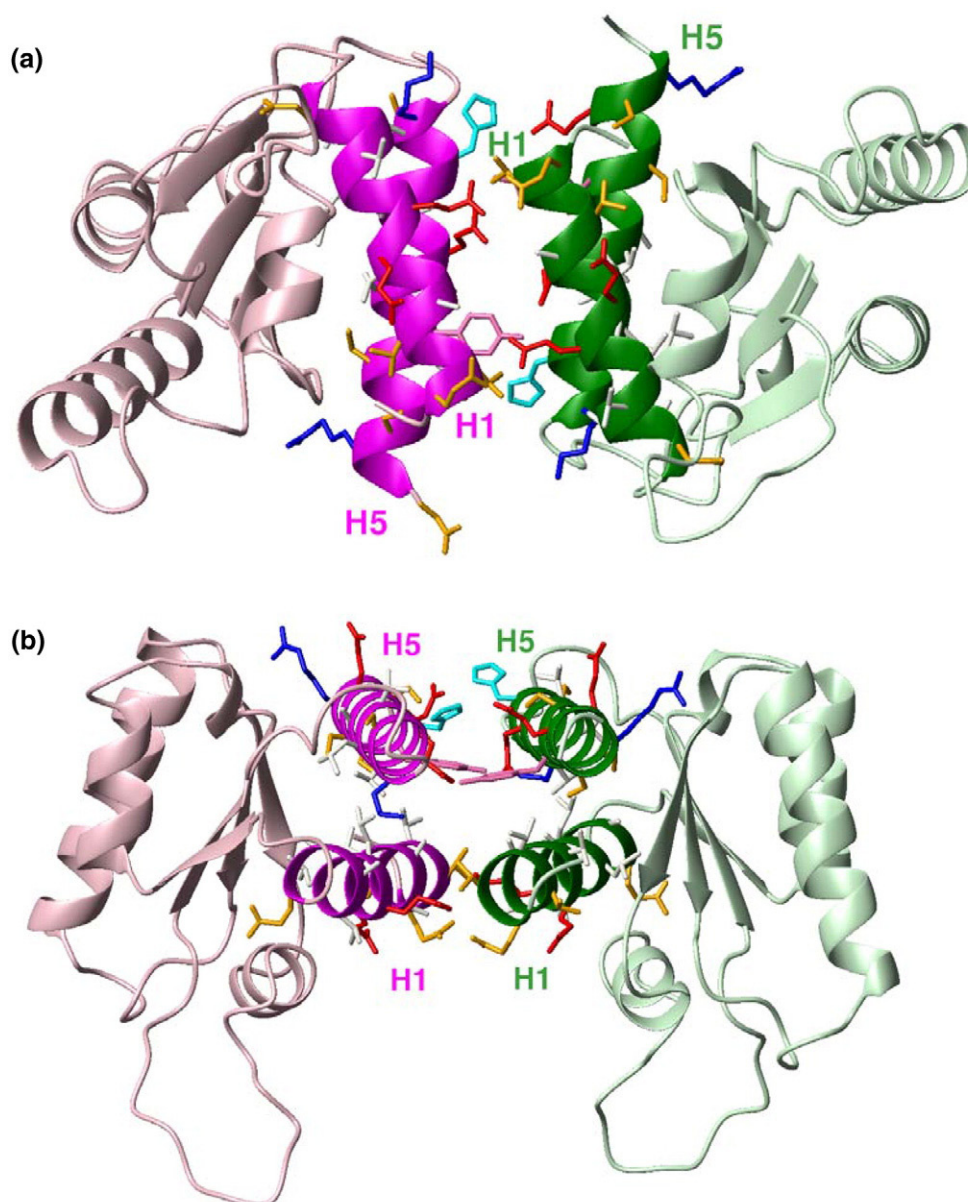
Fig. 8. Two orthogonal views of Gadd45β homodimer three-dimensional model in ribbon representation, rotated around the horizontal axis of the figure ((a) and (b)). One monomer is coloured pink, the other is coloured pale green. Helices H1 and H5 of each monomer are coloured in magenta and dark green, respectively. Residues lying on these helices are shown in stick representation and coloured according to the following scheme: hydrophobic residues, white; acidic residues, red; basic residues, blue, histidine, cyan; and tyrosine, magenta.

Following an approach of self-interaction competition with protein fragments derived from trypsin digestion, we identified two distinct Gadd45β sites involved in self-association that correspond to the predicted H1 and H5 of the protein.[1] Remarkably, these findings partially agree with the previous report on Gadd45α, for which an N-terminal and a C-terminal region have been described as being involved in self-association. However, whilst the C-terminal site (129–165) is virtually overlapping the predicted H5 of Gadd45β, the N-terminal site, identified as residues 33–61,[29] does not match H1 (see the sequence alignments in Fig. 1). Rather, it

overlaps with the Gadd45β sequence predicted as the β1-α2-β2 region (Fig. 1)[1] that is somewhat downstream in the sequence. In the cited study, a large fragment of Gadd45α (residues 20–33) exactly matching the Gadd45β H1, has not been considered; therefore a further comparison between the two different variants is not possible. However, given the high level of sequence homology between the two proteins, especially in this part of the sequences, we cannot rule out an involvement of the missing fragment in Gadd45α auto-association. Noticeably, by looking at the Gadd45β model (Fig. 1 and 8), H1 and H5 are disposed contiguously and arranged

anti-parallel to each other; therefore, they seem to form a large hydrophilic surface that includes the adjacent H4. This observation supports the view that the protein is unable to form higher-order complexes, as it rules out the presence of a second independent site required to allow the oligomerisation to propagate. Moreover, it suggests that auto-association is likely to occur through this unique surface (not involving H4) that, when engaged by a second monomer, could lead to the formation of a compact dimeric structure devoid of further self-interaction sites. The formation of a compact dimer is supported by studies of chemical denaturation with both guanidinium hydrochloride (GdnHCl) and urea, whereby it is seen that the protein, in the presence of up to 1 M denaturants persists in the dimeric form (not shown), whereas it unfolds after reaching higher concentrations.

H4, being parallel with H1, virtually extends the interaction surface towards the protein loop 2 (see Fig. 1), but does not contribute to protein self-association. On the contrary, it is reportedly involved in binding with MKK7.[1] In agreement with previous reports,[1] and in contrast with others,[4,34] Gadd45β and MKK7 interact strongly *in vitro*, exhibiting a K_D of about 13 nM. This value is about eightfold lower than that estimated for Gadd45β self-association, suggesting that the two proteins are able to interact in the presence of monomeric Gadd45β. Consistently, the two proteins interact strongly in the presence of both eH1 and eH5 (see Fig. 7b) that efficiently abrogate the Gadd45β dimerisation. By characterising the recombinant MKK7, we found that, in agreement with other reports,[35,36] the folded kinase is dimeric (see Fig. 4b), suggesting that the interaction between Gadd45β and MKK7 takes place in the context of a large complex comprising at least two Gadd45β and two kinase units (MKK7/Gadd45β:Gadd45β/MKK7). However, the occurrence of such a complex opens the possibility that, under regimens of protein up-regulation, larger aggregates can originate, and indeed, preliminary attempts to detect this complex by gel-filtration analysis have so far invariably shown the presence of high M_r non-covalent complexes (L.T., unpublished results).

Despite their small size, Gadd45 proteins appear to have a modular structure; these and previous findings show that distinct protein regions are involved in interactions with different partners. While the N and C termini are involved in self-association, it similarly appears that a central region, spanning residues A60–A114 and comprising the large acidic patches, is committed to kinase binding and regulation,[1,10,23] and to the interaction with core histones (region 72–124).[37] The same sub-domains, however, have concomitantly a role in the recognition of other partners, such as PCNA,[6] which binds the terminal regions 27–50 and 127–150 of Gadd45α, and nucleophosmin,[28] which interacts with region 61–100 thereby regulating nuclear entry.[28] Therefore, while dissociation of Gadd45 proteins would be required for interaction

with PCNA, a key player of the DNA repair mechanism regulated primarily by Gadd45 proteins, it seems dispensable for binding to kinases, core histones and nucleophosmin. Importantly, all these proteins have a prevailing localisation within cell nuclei, where protein concentration can be considerably higher than in the cytosol and thus self-association needs to be a less disruptive event. Assuming that Gadd45α and Gadd45β have distinct capacities for self-association, this difference would also reflect different properties that could be even more evident at higher concentrations of protein. In this instance, the control of protein over-expression and/or the capacity of more efficient translocation to sub-cellular compartments could be a further critical point that contributes to the different biological properties of the two protein variants.

Experimental Procedures

Materials

Enzymes, TPCK-treated trypsin and other chemicals for buffer preparation, as well as urea and GdnHCl, were purchased from Sigma-Aldrich. pGEX-6P-1 expression vector, PreScission protease, ÅKTA FPLC and columns for affinity, size-exclusion and ion-exchange chromatography were from GE Healthcare, Uppsala, Sweden. pETM expression vectors were from EMBL, Heidelberg. *Pfu* DNA polymerase was purchased from Stratagene. Restriction enzymes BamHI and XhoI and T_4 DNA Ligase were from New England Biolabs, Germany. Perfect Protein Markers were from Novagen, whilst SeeBlue Prestained markers were from Invitrogen. Vivaspin 5 kDa cut-off PES vertical membrane concentrators were from Sartorius, Milan. Non-fat dry milk (NFDM) was from BioRad. Solvents for RP-HPLC analysis were all from Romil, Dublin, Ireland. DNA coding for human Gadd45β and human MKK7 were as described.[1,10] For peptide synthesis, protected N^α-Fmoc-amino acid derivatives and coupling reagents were purchased from Inbios (Pozzuoli, Italy), Sequencing-grade trypsin, DIEA, rink amide MBHA resin and other chemicals were from Sigma-Aldrich (Milano, Italy). Other reagents and chemical suppliers are indicated below.

Solid-phase peptide syntheses were performed on a fully automated peptide synthesiser AAPTECH 348 Ω Advanced Chemtech (Louisville, KY). Preparative RP-HPLC was carried out on a Shimadzu LC-8A, equipped with a SPD-M10 AV detector on a Phenomenex Luna-COMBI HTS C18 column (5 cm×2.12 cm; ID, 10 μm). LC-MS analysis was carried out on an LCQ DECA XP ion trap mass spectrometer (ThermoElectron, Milan, Italy) equipped with an OPTON ESI source, operating at 4.2 kV needle voltage and 320 °C, and with a complete Surveyor HPLC system, consisting of an MS pump, an autosampler and a photodiode array. Narrow-bore 50 mm×2 mm C18 BioBasic LC-MS columns from ThermoElectron were used for these analysis. All ELISA assays to screen the Gadd45β trypsin fractions were carried out using a system consisting of a liquid handler, an automatic arm, a washer and an automated 96-well and 384-well plate reader (Hamilton Robotics, Milano, Italy).

Expression and purification of GADD45β in *Escherichia coli*

Human Gadd45β, hereinafter Gadd45β, was expressed both as pGEX6P-GADD45β as reported previously,[1] and with an N-terminal His$_6$ tag using the vector pET28aGADD45β;[10] in the latter case, recombinant protein expression was optimised in BL21(DE3) bacterial strain induced in the presence of 0.1 mM isopropyl-β-ᴅ-thiogalactopyranoside (IPTG) for 16 h at 22 °C. A 200 mL pellet was re-suspended in 20 mL of cold lysis buffer (25 mM Tris, 500 mM NaCl, 10 mM Imidazole, 1 mM DTT, 0.05% (v/v) Triton X-100, pH 7.5) supplemented with protease inhibitor mixture A (1 mM PMSF, 1.0 μg/mL of aprotinin, 1.0 μg/mL of leupeptin, 1.0 μg/mL of pepstatin, and 1.0 mg/mL of lysozyme) and incubated at room temperature for 30 min. Cells were disrupted by sonication on ice with 10 s on/10 s off cycles for a total of 10 min on. After centrifugation at 15,000 rpm for 30 min at 4 °C, the supernatant was purified on an ÅKTA FPLC chromatography system using a 1 mL HisTrap HP. The column was washed with lysis buffer without Triton and bound protein was eluted using a gradient of 10 mM–500 mM imidazole. Protein elution was monitored by measuring absorbance at 280 nm and the resulting fractions were analyzed by SDS-15% PAGE. The eluted fractions were dialyzed against buffer A (50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.0). MonoQ step, gel-filtration, as well as LC-MS analysis were carried out as described.[1]

Analysis of the protein by gel-filtration was compared with a calibration obtained with marker proteins run on the column under the same conditions. Comparison of chromatograms in the presence and in absence of 1 mM DTT in the running buffer was carried out to show the presence of covalent oligomers. Gel-filtration analyses were carried out on a Superdex 75 10/300 GL column.

Cloning, expression and purification of human MKK7 (1- 400), in *E. coli*

Human MKK7 (1-400), hereinafter MKK7, was expressed as a recombinant protein with an N-terminal GST-tag using the vector pGEx-6P1, allowing the expression of the protein as a GST-fusion product containing a highly specific cleavage site for PreScission Protease upstream of MKK7.[10] The MKK7 cDNA was amplified by PCR using pGEX-2T-MKK7/JNKK2 as template. Recombinant protein expression was optimised in the bacterial strain BL21(DE3)*TrxB,* and induced in the presence of 0.1 mM IPTG for 16 h at 22 °C. The cells from a 200 mL culture were resuspended in 20 mL of cold buffer A, protease inhibitor mixture A was added and the mixture was incubated for 30 min at room temperature. Cells were disrupted by sonication on ice and the total lysate was then centrifuged at 15,000 rpm for 30 min at 4 °C. The resulting supernatant was loaded onto a 1 mL GSTrap FF column equilibrated with 50 mM Tris (pH 8.0), 500 mM NaCl and washed extensively until the absorbance at 280 nm reached baseline. The protein was then eluted with 10 mM GSH dissolved in 50 mM Tris (pH 8.0). Purity was assessed by SDS-PAGE and LC-MS analysis. When required, GST-MKK7 was digested on-column with 160 U of PreScission protease per 1 mL of resin at 4 °C for 5 days. Gel-filtration analysis was carried out on digested MKK7 using a Superose 6 10/30 (GE Healthcare) column previously calibrated with: ovalbumin, 44 kDa; BSA, 66 kDa; transferrin, 81 kDa; catalase, 206 kDa; ferritin, 460 kDa, using 50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.0 as the running buffer.

Digestion with trypsin and peptide fractionation

An aliquot of Gadd45β (1.0 mg; 0.052 μmol) was dissolved in 2.0 mL of 50 mM Tris, 20 mM CaCl$_2$, pH 8.0. TPCK-treated trypsin (Sigma) was added at a final enzyme-substrate ratio of 1:100 and the reaction kept at 37 °C with gentle agitation for 16 h. A protein sample (0.5 μg) was then analyzed by LC-MS/MS to assess protein digestion using a BioBasic 30 mm × 2 mm ID C18 column. The column was equilibrated at 200 μL/min with 5% CH$_3$CN, 0.05% trifluoroacetic acid (TFA), then a gradient of CH$_3$CN from 5%–55% over 65 min was applied and monitored by both photodiode array and MS. The MS analysis was conducted by alternatively recording full mass spectra and data-dependent mass analysis to obtain sequence information from peptide fragmentation. The remaining protein sample was finally injected on a 250 mm × 4.6 mm ID C18 column equilibrated at 1.5 mL/min flow-rate with 5% CH$_3$CN, 0.1%TFA, applying a 5%–55% gradient of CH$_3$CN over 65 min to elute the peptides; 13 fractions of 5 min each were collected from time zero to time 65 min, and were analyzed (4 μL) by LC-MS/MS as reported. Lyophilised fractions were stored at −80 °C.

Circular dichroism (CD)

CD spectra were recorded on a Jasco J-810 spectropolarimeter (JASCO Corp, Milan, Italy) equipped with a Peltier temperature-control system according as described,[1] and native Gadd45β was diluted with water to obtain a final concentration of 5.5×10^{-5} M. A blank run was carried out before every experiment and subtracted from the protein CD spectra. Chemically induced denaturation was carried out on native Gadd45β performing 1 deg.C increments every 2 min from 20 °C to 80 °C, monitoring the CD signal at 222 nm. The temperature was returned to 20 °C to investigate the refolding capacity of the thermally denatured protein. CD spectra were again collected after each 2 deg.C change. To investigate the effects of concentration on protein oligomerisation, serially diluted solutions at concentrations ranging from 5.5×10^{-5} M to 5.5×10^{-6} M were analyzed using cuvettes with increasing path-lengths in order to compensate the signal loss due to dilution. Chemical denaturation experiments were carried out, evaluating the effect of urea and GdnHCl on Gadd45β denaturation as described.[30] Briefly, pH-controlled solutions with different concentrations of urea and GdnHCl, with protein at a constant concentration of 5.5×10^{-5} M were prepared and incubated for 16 h at 20 °C. Chemically induced denaturation was monitored by recording the CD value at 222 nm for each sample. The reversibility of the denaturation was controlled after removal of denaturants from the unfolded protein sample by dialysis overnight. The capacity of the recovered protein to recognise MKK7 was also evaluated by direct ELISA binding. Corresponding blanks were always recorded and subtracted. CD spectra were registered at 25 °C. CD analysis was also done on the synthetic peptides used in the ELISA assays. The concentration of peptides was kept at 1.0×10^{-5} M and a 0.1 cm path-length quartz cuvette was used. Spectra were acquired in a 10 mM sodium phosphate buffer at pH 7.0 and in the presence of increasing concentrations of TFE, up to 20% (v/v).

Non-denaturing gel electrophoresis

The Laemmli discontinuous electrophoresis system without SDS was used for non-denaturing separation.

The mobilities of proteins were determined on gels at 8%, 10%, 12% and 15% (w/v) bis-acrylamide, by loading 18 μg of protein from a 1.8 mg/mL solution (100 μM). Calibration markers were from Sigma-Aldrich. Calibration curves were obtained as described,[38] (and see the Sigma technical bulletin no. MKR-137).

Peptide synthesis and purification

Peptides corresponding to different regions of Gadd45β and MKK7 were designed on the basis of a predicted model,[1] and prepared by solid-phase peptide synthesis as C-terminally amidated and N-terminally acetylated derivatives following standard Fmoc chemistry protocols. A Rink-amide MBHA resin (substitution 1.1 mmol/g) and amino acid derivatives with standard protections were used in all syntheses. Cleavage from the solid support, performed by treatment with a TFA/triisopropylsilane (TIS)/water (90:5:5, by vol.) mixture for 90 min at room temperature, afforded the crude peptides that were precipitated in cold ether, dissolved in a water/acetonitrile (1:1, v/v) mixture and lyophilised. Products were purified by RP-HPLC using a C18 Jupiter column (50 mm × 22 mm) applying a linear gradient of 5%–70% TFA (0.1% in acetonitrile) (with 95%–30% TFA (0.1% in water)) over 30 minutes. Peptide purity and integrity were confirmed by LC-MS mass measurements using a Surveyor LC system coupled to an LCQ Deca XP mass spectrometer equipped with an OPTON ESI source. Characterisation was done under standard conditions of peptide analysis. Peptides were designed on the basis of the predicted Gadd45β secondary structure described in Ref. 1 and corresponded to eH1 (A12–R35, AAQKMQTV-TAAVEELLVAAQRQDR), eH4 (R91-E104, RVSGMQRLA-QLLGE), short H5 (S132-R146, SHGLVEVASYCEESR), and eH5 (A129-N148, AWKSHGLVEVASYCEESRGN). The peptide GPVWKMRFRKTGHVIAVKQMRRSGN, corresponding to fragment G132-N156 of MKK7,[10] and the fragment A60-D86 of Gadd45β (sequence: AIDEEEE-DDIALQIHFTLIQSFCCDND), corresponding to the shortest region of Gadd45β still able to bind to and block MKK7,[1] were also prepared and purified. The highly acidic peptide Gadd45β (A60-D86) was purified by RP-HPLC using a C18 Jupiter column (50 mm × 22 mm) applying a linear gradient of 5% to 70% acetonitrile in 10 mM phosphate buffer pH 7.0, over 30 minutes.

Biotinylation of Gadd45β

Fractions of Gadd45β (1 mg/ml) were biotinylated using an EZ Link NHS-LC-biotin kit (Pierce) essentially according to the manufacturer's instructions but with slight modifications. One volume of 2 mg/mL NHS-LC-biotin was added to 20 volumes of protein and incubated on ice for 1 h; the reaction was then stopped by addition of one volume of 50 mM glycine. Biotinylated samples were dialyzed against buffer B (25 mM Tris, 150 mM NaCl 1 mM EDTA, 1 mM DTT, pH 7.5) to remove excess glycine and free biotin, and stored at −80 °C. The incorporation of one to two biotin moieties per molecule of protein was recorded by LC-MS analysis.

Gadd45β self-association and competition ELISA assays

An ELISA-like assay was used to monitor Gadd45β dimerisation. For this purpose, Gadd45β at concentration of 0.52 μM in buffer B was dispensed into a 96-well microtiter plate. Some wells were filled with buffer alone and were used as blanks. After incubation for 16 h at 4 °C, the solutions were removed and the wells filled with 350 μL of a 1% (w/v) solution of NFDM in PBS. The plate was incubated for 1 h at 37 °C in the dark. After washing with buffer T-PBS (PBS with 0.004% (v/v) Tween), the wells were filled with 100 μL of biotinylated Gadd45β at concentrations ranging between 16 μM and 2.1 μM. Each datum point was performed in triplicate. Following incubation for 1 h in the dark at 37 °C the solutions were removed and the wells again washed with T-PBS. Then 100 μL of 1:1000 horseradish peroxidase-conjugated streptavidin dissolved in buffer was added to each well and the plate incubated for 1 h at 37 °C in the dark. After removal of the enzyme solution and washing, 100 μL of the chromogenic substrate *o*-phenylendiamine (0.4 mg/mL in 50 mM sodium phosphate-citrate buffer, containing 0.4 mg/mL of urea in hydrogen peroxide) was added and the colour was allowed to develop in the dark for 5 min. The reaction was stopped by adding 50 μL of 2.5 M H_2SO_4. The absorbance at 490 nm was measured in all wells and the values were averaged after subtracting the corresponding blanks. For the competition experiments, 100 μL aliquots of 0.52 μM His_6-Gadd45β were coated on the wells of a microtiter plate and 0.26 μM biotinylated His_6-Gadd45β (molar ratio 1:0.5) was used throughout (pre-saturation condition). Peptides (0.52 μM) used as competitors were used at a molar ratio of 1:1 to coated unbiotinylated protein and pre-incubated with 0.26 μM biotinylated Gadd45β for 30 min at 4 °C before addition to each well. Peptides derived from the trypsin digestion were used at a nominal concentration of 0.52 μM, calculated assuming a 100% trypsin cleavage and a 100% recovery from the HPLC fractionation. GST-MKK7, Gadd45β eH1, eH4, and eH5, and the synthetic peptides MKK7(G132-N156) and Gadd45β(A60-D86), were always used at a concentration of 0.52 μM. ELISA assays were carried out at least in duplicate. Competition results are reported as (B/B_0) X 100, where B is the average absorbance from the triplicate data points for a given analyte and B_0 is the average absorbance determined without competitor.

Dose dependent competition assay

Dose-dependent competition assays were carried out to monitor the Gadd45β region of dimerisation. For this purpose, 100 μl of 0.26 μM Gadd45β in buffer B was coated on microtiter wells overnight at 4 °C. After blocking with NFDM for 1 h and washing with TPBS, increasing concentrations of competitors (Gadd45β (A12-R35), Gadd45β (R91-E104), Gadd45β (A129-N148), MKK7 (G132-N156) and the whole kinase protein GST-MKK7 ranging from 0 to 0.52 μM were pre-incubated with 0.13 μM biotinylated Gadd45β for 30 min before addition to each well. The subsequent steps of the ELISA assays were carried out as described.

Gadd45β -MKK7 association

Association between Gadd45β and MKK7 was investigated by ELISA assays by coating the GST-fused full-length kinase for 16 h at 4 °C, at a concentration of 42 nM. After extensive washing with T-PBS buffer and blocking with NFDM (1 h, 37 °C, 350 μL), wells were incubated with different solutions of biotinylated Gadd45β at concentrations ranging from 8.4 nM to 168 nM. Bound

protein was then detected as described above. K_D values were estimated as the concentration of Gadd45β able to give half of the saturation signal. Binding competition assays were performed by coating GST-MKK7 at 42 nM as described, a concentration of biotinylated Gadd45β of 21 nM (pre-saturation conditions) and using the competitors (synthetic eH1, eH5, short H5, eH4, Gadd45β(A60-D86), MKK7(G132-N156) and GST-MKK7) at a 2:1 competitor to soluble protein ratio (concentration of competitors 42 nM).

Modelling of Gadd45β homodimers

The model-building procedure for Gadd45β protein was as described.[1] To build Gadd45β homodimers, the first ten residues of the unstructured N-terminal region were removed. The resulting models obtained from manual docking were completed by addition of all hydrogen atoms and underwent energy minimisation with the NAMD[39] package using the Charmm22 force field.[40] Molecular dynamics simulations were run in solvent by confining the minimised complexes in rectangular TIP3P water boxes, with a minimal distance from the solute to the box wall of 1.2 nm. Counterions (Na$^+$) were added to neutralise the system. The particle mesh Ewald method was applied to calculate long-range electrostatics interactions, setting the non-bonded cutoff to 14 Å. The solvated molecules were then energy minimised through 1000 steps with solute atoms restrained to their starting positions using a force constant of 1 kcal mol^{-1} Å^{-1} before MD simulations. After this, the molecules were submitted to 50 ps of restrained MD (1 kcal mol^{-1} Å^{-1}) at constant volume, gradual heating to 310 K, followed by 50 ps of restrained MD (1 kcal mol^{-1} Å^{-1}) at constant pressure to adjust the system density. Production runs were carried out for 200 ps using a time-step of 1 fs. Snapshots from the production run were saved every 1000 steps and analyzed with the NAMD program. Model figures were made with MOLMOL.[41]

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