

Insights into the Structural Basis of the GADD45 β -mediated Inactivation of the JNK Kinase, MKK7/JNKK2^{*S}

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Salvatore Papa[‡], Simona M. Monti[§], Rosa Maria Vitale[§], Concetta Bubici^{‡1}, Shanthi Jayawardena[‡], Kelleen Alvarez[‡], Enrico De Smaele^{‡2}, Nina Dathan[§], Carlo Pedone[§], Menotti Ruvo^{§3}, and Guido Franzoso^{‡4}

From [‡]The Ben May Institute for Cancer Research, University of Chicago, Chicago, Illinois 60637 and the [§]Istituto di Biostrutture e Bioimmagini, Consiglio Nazionale delle Ricerche, Via Mezzocannone 16, 80134 Napoli, Italy

NF- κ B/Rel factors control programmed cell death (PCD), and this control is crucial to oncogenesis, cancer chemoresistance, and antagonism of tumor necrosis factor (TNF) α -induced killing. With TNF α , NF- κ B-mediated protection involves suppression of the c-Jun-N-terminal kinase (JNK) cascade, and we have identified Gadd45 β , a member of the Gadd45 family, as a pivotal effector of this activity of NF- κ B. Inhibition of TNF α -induced JNK signaling by Gadd45 β depends on direct targeting of the JNK kinase, MKK7/JNKK2. The mechanism by which Gadd45 β blunts MKK7, however, is unknown. Here we show that Gadd45 β is a structured protein with a predicted four-stranded β -sheet core, five α -helices, and two acidic loops. Association of Gadd45 β with MKK7 involves a network of interactions mediated by its putative helices α 3 and α 4 and loops 1 and 2. Whereas α 3 appears to primarily mediate docking to MKK7, loop 1 and α 4-loop 2 seemingly afford kinase inactivation by engaging the ATP-binding site and causing conformational changes that impede catalytic function. These data provide a basis for Gadd45 β -mediated blockade of MKK7, and ultimately, TNF α -induced PCD. They also have important implications for treatment of widespread diseases.

NF- κ B/Rel transcription factors promote cell survival, and this activity of NF- κ B counters programmed cell death (PCD)⁵ elicited by the pro-inflammatory cytokine tumor necrosis factor (TNF) α (1, 2). This inhibition of TNF α -induced PCD by NF- κ B is crucial for sustaining chronic inflammation and

tumor progression (2, 3). NF- κ B afforded protection also serves immunity, lymphopoiesis, and cancer chemoresistance (1, 4, 5). We and others have shown that, downstream of TNF-Rs, the NF- κ B-mediated antagonism of PCD involves a suppression of the sustained activation of the c-Jun N-terminal kinase (JNK) cascade (6, 7). This is one of the major mitogen-activated protein kinase (MAPK) pathways, and like other such pathways, it transduces signals through phosphorylation of hierarchically ordered kinase modules to elicit an appropriate cellular response (8). Prolonged activation of JNK by TNF α was shown to elicit PCD by causing Itch-dependent proteolysis of the caspase-8 antagonist, c-FLIP_L, and cleavage of Bcl-2-like factor, Bid, into proapoptotic jBid (2, 9, 10). Thus, the biological outcome of the triggering of TNF-Rs is determined by a delicate balance between the opposing activities of the JNK and NF- κ B pathways.

The inhibitory activity that NF- κ B exerts on the JNK cascade involves an up-regulation of target genes (1), and we have identified Gadd45 β , a member of the Gadd45 family of structurally related factors (11), as the product of one of such genes (6). Gadd45 β is induced rapidly by TNF α through a mechanism that requires NF- κ B, its ectopic expression blocks TNF α -elicited JNK activity and PCD in NF- κ B null cells, and gene targeted- or antisense-mediated inactivation of endogenous *gadd45 β* impairs the NF- κ B-mediated control of JNK signaling and PCD downstream of TNF-Rs (2, 6, 11). The Gadd45 β -afforded blockade of this signaling depends on a direct targeting and inactivation of the MAPKK, MKK7/JNKK2, a selective and essential inducer of JNK following exposure to TNF α (11). Indeed, the Gadd45 β -MKK7 interaction is a crucial molecular link between the NF- κ B and JNK pathways, and accordingly, a key checkpoint controlling cell fate downstream of TNF-Rs (2, 11). The relevance of this link is underscored by the ability of cell-permeable peptides selectively interfering with the Gadd45 β -mediated suppression of MKK7 to hinder NF- κ B afforded cytoprotection against TNF α -induced killing (11), and the ability of short hairpin RNAs knocking-down *MKK7* to halt this killing in NF- κ B null cells (9). Knock-out studies confirmed the essential role that Gadd45 β plays in blocking TNF α -induced PCD in certain tissues (2, 11). Other such studies in mice also uncovered a requirement for Gadd45 β in skeleton morphogenesis, cell resistance to stress- and anticancer drugs-induced killing, and differentiation and function of T-helper 1 (T_H1) T cells (11–14).

In many instances, the biological activity of Gadd45 factors has been linked to regulation of protein kinases, such as MAPK

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² Current address: Dept. of Experimental Medicine and Pathology, University of Rome "La Sapienza," Viale Regina Elena 324, 00161 Rome, Italy.

³ To whom correspondence may be addressed. Tel.: 39-081-2536644; Fax: 39-081-2534574; E-mail: menotti.ruvo@unina.it.

⁴ To whom correspondence may be addressed. Tel.: 773-834-0020; Fax: 773-702-3701; E-mail: gfranzos@midway.uchicago.edu.

⁵ The abbreviations used are: PCD, programmed cell death; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; eGFP, enhanced green fluorescent protein; PTD, protein transduction domain; 4-VP, 4-vinylpyridine; LC-MS, liquid chromatography-mass spectrometry; CD, circular dichroism; FL, full-length.

Basis for Inhibition of MKK7 by Gadd45 β

pathway kinases (15–17). We showed for instance that Gadd45 β binds directly to MKK7, and that this binding is sufficient alone to cause inactivation of this kinase. As other Gadd45 family proteins, however, Gadd45 β lacks enzymatic activity such as phosphatase activity (16, 17), and so, the basis for its ability to block MKK7 remains unknown. To understand this basis, here we used a combined structural and functional approach. Using this approach, we constructed a model predicting that Gadd45 β is a structured protein, consisting essentially of a central four-stranded β -sheet core surrounded by five α -helices and two acidic loops. In this model, contact of Gadd45 β with MKK7 involves an extensive network of interactions mediated by its α -helices α 3 (Ile⁶⁹-Asn⁸⁵) and α 4 (Met⁹⁵-Leu¹⁰²) and loops 1 and 2 (Ile⁶¹-Asp⁶⁸ and Gly¹⁰³-Leu¹¹⁷, respectively). Indeed, we found that whereas α 3 plays a key role in docking of Gadd45 β to MKK7, the loop 1 and α 4-loop 2 regions of Gadd45 β are essential for kinase inactivation. These regions appear in fact to establish several bonds with residues in the catalytic pocket and the β 3-helix C loop of MKK7, preventing access of the kinase to ATP and inducing conformational changes that stabilize this kinase in an inactive conformation. These data provide a mechanism for Gadd45 β -mediated suppression of MKK7, and ultimately, of TNF α -induced PCD (2). Given the key role that inappropriate blockade of PCD by NF- κ B plays in chronic inflammation and cancer (1, 4, 5), they also have potential implications for development of new treatments for these diseases.

EXPERIMENTAL PROCEDURES

Protein Purification and Binding Assays—Glutathione S-transferase (GST) proteins used Figs. 1–4 were purified from bacterial lysates using glutathione beads (Sigma) as detailed elsewhere (11). Purity was verified by Coomassie Blue staining (Figs. 3E and 4C). For binding assays, *in vitro* translated proteins (5 μ l) were incubated with GST (10 μ l) proteins for 20 min and then pulled down with glutathione beads as described previously (11). Prior to loading onto gels, beads were washed extensively in binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Triton X-100; Figs. 1, B, D, and F, and 2B). In Fig. 2D and supplementary Fig. S5B, higher stringency was achieved by performing GST precipitations using 0.4% Triton X-100, 5 μ l of translated proteins, and 4 μ l of GST-coupled beads.

Kinase Assays—MKK7 kinase assays were performed as detailed elsewhere (11). Briefly, active FLAG-MKK7 was immunoprecipitated with an anti-FLAG antibody (Sigma) from lysates of HEK-293 cells treated with human TNF α (2,000 units/ml; Peprotech) for 10 min and then subjected to *in vitro* kinase reaction for 20 min at 30 °C. Preincubation with recombinant GST or GST-Gadd45 β proteins was for an additional 10 min (Figs. 3, B and D, and 4B).

Additional Methods—A detailed description of additional materials and methods, including cell treatments and death assays, plasmids, human (h)Gadd45 β purification, native gel electrophoresis, gel filtration, LC-MS, circular dichroism (CD), limited proteolysis and alkylation analyses, and modeling can be found in the supplementary data.

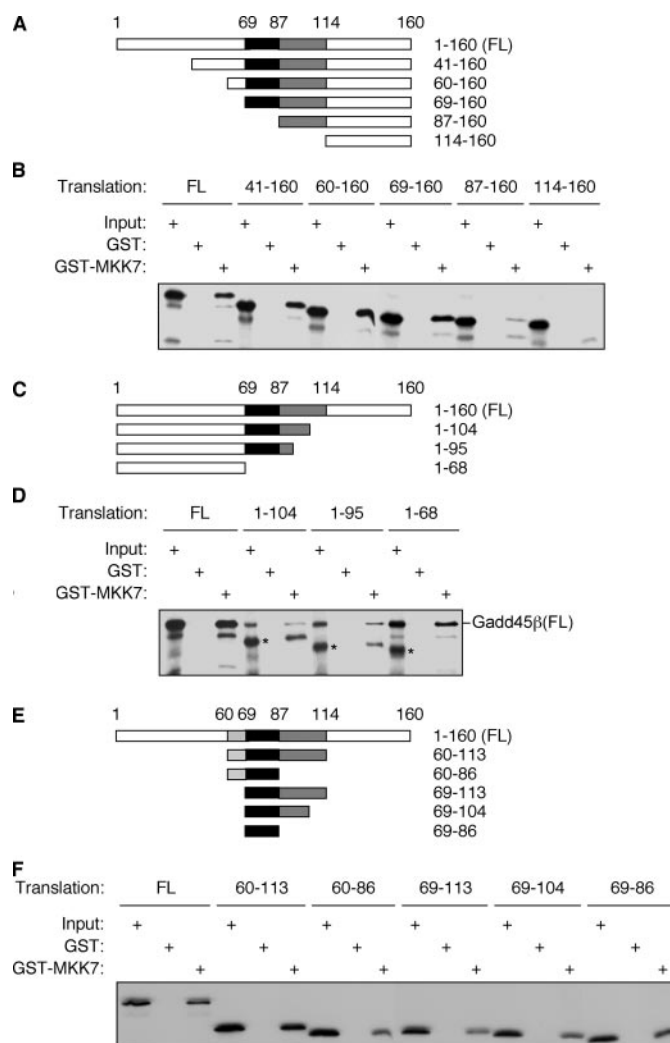


FIGURE 1. Gadd45 β interacts with MKK7 through an extended region comprising peptide 69–86. A, C, and E, representation of the N- and C-terminal truncations and internal Gadd45 β polypeptides used for binding assays, respectively. Numbers indicate amino acid residues. Region 69–86 is shown in black; other MKK7-binding regions are in gray. B, D, and F, pull-down assays showing binding of GST- and GST-MKK7-coated beads to the ³⁵S-labeled, *in vitro* translated proteins depicted in A, C, and E, respectively. Shown is 40% of the inputs. Asterisks in D and F mark proteins synthesized from linearized pBS-Gadd45 β (FL) templates. Full-length (FL) Gadd45 β derived from undigested plasmids is also indicated.

RESULTS

Identification of the Regions of Gadd45 β Contacting MKK7—Gadd45 proteins have no known enzymatic activity (16, 17). Thus, to determine the basis for Gadd45 β -mediated inactivation of MKK7, we mapped the region(s) of Gadd45 β involved in interaction with this kinase. Plasmids encoding N- or C-terminal truncated Gadd45 β proteins were translated *in vitro* (Fig. 1, A and C, respectively), and the resulting products were tested for their ability to bind to GST-MKK7 fusion proteins in GST pull-down assays (Fig. 1, B and D). GST served as negative control. Full-length (FL) Gadd45 β bound strongly to GST-MKK7, but not to GST (Fig. 1B), as shown previously (11). Gadd45 β -(41–160), Gadd45 β -(60–160), and Gadd45 β -(69–160) also interacted with the fusion protein with high affinity (Fig. 1B; compare the fractions of precipitated products relative to their inputs), suggesting that residues 1–68 are dispensable for tight

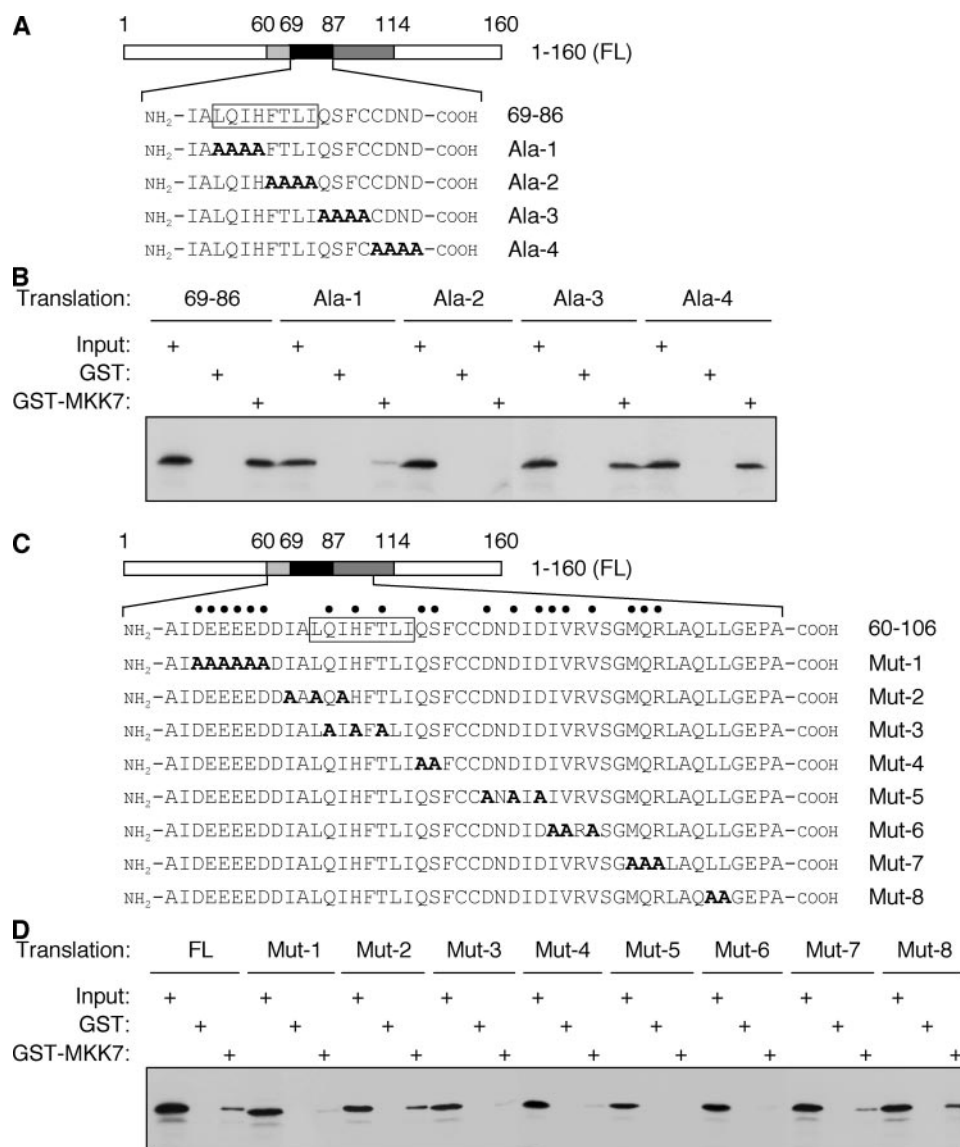


FIGURE 2. Identification of Gadd45β residues mediating binding to MKK7. A and C, representation of the Gadd45β(69–87) and Gadd45β(FL) alanine-scanning mutants used for binding assays, respectively. MKK7-binding regions are in black or gray, with darkness correlating with the apparent relevance to this binding. Mutagenized residues are shown; alanine substitutions are bold. The Leu⁷¹–Ile⁷⁸ region is boxed; residues contributing to binding are dotted. B and D, pull-down assays showing binding of GST- or GST-MKK7-coated beads to the ³⁵S-labeled, *in vitro* translated products depicted in A and C, respectively. Shown is 40% of the inputs. In B and D different stringency was used (see “Experimental Procedures”).

binding of Gadd45β to MKK7. Notably, removal of an additional 18 amino acids severely compromised this binding (Fig. 1B, see 87–160), whereas further truncations also involving residues Ile⁸⁷–Glu¹¹³ abolished it (Fig. 1B, see 114–160). These data suggest that region Ile⁶⁹–Asp⁸⁶ is essential for association of Gadd45β with MKK7 and that the adjacent region Ile⁸⁷–Glu¹¹³ also contributes to this association.

Analysis of C-terminal truncations confirmed the presence of a MKK7 interaction region between Gadd45β residues 69 and 95 (Fig. 1D, compare 1–95 and 1–68). Full-length Gadd45β, yielded by incomplete restriction digestion of pBS-Gadd45β templates (Fig. 1D), served as internal positive control. Together, these findings suggest that Gadd45β interacts with MKK7 primarily through region Ile⁶⁹–Asp⁸⁶, and that additional contacts are made through Ile⁸⁷–Glu¹¹³.

These data do not exclude existence of additional MKK7-binding regions within Gadd45β (discussed below). The importance of the Ile⁶⁹–Asp⁸⁶ region for this binding, however, was corroborated by findings with internal Gadd45β peptides (Fig. 1E). As shown in Fig. 1F, Gadd45β(60–113) interacted with GST-MKK7 with high affinity (comparable with that of FL), and Gadd45β(69–86) virtually recapitulated this interaction, suggesting that Ile⁶⁹–Asp⁸⁶ is sufficient alone for tight association of Gadd45β with MKK7 (compare 69–86 to FL).

Delineation of Residues Involved in Interaction of Gadd45β with MKK7—To further delineate which residues of Gadd45β are most critical for binding to MKK7, we generated alanine-scanning mutants of region Ile⁶⁹–Asp⁸⁶ (Fig. 2A). As shown in Fig. 2B, mutation of residues Leu⁷¹–His⁷⁴ or Phe⁷⁵–Ile⁷⁸ (Ala-1 and Ala-2, respectively) severely compromised association of Gadd45β(69–86) with MKK7, whereas mutation of residues Gln⁷⁹–Cys⁸² or Cys⁸³–Asp⁸⁶ impacted on this association only modestly (Fig. 2B, Ala-3 and Ala-4, respectively), suggesting that contact with MKK7 is primarily mediated through the Leu⁷¹–Ile⁷⁸ segment of region Ile⁶⁹–Asp⁸⁶.

Next, we introduced select mutations within the broader region Ala⁶⁰–Ala¹⁰⁶ in the context of Gadd45β(FL) (Fig. 2C), because additional residues within this region appeared to be involved in association with MKK7 (discussed above; see Fig. 1). As shown in Fig. 2D, alanine substitutions of acidic residues Asp⁶²–Asp⁶⁷ (Mut-1) significantly weakened Gadd45β interaction with MKK7 (compare with FL). Conversely, mutation of Ile⁶⁹, Leu⁷¹, and Ile⁷³ (Mut-2) had no effect on this interaction, suggesting that the binding impairment seen with Ala¹ (Fig. 2B) was due to ablation of Gln⁷² and/or His⁷⁴, and not of Leu⁷¹ or Ile⁷³. Indeed, substitution of the former residues, along with that of Thr⁷⁶ severely compromised Gadd45β association with MKK7 (Fig. 2D, Mut-3). Binding was also impaired by mutation of Asp⁸⁴/Asp⁸⁶/Asp⁸⁸ (Mut-5) or Ile⁸⁹/Val⁹⁰/Val⁹² (Mut-6), and to a lesser extent, of Gln⁷⁹/Ser⁸⁰ (Mut-4) or Met⁹⁵/Gln⁹⁶/Arg⁹⁷ (Mut-7) (Fig. 2D). Mut-8 precipitated with GST-MKK7 with efficiency comparable with that of Gadd45β(FL).

Notably, consistent with the notion that Gadd45β contacts MKK7 through an extended surface, beyond that created by

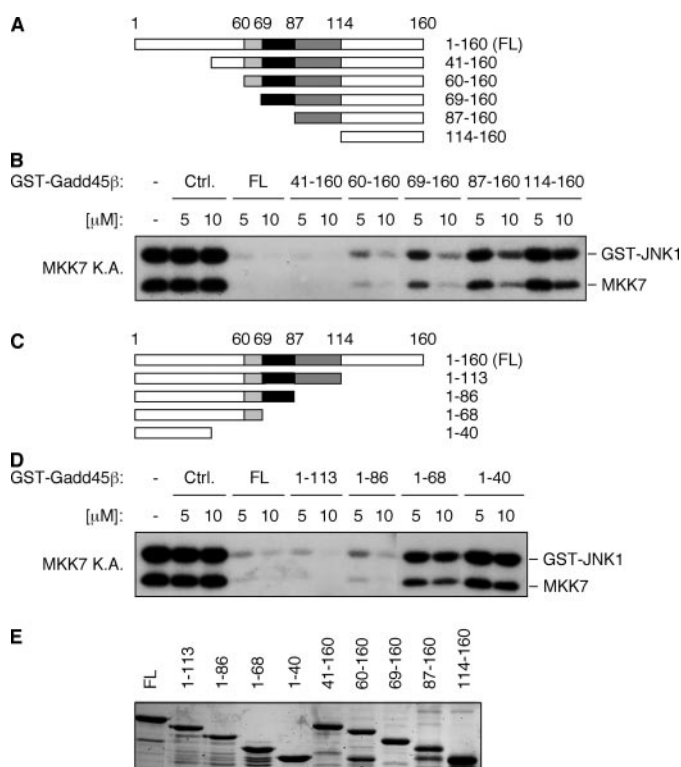


FIGURE 3. Inactivation of MKK7 involves distinct regions of Gadd45 β . A and C, representation of the N- and C-terminal truncations of Gadd45 β used for kinase assays, respectively. Numbers indicate amino acid residues of the Gadd45 β truncations used. MKK7-binding regions are colored as in Fig. 2A. B and D, kinase assays (K.A.) showing inhibition of active MKK7 by the indicated N- and C-terminal truncated GST-Gadd45 β polypeptides. Immunoprecipitated MKK7, GST-JNK1 substrate, and concentrations of GST (Ctrl.) and GST-Gadd45 β proteins are shown. (–), no inhibitor; (FL), GST-Gadd45 β (FL). E, Coomassie Brilliant Blue staining showing purity of the proteins used in B and D.

Ile⁶⁹–Asp⁸⁶, alanine substitutions within Gadd45 β (FL) had only partial inhibitory effects on the interaction of these proteins (Fig. 2D and data not shown), whereas the inhibitory effects of mutations introduced in the context of Gadd45 β -(69–86) were virtually complete (Fig. 2B, Ala-2, and Ala-1). It is also noteworthy that, with the former mutants, binding assays were performed at higher stringency (see “Experimental Procedures”), which may explain in part why ablation of certain residues appeared to have more dramatic effects in the context of Gadd45 β (FL) than in that of Gadd45 β -(69–86) (e.g. see mutation of Gln⁷⁹/Ser⁸⁰; note binding of Mut-4 (Fig. 2D) and Ala-3 (Fig. 2B); see also Mut-5 (Fig. 2D) and Ala-4 (Fig. 2B), both containing mutations of Asp⁸⁴/Asp⁸⁶). It is also possible, however, that certain mutations had conformational effects that could be revealed only in the context of a full-length polypeptide (further discussed below; see also “Discussion”). In summary, the data suggest that binding of Gadd45 β to MKK7 primarily involves Gln⁷² and/or His⁷⁴, as well as residues within peptide Phe⁷⁵–Ile⁷⁸ such as Thr⁷⁶. They also suggest an involvement in this binding (either in direct contact with MKK7 or in maintenance of Gadd45 β conformational stability) of additional residues both within and outside region Ile⁶⁹–Asp⁸⁶ (i.e. Gln⁷⁹, Ser⁸⁰, Asp⁸⁴ and/or Asp⁸⁶, within; and Asp⁶²–Asp⁶⁷, Asp⁸⁸, Ile⁸⁹, Val⁹⁰, Val⁹², Met⁹⁵, Gln⁹⁶, and/or Arg⁹⁷, outside).

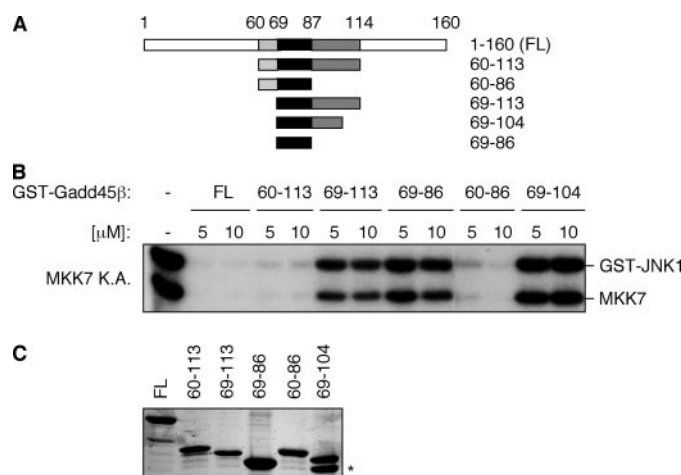


FIGURE 4. Gadd45 β regions 60–68 and 87–113 contain MKK7 inhibitory modules. A, representation of the internal Gadd45 β fragments used for kinase assays. Numbers and black/gray colors are used as in Fig. 3. B, kinase assays (K.A.) showing inhibition of active MKK7 by the indicated GST-Gadd45 β proteins. Immunoprecipitated MKK7, GST-JNK1 substrate, and GST-Gadd45 β protein concentrations are shown. (–), no inhibitor; (FL), GST-Gadd45 β (FL); asterisk, truncated product. C, Coomassie Brilliant Blue staining showing purity of the proteins used in B.

Regions of Gadd45 β Mediating MKK7 Inactivation—To delineate regions of Gadd45 β involved in inhibition of MKK7, we examined the activity of N- and C-terminal Gadd45 β truncations in MKK7 kinase assays, *in vitro* (Fig. 3, A and C, respectively). Active FLAG-MKK7 was isolated from TNF α -treated 293 cells, and kinase assays were performed in the presence of purified GST-Gadd45 β proteins or control GST (Fig. 3, B and D; see also Fig. 3E). As shown previously, phosphorylation of GST-JNK1 by MKK7, as well as autophosphorylation, were effectively blocked by Gadd45 β (FL) but not by GST (Ctrl.), in a dose-dependent manner (Fig. 3, B and D; see also Ref. 11). The inhibitory activity of Gadd45 β was virtually unaffected by removal of 40 N-terminal residues, and was affected only marginally by removal of an additional 19 amino acids (Fig. 3B, 41–160 and 60–160, respectively), suggesting that region 1–59 is dispensable for effective blockade of MKK7 by Gadd45 β . Notably, larger deletions progressively hindered the ability of Gadd45 β to inhibit kinase function, with Gadd45 β -(114–160) proteins virtually lacking any such ability (Fig. 3B). Gadd45 β -(69–160) and, albeit to a lesser extent, Gadd45 β -(87–160) retained instead significant inhibitory activity *in vitro*, but only at high concentrations.

Findings were confirmed by analysis of C-terminal Gadd45 β truncations (Fig. 3, C and D). Deletion of residues 114–160 had no effect on Gadd45 β afforded blockade of MKK7 (Fig. 3D, 1–113), and a further deletion of residues 87–160 impacted upon this blockade only modestly, albeit reproducibly (Fig. 3D, 1–86; see 5 μ M). In contrast, a Gadd45 β -(1–68) protein exhibited only weak inhibitory activity, and Gadd45 β -(1–40) had virtually no such activity (Fig. 3D). These findings correlate with the physical interaction data shown in Fig. 1B, and suggest that Gadd45 β -mediated inactivation of MKK7 involves, in addition to the MKK7-binding peptide Ile⁶⁹–Asp⁸⁶ (Fig. 1F), two regions spanning Tyr⁴¹–Asp⁶⁸ and Ile⁸⁷–Glu¹¹³.

To further delineate the role of these regions in inhibition of MKK7 and determine whether these are also sufficient for this

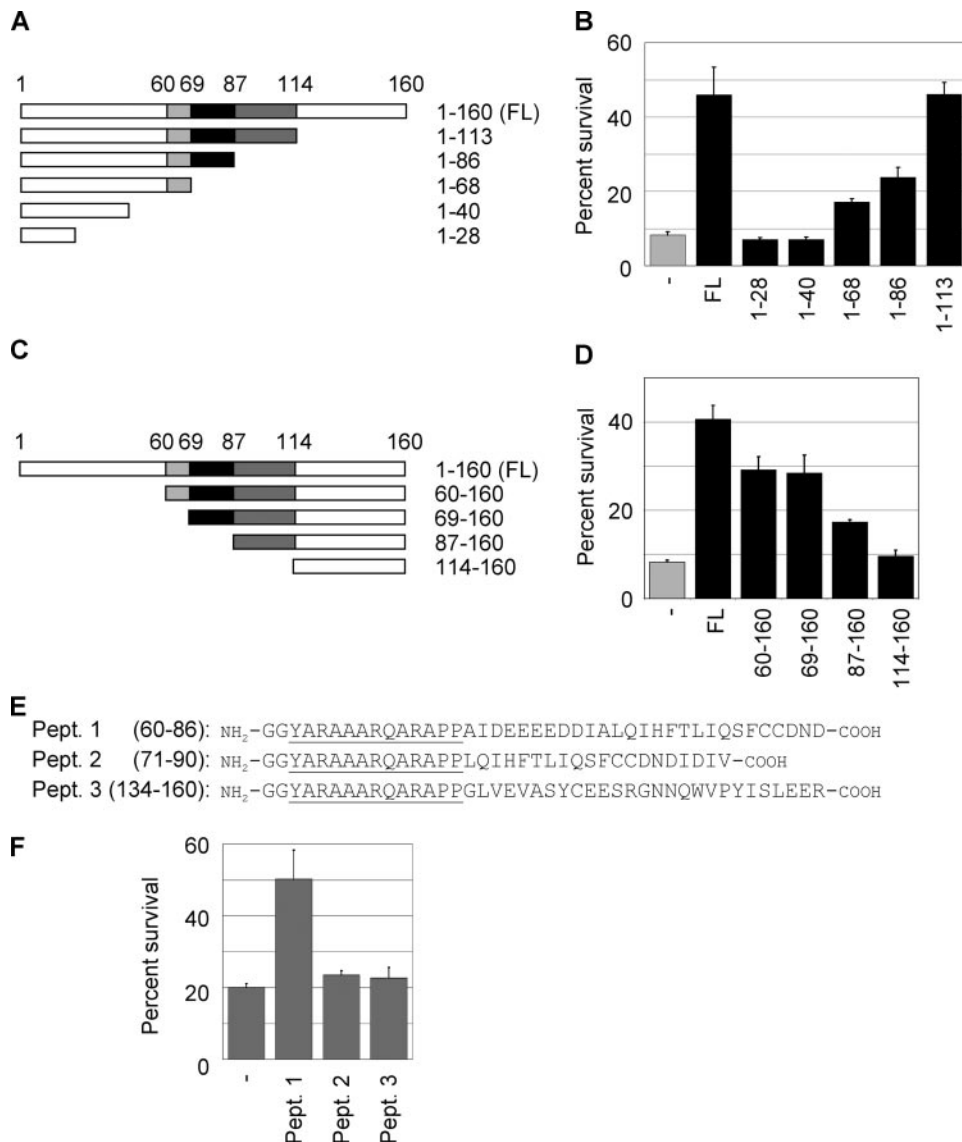


FIGURE 5. Gadd45 β -afforded protection involves the same regions that mediate inhibition of MKK7. A and C, representation of the N- and C-terminal Gadd45 β truncations used in cytoprotection assays, respectively. Numbers and black/gray colors are used as in Fig. 3. B and D, PI nuclear staining assays showing survival of *RelA*^{-/-} cells expressing the indicated C- and N-terminal truncated Gadd45 β proteins fused to eGFP, respectively, following treatment with TNF α plus low doses of cycloheximide (0.1 μ g/ml). Percent survival reflects the fraction of live cells (i.e. DNA content $\geq G_1$) in TNF α -treated cultures relative to control cultures treated with cycloheximide alone. (—), pEGFP; (FL), pEGFP-Gadd45 β (FL). E, sequences of the PTD4 fusion peptides of Gadd45 β used in F. PTD4 and the PP linker are underlined. Numbers designate the Gadd45 β fragments fused to PTD4. F, viability of *RelA*^{-/-} cells exposed to the indicated PTD4 fusion peptides or Me₂SO (—) and treated with TNF α plus 0.1 μ g/ml cycloheximide. Percent survival reflects the fraction of live (i.e. adherent) cells in TNF α -treated cultures relative to control cultures treated with cycloheximide alone. Values in B, D, and F represent the mean (\pm S.D.) of three experiments. Intracellular peptide incorporation was verified through flow cytometry and confocal microscopy.

activity, we examined internal Gadd45 β fragments (Fig. 4, A, see also C). As shown in Fig. 4B, Gadd45 β -(60–86) exhibited strong inhibitory activity *in vitro*, even though at lower concentrations (i.e. 5 μ M) this activity was somewhat weaker than that of Gadd45 β (FL). Of interest, removal of the acidic stretch at Asp⁶²–Asp⁶⁸ completely abolished kinase inactivation (Fig. 4B, compare 69–86 and 60–86), suggesting that this stretch is crucial for inhibitory function. Notably, however, its relevance to this function was markedly less apparent in the context of longer Gadd45 β polypeptides, extending C-terminal to either Glu¹¹³ or Arg¹⁶⁰. Indeed, these polypeptides, but not those

extending only to Glu¹⁰⁴, were capable of affording significant suppression of MKK7 even in the absence of the Asp⁶²–Asp⁶⁸ stretch (Fig. 4B, compare 69–86, 69–104, and 69–113; see also Fig. 3B, note the inhibitory activity of 69–160). These data are consistent with those shown in Fig. 3 and further support existence of a second MKK7 inhibitory module within Pro¹⁰⁵–Glu¹¹³. Activity of this module was especially evident at lower protein concentrations (i.e. 5 μ M or lower; see Figs. 3B and 4B; data not shown), suggesting that this plays an important physiologic role.

Altogether, the data indicate that high affinity binding of Gadd45 β to MKK7 is mediated through region Ile⁶⁹–Asp⁸⁶, being both necessary and sufficient for this binding (Fig. 1). Association of Ile⁶⁹–Asp⁸⁶ with MKK7, however, is insufficient alone to inhibit kinase activity (Fig. 4B). The suppressive action of Gadd45 β on MKK7 depends in fact on establishment of further contacts involving the two inhibitory modules at Ala⁶⁰–Asp⁶⁸ and Glu¹⁰⁴–Glu¹¹³.

Gadd45 β Regions Mediating Suppression of TNF α -induced PCD—To tighten the link between Gadd45 β -mediated suppression of MKK7 and antagonism of TNF α -induced PCD (2), we examined whether these activities of Gadd45 β were mediated by the same peptidic regions. To this end, C- and N-terminal truncations of Gadd45 β (Fig. 5, A and C, respectively) were tested in cytoprotection assays in *RelA*^{-/-} cells. As shown previously (6), upon expression of a fusion protein of enhanced green fluorescent protein (eGFP) and Gadd45 β (FL), these

cells were effectively rescued from TNF α -induced killing, with more than 50% of eGFP-Gadd45 β (FL)-expressing cells being viable at 12.5 h (Fig. 5B). Cells expressing eGFP(—) remained susceptible to this killing, as expected. Deletion of residues 114–160 had no apparent effect on the ability of Gadd45 β to counter TNF α -induced PCD (Fig. 5B, 1–113), whereas larger deletions progressively impaired this ability, with a Gadd45 β protein spanning residues 1–40 completely lacking such ability (Fig. 5B, 1–40, also 1–28). Hence, as for inhibition of MKK7, *in vitro* (Figs. 3 and 4), Gadd45 β depends for antiapoptotic function on a peptidic segment comprised between Tyr⁴¹ and Asp¹¹³.

Consistent with this notion, removal of residues 1–113 abolished the ability of Gadd45 β to blunt TNF α -induced PCD, whereas a Gadd45 β -(87–160) protein retained weak, but significant protective activity (Fig. 5D, 114–160 and 87–160, respectively). Deletion of residues 1–68 yielded a protein that could effectively counter TNF α cytotoxicity, but was significantly less effective than Gadd45 β (FL) in this action (Fig. 5D, compare 69–160 and FL). These data are consistent with the presence of a critical protective module within Ile⁶⁹–Glu¹¹³, a region also required for inhibition of MKK7 (Figs. 3 and 4). They also suggest the existence of a second protective module within Met¹–Asp⁶⁸, a region harboring the Asp⁶²–Asp⁶⁸ peptide, also involved in MKK7 inactivation (Fig. 4B).

Surprisingly, however, removal of residues 1–59, which do not contain this Asp⁶²–Asp⁶⁸ peptide, impacted on the ability of Gadd45 β to counter TNF α -elicited PCD to an extent similar to that of deletion of region 1–68 (Fig. 5D, see 60–160 and 69–160). To clarify the involvement of Asp⁶²–Asp⁶⁸ in cytoprotection, we therefore tested the activity of a synthetic protein transduction domain (PTD) 4-Gadd45 β -(60–86) peptide in *RelA*^{-/-} cells. This peptide consisted of region Asp⁶²–Asp⁶⁸ and the MKK7-binding region, Ile⁶⁹–Asp⁸⁶, fused to a PTD4 peptide (Fig. 5E, peptide 1), which enables transduction through biological membranes (18). As shown in Fig. 5F, exposure to peptide 1, but not to control peptides 2 or 3 (71–90 and 134–160), which fail to block MKK7 *in vitro* (Figs. 1F and 4B; data not shown), effectively rescued *RelA*^{-/-} cells from TNF α -induced PCD. Me₂SO-treated cells remained susceptible to this PCD, as expected (Fig. 5B). Peptides entered cells with comparable efficiency, shown by flow cytometry and confocal microscopy (data not shown). These data indicate that the Asp⁶²–Asp⁶⁸ module can afford significant protection against TNF α -inflicted killing, regardless of the presence of Ile⁸⁷–Glu¹¹³.

We concluded that Gadd45 β -mediated protection involves participation of two distinct peptidic regions at Ala⁶⁰–Asp⁶⁸ and Ile⁸⁷–Asp¹¹³. Activity of these regions is markedly increased by the presence of peptide Ile⁶⁹–Asp⁸⁶, containing a key MKK7-binding module (Figs. 1 and 2). Although they are capable of acting somewhat independently, regions Ala⁶⁰–Asp⁶⁸ and Ile⁸⁷–Asp¹¹³ are likely to function cooperatively *in vivo* to maximize suppression of TNF α -induced PCD. Hence, the ability of Gadd45 β to counter TNF α -induced PCD tightly correlates with its ability to block MKK7 (Figs. 1–4), further validating the view that these two activities of Gadd45 β are inseparable from one another (2).

Characterization of the Structure of Gadd45 β —To investigate the structure of Gadd45 β , a recombinant protein of human (h)Gadd45 β was purified from bacterial lysates, upon removal of the fused GST tag by PreScission protease (Amersham Biosciences). The isolated protein was ~98% pure and had an apparent molecular mass of 18,096.8 \pm 0.5 Da (inclusive of the GPLGS linker left from proteolytic cleavage), as shown by LC-MS analyses (supplementary Fig. S1, data not shown). Further studies involving native gel electrophoresis and gel filtration chromatography established that, like Gadd45 α (19), Gadd45 β exists primarily as a dimer, assembled through non-

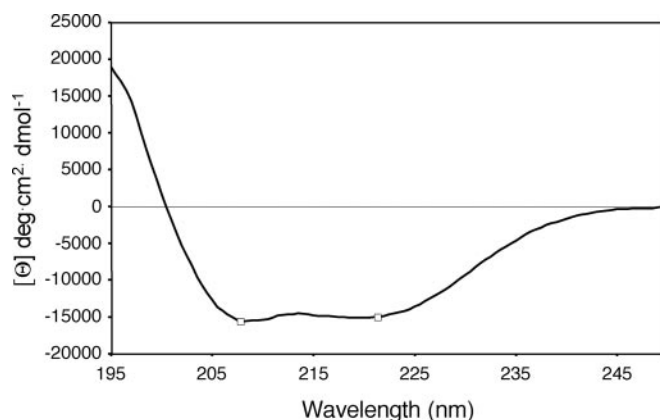


FIGURE 6. CD spectrum of purified hGadd45 β in the far UV region. Shown is the profile of hGadd45 β in the far UV region. The two negative peaks at 209 and 219 nm, indicative of high α -helical content, are indicated by open squares.

covalent bonds.⁶ Lack of inter- and intramolecular disulfide bonds was further confirmed through alkylation of cysteine residues with 4-vinylpyridine (4-VP) followed by LC-MS analysis of tryptic hGadd45 β fragments (see below).

To assess the secondary structure of Gadd45 β , we then used CD recording protein spectra in the far UV region (*i.e.* 195–250 nm). As shown in Fig. 6, the CD spectrum of native hGadd45 β exhibited two negative peaks at 209 and 219 nm and one positive peak at 195 nm, indicative of a predominantly folded structure with a high α -helical content (*i.e.* ~45%) (20). This structure was also highly stable, because its CD spectrum was not significantly affected by exposure to either denaturing agents such as guanidinium hydrochloride (1 M) or structuring solvents such as trifluoroethanol (50% v/v) (data not shown).

To extend these studies, we then probed chain flexibility and accessibility of exposed residues to solvent by performing limited proteolysis assays, using several enzymes (21). Time progression analyses of trypsin digestion showed that the final proteolytic event occurred at Arg⁹¹, with peaks produced by this event being recorded by LC-MS only at 720 min (supplementary Fig. S2A). This inaccessibility to trypsin suggested that this residue was either buried within the protein or contained within a non-flexible structure (see below; Fig. 7, B and C). Cleavages at Arg³², Lys⁴⁵, Lys¹³¹, and Arg¹⁴⁶ also proceeded slowly, whereas cleavage at other sites occurred more readily (supplementary Fig. S2A), suggesting that unlike the former residues, the latter sites were exposed to the outer surface of the protein (see Fig. 7, B and C). Findings were extended by the use of α -chymotrypsin. As shown in supplementary Fig. S2B, kinetic analyses of the digested products revealed that hydrophobic residues Leu²⁷, Leu⁵⁶, and, to a lesser extent, Trp¹³⁰ and Tyr¹⁴¹ were exposed to the enzyme. Chain flexibility at the N and C termini of Gadd45 β was finally confirmed by the use of Glu-C. Digestion with this enzyme overall proceeded slowly, with complete cleavages being recorded at the N and C termini of the protein only after 10 h (supplementary Fig. S2C). The first of these cleavages occurred at Glu²⁵, followed by two other cleavages at Glu⁴² and Glu¹³⁷. The central core of the protein

⁶ L. Tornatore and M. Ruvo, manuscript in preparation.

was unaffected by the enzyme, throughout the course of the reaction.

Next, we assessed accessibility of cysteine residues to solvent by performing limited thiol alkylation reactions using 4-VP and purified hGadd45 β . As shown in supplementary Fig. S3A, following these reactions, $\sim 90\%$ of the protein harbored two molecules of 4-VP ($18,310.4 \pm 0.5$ Da), whereas the remaining 10% incorporated three such molecules ($18,416.3 \pm 0.5$ Da). Proteolysis with trypsin of these partially alkylated products followed by LC-MS analysis revealed that residue Cys⁹ (within Thr²-Lys¹⁵) and one of three cysteines within Leu⁴⁶-Arg⁹¹ (i.e. Cys⁵⁷, Cys⁸², or Cys⁸³) were consistently alkylated in virtually all products (supplementary Fig. S3B). Conversely, fragment Ser^{132–146}, containing Cys¹⁴², was alkylated only partially, because unmodified peptide was also detected readily (see supplementary Fig. S3B). To precisely delineate which cysteine within Leu⁴⁶-Arg⁹¹ was alkylated by 4-VP, we then used α -chymotrypsin. Combined LC-MS and MS/MS analyses of α -chymotrypsin-digested products revealed the presence of a monoalkylated Cys⁸²-Met⁹⁵ peptide, which upon fragmentation, was found to be derivatized on Cys⁸³, establishing reactivity of this residue (data not shown). We concluded that, among the six cysteines of Gadd45 β , accessibility to solvent was highest for Cys⁹ and Cys⁸³, followed by Cys¹⁴². The other three cysteines were instead unreactive to 4-VP, suggesting that they were buried within the protein.

Modeling of Gadd45 β —A search of the NCBI Conserved Domain Data Base using reverse position-specific BLAST and the FUGUE server (22, 23) identified Gadd45 proteins as members of the L7Ae/L30e/S12e family of structurally related, ribosomal polypeptides (24). Despite low sequence identity ($<20\%$), Gadd45 β exhibits a high degree of similarity in secondary structure profile with spliceosomal protein 15.5K (25), a member of this family, with gaps/insertions between these proteins being distributed preferentially in loop regions (Fig. 7A). The selection of 15.5K as template along with introduction of secondary structure restraints, based both on experimental data yielded by limited proteolysis and alkylation analyses (Fig. 6 and supplementary Figs. S2A–C and S3A–B, data not shown) and secondary structure predictions, enabled an accurate modeling of the structure of Gadd45 β (Fig. 7A, further details can be found in the supplementary data, Materials and Methods section; see also Ref. 26).

The model indicates that hGadd45 β is essentially constituted by a series of alternating α -helices and β -strands, which form a α - β - α sandwich-like structure (Fig. 7, B and C). This is characterized by a central four-stranded β -sheet core consisting of three antiparallel and one parallel β -strands, ordered as follows: Leu³⁶-Val³⁸ (β 1), His¹¹⁸-Thr¹²³ (β 4), Val⁵⁴-Ala⁶⁰ (β 2), Asn⁸⁸-Val⁹² (β 3). The five α -helices are oriented such that Gln¹⁷-Gln³³ (α 1), Met⁹⁵-Leu¹⁰² (α 4), and Lys¹³¹-Arg¹⁴⁶ (α 5) lie on one side of the β -sheet, and Val⁴⁰-Val⁴⁹ (α 2) and Ile⁶⁹-Asn⁸⁵ (α 3) pack against the other. The main insertions relative to template are located in the acidic loops at Ile⁶¹-Asp⁶⁸ and Gly¹⁰³-Leu¹¹⁷ (loop 1 and 2, respectively). MD trajectory analysis of both human and mGadd45 β further indicated that the fold of the protein is stabilized by several H-bonds and salt bridges between the side chains of the follow-

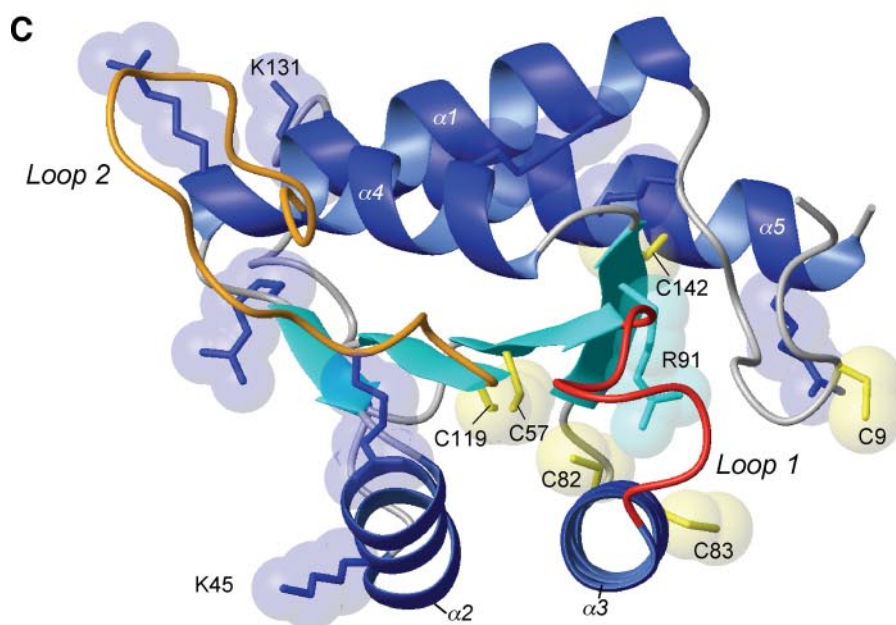
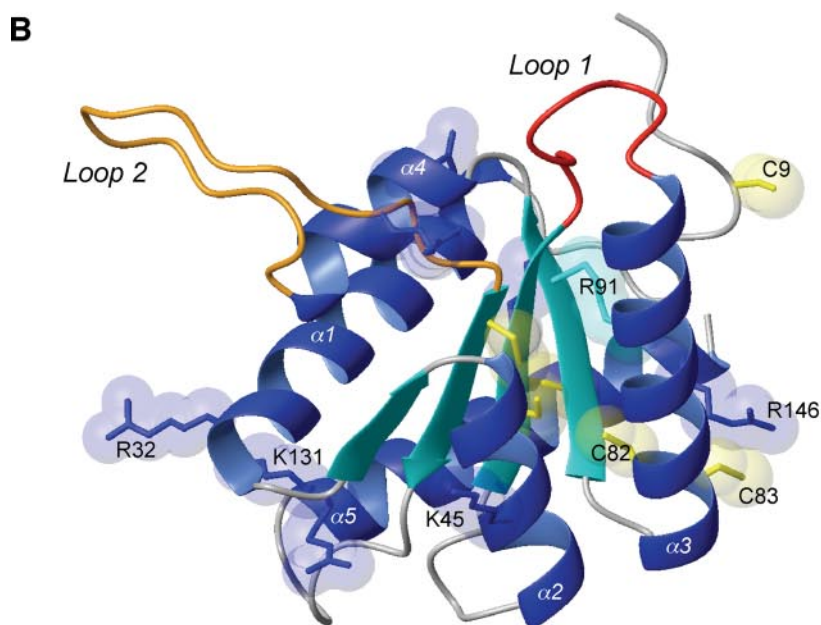
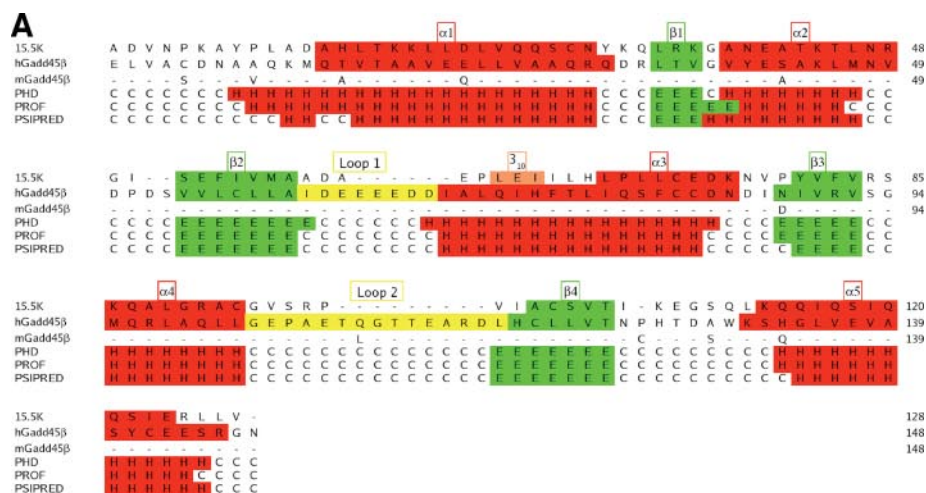
ing charged/polar residues: Arg⁹¹ (β 3) and Gln⁷⁹ (α 3), Arg¹⁴⁶ (α 5) and Asp⁸⁶ (α 3- β 3 loop)/Asn⁸⁸ (β 3), Lys¹³¹ (α 5) and Glu²⁵ (α 1), Arg⁹⁷ (α 4), and Gln¹⁷/Glu²⁴ (α 1).

This model is fully consistent with the experimental data, including the limited proteolysis and alkylation data (Fig. 6 and supplementary Figs. S2A–C and S3A–B; data not shown). It shows in fact that the six cysteines of hGadd45 β are too far apart from each other to engage in disulfide bridges, explaining why no such bridges are found in the pure protein.⁶ It also explains the ready accessibility to alkylation of Cys⁹ and Cys⁸³ (supplementary Fig. S3 and data not shown), which exhibit a solvent accessible surface of $>40\%$ (Fig. 7, B and C), as well as the modest reactivity of Cys¹⁴² (supplementary Fig. S3B and data not shown), exhibiting a solvent accessible surface value of $\sim 3\%$. In further agreement with the data, the remaining three cysteines of Gadd45 β appear to be buried instead within the protein (solvent accessible surface $<3\%$), two lying within the β -sheet core and one within α 3 (Fig. 7, B and C). Robustness of the model was further validated by the virtual inaccessibility to proteases of Arg⁹¹ (β 3) (supplementary Fig. S2A–C), buried in fact within the protein, and the presence of Arg³², Lys⁴⁵, Lys¹³¹, and Arg¹⁴⁶ within highly organized, α -helical structures (Fig. 7, A–C).

Modeling of MKK7—MKK7 is a member of the protein kinase family, the largest family of polypeptides folding into a topologically defined, two-lobe structure (27). The smaller, N-terminal lobe of these proteins essentially consists of a five-stranded β -sheet structure and a so-called helix C, and is involved in nucleotide anchoring and orientation (27, 28). The C-terminal lobe comprises instead a predominantly α -helical structure containing six major α -helices, three β -strands, and the catalytic and activation loops, and is primarily responsible for substrate binding and phosphotransfer initiation (Fig. 8 and supplementary Fig. S4). The deep cleft between the two lobes hosts the ATP binding site. Twelve residues within the kinase domain are nearly invariant throughout the protein kinase superfamily (supplementary Fig. S4), these residues being crucial for catalytic function (27, 28).

To model the kinase domain of MKK7 (Tyr¹¹³-Met³⁹⁸), we used as template the crystallographic structure of the corresponding domain of TAO2 (31.1% identity) (29), instead of that of MEK-1 (30), despite that this had a higher sequence identity with MKK7 (i.e. 39.4%). This is because the x-ray structure of MEK-1 was resolved in the presence of a non-competitive antagonist, which caused a peculiar deformation of the catalytic site (30). The sequence of MEK-1 was, nevertheless, utilized for multiple alignment with TAO2 and MKK7, to facilitate correct positioning of conserved residues during model building (supplementary Fig. S4). The backbone-atom best fit of the final model of MKK7 on the chosen template yielded a root mean square deviation value of 0.359 Å.

Expectedly, this model shows that the structure of the kinase domain of MKK7 is very similar to that of other such domains of MAPKs (Fig. 8; also supplementary Fig. S4) (27, 30). Indeed, in this model, the ATP-binding site of MKK7 is formed by a number of highly conserved residues (27, 30). The backbone amide of Thr¹³⁰ appears to be involved in H bonds with oxygen atoms of the phosphate groups of ATP. Furthermore, Lys¹⁴⁹,



the invariant residue within $\beta 3$, known to play a crucial role in anchorage and orientation of ATP in other protein kinases, contacts in the modeled MKK7 the three-phosphate group of the nucleotide (Fig. 8) and forms a salt bridge with the carboxyl group of the nearly invariant residue Asp¹⁶⁶. In the model, Glu¹⁹⁷ and Met¹⁹⁹ too contribute to ATP anchorage by forming H bonds with the adenine ring of the nucleotide. Met¹²⁶, Val¹³⁴, Ala¹⁴⁷, Met¹⁹⁶, Leu¹⁹⁸, Met¹⁹⁹, and Leu²⁵⁰ further participate in formation of the hydrophobic pocket that accommodates this ring.

Modeling of the MKK7-Gadd45 β Complex—The modeled kinase domain of MKK7 appears to be positively charged, bearing a net value of +6 (supplementary Fig. S4). Mapping of the electrostatic potential onto the molecular surface showed a localized, positive patch at the interface between the N- and C-terminal lobes, in correspondence of the ATP-binding site (Fig. 8, data not shown). In contrast, Gadd45 β is a highly negatively charged protein (net value of −16), and the largest acidic regions on its surface are those primarily formed by its N terminus and those modeled as loops 1 and 2 (Fig. 7, A–C, data not shown). Loop 1, in particular, is a stretch of all-negative residues that could potentially interact with residues of MKK7 involved in binding to the three-phosphate group of ATP (27, 30). This hypothesis is strongly supported by experimental evidence, because the basic residue-rich region 132–156 (comprising $\beta 2$ and $\beta 3$) was previously identified as a dominant Gadd45 β -binding site of MKK7 (11). Indeed, screening of 2,000 different arrangements of the MKK7-Gadd45 β complex generated with the ZDOCK program (31) confirmed preferential clustering of models exhibiting loop 1 in close proximity of the ATP-binding site of MKK7.

To further refine these models, we then took into account the finding that truncations of MKK7 spanning residues 63–401, 91–401, and

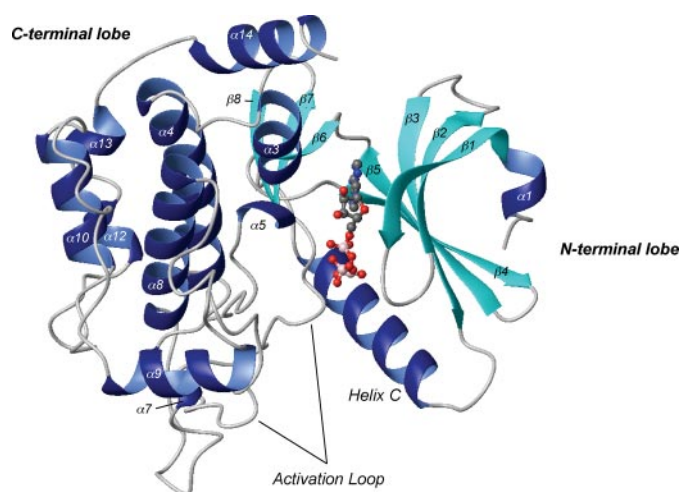


FIGURE 8. Model of the kinase domain of MKK7 (Tyr¹¹³–Met³⁹⁸) bound to ATP, in ribbon representation. ATP is in ball and stick representation. α -Helices are in blue, and β -strands are in cyan, as indicated.

132–401 bind to Gadd45 β with affinity seemingly comparable with that of full-length MKK7 (11). We also accounted for the aforementioned crucial importance for this binding of β 2– β 3 of MKK7 (11). The dispensability of β 1 for association of MKK7 with Gadd45 β was instead likely due to the high relative content in acidic residues of this strand (supplementary Fig. S4). It is plausible, in fact, that to enable interaction of β 2– β 3 with Gadd45 β and insertion of loop 1 into the catalytic cleft of MKK7, β 1, may have to unfold, as seen in the interaction of cyclin-dependent kinase 2 with its inhibitor, p27^{KIP1} (Ref. 32, see “Discussion”). In light of these findings and considerations, we performed docking of Gadd45 β onto a truncated MKK7 protein spanning residues 129–398, rather than onto a full-length kinase domain, as done previously with the cyclin-dependent kinase 2-p27^{KIP1} complex (32). We also removed 10 residues from the N terminus of Gadd45 β , as these were both unstructured and dispensable for interaction with MKK7 (Figs. 7, A–C, and 1, respectively). The MD-refined complex that exhibited the lowest potential energy, strongest intermolecular H-bond interactions, and highest hydrophobic/hydrophilic complementarity, along with the lowest accessible surface for residues contacting ATP, is depicted in Fig. 9B.

Notably, this modeled complex fully accounts for the *in vitro* binding data, including the site-directed mutagenesis data (Figs. 1 and 2), as well as the functional data shown in Figs. 3 and 4 (see “Discussion”). Specifically, it suggests that Gln⁷² and Thr⁷⁶ (α 3) of Gadd45 β are involved in H-bonds with Lys²⁰⁵ and Lys²⁰⁸ (α 3) of MKK7, respectively (Fig. 9, B and C). It also suggests that Gln⁹⁶ (α 4) and Asp¹¹⁶ (loop 2) of Gadd45 β interact with the guanidinium group of the side chain of Arg¹⁵² (β 3) of MKK7, that Gln¹⁰⁹ (loop 2) is

H-bonded to MKK7 Arg¹⁵³ (β 3), and that Glu¹¹³ (also loop 2) is salt-bridged to the side chain of Lys¹⁵⁷ (helix C- β 3 loop) (Fig. 9C). Several contacts appear also to be made between loop 1 of Gadd45 β and residues in the catalytic pocket of MKK7. Indeed, the model shows that Asp⁶² and Glu⁶⁴ in this loop form H-bonds with the backbone amide groups of Gly¹²⁹, Thr¹³⁰, Cys¹³¹ (all within the β 1– β 2 loop), and Val¹³⁴ (within β 2) of MKK7, whereas Glu⁶⁵ and Glu⁶⁶ (also in loop 1) are salt-bridged to the side chains of MKK7 Lys¹⁴⁹ (β 3) and Arg¹⁶² (helix C), respectively (Fig. 9C). Finally, Met⁹⁵ and Gln⁹⁶ (α 4) of Gadd45 β are depicted to form van der Waals interactions with the side chains of Pro¹³³ (β 2) and Trp¹³⁵ (β 2) of MKK7, respectively (Fig. 9C).

To further validate the proposed model of the MKK7-Gadd45 β complex, we tested its prediction that residues within putative Gadd45 β acidic loops 1 and 2 (*i.e.* Glu⁶⁵, Glu⁶⁶, and Glu¹¹³) directly contact basic residues within the catalytic pocket of MKK7 (*i.e.* Lys¹⁴⁹, Lys¹⁵⁷, and Arg¹⁶²). To this end, we performed GST pull-down assays using the “swapped” MKK7 and Gadd45 β mutants depicted in supplementary Fig. S5A and S5B. As shown in supplementary Fig. S5B, the MKK7(K149E,K157E,R162E) and MKK7(K149E,R162E) mutants failed to bind to wild-type Gadd45 β (FL) proteins (*lanes* 3 and 12, respectively), under conditions that enabled strong binding of these proteins to wild-type MKK7 (GST-MKK7) (see *lanes* 2 and 11). Remarkably, swapping mutations of cognate acidic residues of Gadd45 β , predicted by our model to interact via salt bridges with the mutated basic amino acids of MKK7, partially restored binding of mutant MKK7 proteins to Gadd45 β polypeptides (supplementary Fig. S5B; compare binding of Gadd45 β (E65R,E66R,E113R) to wild-type and MKK7 (K149E,K157E,R162E) proteins (*lanes* 3 and 9, respectively); also compare binding of Gadd45 β (E65R,E66R) to wild-type and MKK7 (K149E,R162E) proteins (*lanes* 12 and 18, respectively)). Rescue by these mutations was, nevertheless, incomplete, possibly due to conformational distortions caused by changes in the electrostatic charge of the targeted proteins. In contrast to what was seen with mutated MKK7 proteins, introduction of the aforementioned E65R,E66R,E113R and E65R,E66R mutations markedly impaired binding of wild-type MKK7 to Gadd45 β polypeptides (supplementary Fig. S5B; compare *lanes* 2 and 11 (FL) with *lanes* 8 and 17 (E65R,E66R,E113R and E65R,E66R, respectively)). Somewhat expectedly, wild-type MKK7 retained weak binding activity for mutated Gadd45 β polypeptides, likely owed to its preserved ability to form other interactions with these polypeptides (see Fig. 9). Under the conditions used, none of the tested MKK7 proteins exhibited significant association with Gadd45 β (Mut-1) (supplementary Fig. S5B; *lanes* 4–6 and 13–15; see also Fig. 2D). Together, these data further validate our proposed model of the Gadd45 β -MKK7 com-

FIGURE 7. A, sequence alignment of human and murine Gadd45 β with 15.5 K. Observed (with 15.5K) and predicted (with Gadd45 β ; based on the PHD, PROF, and PSIPRED programs) secondary structure elements are highlighted: red, α -helix; green, β -strand; orange, 3_{10} helix. Loops 1 and 2 are in yellow. Highlighted residues in the sequence of hGadd45 β take into account the revisions made in the final models depicted in B and C. The output of each secondary-structure prediction program used with hGadd45 β (*i.e.* PHD, PROF, and PSIPRED) is shown. H, α -helix; E, β -strand; C, random coil. B and C, different views of the three-dimensional model of hGadd45 β in ribbon representation. Loops 1 and 2 are shown in red and orange, respectively; α -helices, blue; β -strands, cyan. Arg and Lys residues that were experimentally shown to be exposed to solvent are depicted as blue stick bonds and transparent van der Waals surfaces. Arg⁹¹ (shown to be buried within the protein) is in cyan. Cys residues are in yellow.

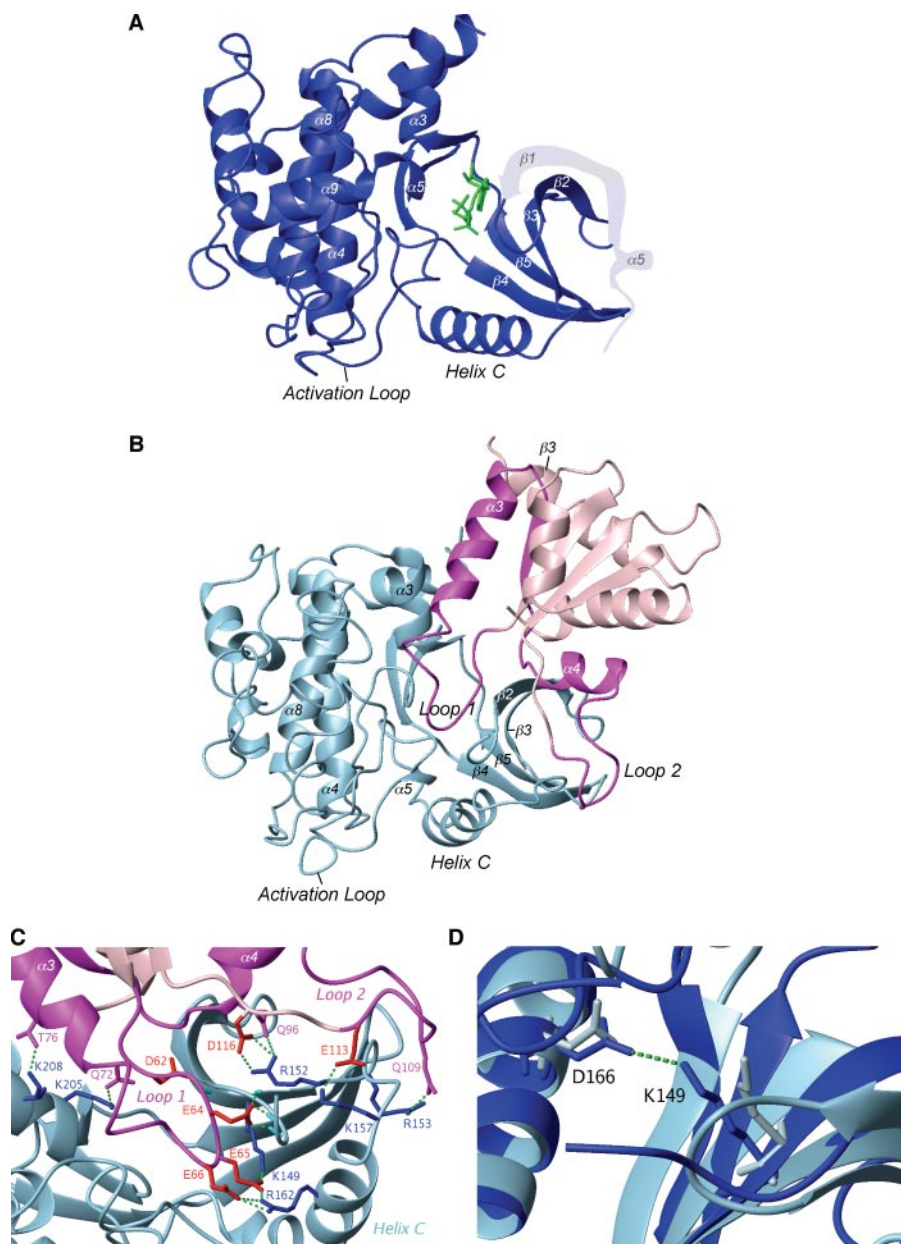


FIGURE 9. A, model of the kinase domain of MKK7 in ribbon representation and complexed to ATP, depicted as green bond sticks. MKK7 is shown in blue, with $\beta 1$ (Tyr¹¹³-Ser¹²⁸) (not included in the model) being transparent. B, model of the Gadd45 β -MKK7 complex following MD simulation in ribbon representation, and C, details of the MKK7-Gadd45 β interaction. MKK7 is in light blue; Gadd45 β is in pink, with the 60–114 region shown in magenta. In C, polar residues involved in intermolecular side chain-side chain interactions at the MKK7-Gadd45 β interface are in bond stick representation. Among these, acidic residues of Gadd45 β are in red; basic residues of MKK7 are in blue. Other polar residues are colored according to their ribbon. H-bonds are depicted as green dotted lines. D, details of the interaction between the side chains of Lys¹⁴⁹ and Asp¹⁶⁶, shown in stick representation and colored according to their ribbon. Shown are the models of MKK7, prior to (*i.e.* in active conformation; blue) and after (*i.e.* in the Gadd45 β -bound, inactive conformation; light blue) MD simulation, superimposed at the backbone atoms level. The H-bond between Asp¹⁶⁶ and Lys¹⁴⁹ in the active kinase is shown in green.

plex and underscore the importance of the predicted salt bridges between residues Lys¹⁴⁹, Lys¹⁵⁷, and Arg¹⁶² of MKK7 and glutamic residues of putative loops 1 and 2 of Gadd45 β in formation of this complex.

In summary, the model suggests that Gadd45 β establishes an extensive network of interactions with MKK7: the first two turns of Gadd45 β $\alpha 3$ interact with $\alpha 3$ of MKK7, thereby enabling insertion of loop 1 into the catalytic cleft; loop 2 of

Gadd45 β interacts instead with $\beta 3$ and the two loops of MKK7 linking $\beta 3$ to helix C and $\beta 4$ to $\beta 5$ (Fig. 9B). This model is strongly supported by experimental data and provides a mechanism for Gadd45 β -mediated blockade of MKK7.

DISCUSSION

Here, we propose a mechanism for the Gadd45 β -mediated suppression of MKK7, and ultimately, of TNF α -induced PCD (Fig. 5). Gadd45 β is a structured protein, predicted to have a four-stranded β -sheet core surrounded by five α -helices and two acidic loops, a prediction fully consistent with CD, limited proteolysis, and alkylation studies (Figs. 6 and 7; supplementary Figs. S2 and S3; data not shown). Furthermore, site-directed mutagenesis data and the presented optimized model of the MKK7-Gadd45 β complex suggest that this protein associates with MKK7 through an extensive network of interactions mediated by residues comprised within its putative $\alpha 3$, loop 1 and $\alpha 4$ -loop 2 regions (Fig. 9, also Figs. 1 and 2), ultimately preventing binding of ATP and causing conformational changes that are incompatible with MKK7 catalytic function (Figs. 8 and 9, also Figs. 3 and 4).

Proposed Mechanism for Gadd45 β -mediated Inhibition of MKK7—The final model, supported by extensive biochemical and site-directed mutagenesis data and molecular docking analyses, enables elaboration of a mechanism for Gadd45 β -afforded inhibition of MKK7 (11). This model suggests that anchoring of Gadd45 β to MKK7 is primarily mediated by electrostatic interactions and H-bonds formed between charged/polar residues positioned at the interface between the two proteins. Several contacts are seemingly established here

between Lys¹⁴⁹, Lys¹⁵⁷, and Arg¹⁶² on the kinase active site and Glu⁶⁵, Glu⁶⁶, Glu¹¹³ on loops 1 and 2 of Gadd45 β ; further interactions appear to be established between Gln⁷² and Thr⁷⁶ ($\alpha 3$) of Gadd45 β and lysine residues within $\alpha 3$ of MKK7 (Fig. 9C), and between residues within $\alpha 4$ and loop 2 (Gln⁹⁶, Gln¹⁰⁹, and Asp¹¹⁶) of Gadd45 β and $\beta 3$ and the helix C- $\beta 3$ loop of MKK7 (Arg¹⁵² and Arg¹⁵³). Finally, van der Waals interactions appear to be formed between Met⁹⁵/Gln⁹⁶ ($\alpha 4$) of Gadd45 β and Pro¹³³/Trp¹³⁵ ($\beta 2$) of MKK7, and between

Trp¹¹²/Glu¹¹³ (loop 2) of Gadd45 β and Thr¹⁹⁰/Asn¹⁸⁹ (β 4- β 5 loop) of MKK7 (Fig. 9C). Analysis of the model also suggests that, during formation of the Gadd45 β -MKK7 complex, interactions between Gadd45 β and β 2, β 3, and helix C- β 3 loop of MKK7 cause the β 1 strand of the kinase to unfold and move away from the catalytic pocket, pushed by electrostatic repulsion by Gadd45 β , it too being negatively charged (Figs. 7A and 9, B and C). This displacement of β 1 would then enable insertion of acidic loop 1 into the catalytic pocket, where this could engage in H-bonds and polar interactions with Lys¹⁴⁹ and other residues normally binding to the three-phosphate group of ATP (Fig. 9, B and C). Notably, this engagement of the ATP-binding site by loop 1 seems to prevent access of the kinase to ATP (11).

Notably, analysis of the three-dimensional models of MKK7 in its Gadd45 β -bound and unbound states, raises the intriguing possibility that association with Gadd45 β also causes the kinase to undergo conformational changes that further secure its inactivation. Indeed, as a result of interaction of α 4-loop 2 of Gadd45 β with β 3 and the β 3-helix C and β 4- β 5 loops of MKK7 and of occupancy of the catalytic pocket by loop 1, helix C of MKK7 appears to be pushed away from this pocket, to a position kinases normally have in their inactive conformation (Fig. 9D, see also Fig. 9B). Interestingly, however, the predicted overall conformation of Gadd45 β -bound MKK7 is distinct from that seen in the typical, or "open," inactive conformation, because in the proposed model of the Gadd45 β -MKK7 complex, the kinase activation loop retains its "active" position, that is away from the catalytic cleft, occupied in the complex by loop 1 (Fig. 9, A and B) (see also Refs. 27, 28, and 30). Yet, as seen in the typical open conformation, the key intramolecular bond between the invariant MKK7 residues, Lys¹⁴⁹ (β 3) and Asp¹⁶⁶ (helix C), is disrupted by the interaction with Gadd45 β (Fig. 9D and supplementary Figs. S4 and S5). Of interest, the aforementioned Gadd45 β -induced conformational changes of MKK7 closely resemble those elicited on cyclin-dependent kinase 2 by its inhibitor p27^{KIP1} (32). In keeping with the analogy with the proposed mechanism for Gadd45 β -mediated blockade of MKK7, p27^{KIP1} too targets the kinase ATP-binding site, preventing engagement of this site by ATP (32). Moreover, it too enters the catalytic pocket via displacement of strand β 1 (32). It is noteworthy, however, that whereas the loop 1 of Gadd45 β is predicted to contact residues in this pocket that interact with the three-phosphate group of ATP, p27^{KIP1} engages residues of this pocket that normally accommodate the adenine ring of ATP (Fig. 9, B and C) (32).

Experimental Validation of the Model—This model for Gadd45 β -mediated blockade of MKK7 is supported by compelling experimental evidence. It accounts for previous findings that region 132–156 of MKK7 (so-called peptide P1; comprising β 2, β 3, and the β 3-helix C loop) is critical for anchoring to Gadd45 β (supplementary Fig. S4). Consistently, soluble P1 effectively interferes with Gadd45 β -mediated inhibition of MKK7, both *in vitro* and *in vivo* (11). The model also explains the ability of Gadd45 β to block access of MKK7 to ATP, as well as the dispensability of MKK7 region 1–131 (comprising β 1) for binding of the kinase to Gadd45 β (Ref. 11, discussed above). Furthermore, both the model of MKK7 and Gadd45 β were built on suitable structural templates, and robustness

of both, as well as that of the model of the Gadd45 β -MKK7 complex, was validated through extensive experimentation, including structural (Fig. 6 and supplementary Figs. S2 and S3; data not shown) and functional analyses (Figs. 1–4 and supplementary Fig. S5).

In vitro studies confirmed that region Ile⁶⁹–Asp⁸⁶ (α 3) is both required and sufficient for high affinity binding of Gadd45 β to MKK7 (Figs. 1 and 2), and site-directed mutagenesis showed the importance of Gln⁷² and Thr⁷⁶ within this region for this binding (Fig. 2, D, Mut-3; B, Ala-1 and Ala-2; see Fig. 9C). These data also supported a key role for α 3 in docking of Gadd45 β to MKK7 (see above). Moreover, they confirmed the model prediction that region Ile⁶⁹–Asp⁸⁶ (α 3) is insufficient alone to cause kinase inactivation (Fig. 4B, 69–86), as this region contacts MKK7 at the outer brim of the catalytic pocket (Fig. 9, B and C). As also predicted by the model, Gadd45 β inhibitory function requires at least one of two modules located within loop 1 and α 4-loop 2 (Figs. 3 and 4). The key role of loop 1 in this function was shown by the finding that only peptide Ala⁶⁰–Asp⁸⁶ (loop 1- α 3), and not Ile⁶⁹–Asp⁸⁶ (α 3 alone), could afford blockade of MKK7, *in vitro* (Fig. 4B; also Fig. 3D, 1–86). Consistently, this loop was also capable of stabilizing association of Gadd45 β with MKK7, as mutation of Asp⁶²–Asp⁶⁷ significantly weakened this association (Fig. 2D, Mut-1). Finally, the role of predicted salt bridge interactions between glutamic residues within putative loops 1 and 2 of Gadd45 β (*i.e.* Glu⁶⁵, Glu⁶⁶, and Glu¹¹³) and basic residues within the catalytic pocket of MKK7 (*i.e.* Lys¹⁴⁹, Lys¹⁵⁷, and Arg¹⁶²) were confirmed in GST pull-down assays performed with swapped protein mutants (supplementary Fig. 5).

The model also provides a basis for the observed inhibitory effects of loop 2 (Figs. 3, B, 69–160; and 4, B, 69–113). It shows in fact that by contacting the β 3-helix C region of MKK7, this loop could cause conformational changes that impede catalytic function (Fig. 9, A–D). To cause these changes, however, loop 2 has to be positioned correctly in proximity of helix C, explaining why Gadd45 β truncations lacking the docking module, α 3, only have weak inhibitory effects despite the presence of α 4-loop 2 (Fig. 3B, 87–160; see also Fig. 9, B and C). In line with the ability of this α 4-loop 2 region to interact with MKK7, Gadd45 β proteins containing this region can bind, albeit weakly, to MKK7 even in the absence of α 3 (Fig. 1B, 87–160).

Finally, in addition to predicting residues contacting MKK7 (discussed above, Mut-1/Mut-3; Figs. 2, C–D, and 9C), the model confirms a role for others in maintenance of the global protein fold. It shows in fact that Gln⁷⁹ and Asp⁸⁶ (mutated in Mut-4 and Mut-5, respectively) could participate in intramolecular H-bonds that may stabilize the fold of Gadd45 β (data not shown) and that Ile⁸⁹/Val⁹⁰/Val⁹² (mutated in Mut-6) are likely to be required for the secondary structure of β 3 (Fig. 7A), which explains the marked binding impairment observed *in vitro* with Mut-4, Mut-5, and Mut-6 (Fig. 2D). Also, consistent with this view, is the finding that mutation of Gln⁷⁹ and Asp⁸⁶ appeared to have more dramatic effects in the context of Gadd45 β (FL) than in that of Gadd45 β -(60–86) (Fig. 2, B and D). The model finally illustrates how the partial binding defect

seen with Mut-7 is owed to involvement of Met⁹⁵/Gln⁹⁶ in direct contact with MKK7 (Figs. 2D and 9C).

Relevance to Gadd45 Family Protein Functions and NF-κB—Gadd45 factors are emerging as a new class of kinase regulators, as they have been shown to modulate activity of various such enzymes, including Cdc2 and MAPK pathway kinases such as MEKK4, ASK1, MKK7, and p38 (11, 15, 17, 33–35). The kinase-binding region of these factors was previously delineated only for the Gadd45α-Cdc2 complex and was mapped to the highly conserved region of Gadd45α, Val⁶⁹–Glu⁸⁴ (33), corresponding to the MKK7-binding module, α3, of Gadd45β (81.2% identity among the two regions; supplementary Fig. S6). Interestingly, the MKK7-binding regions of Gadd45β at loop 1 and α4-loop 2 are significantly less conserved among Gadd45 factors (supplementary Fig. S6), suggesting that, whereas α3 represents a common kinase-docking module, possibly explaining the promiscuity of these factors for kinase enzymes, the loop regions may account for specificity of the inhibitory action Gadd45 factors.

Additional conserved structures among Gadd45 proteins are the two dimerization modules. With Gadd45α, these modules were mapped to Gln³³–Ala⁶¹ and Ser¹³²–Arg¹⁶⁵ (19), corresponding to Gln³³–Ile⁶¹ (β1-α2-β2) and Thr¹²⁷–Arg¹⁶⁰ (β4/α5 loop-α5), respectively, of hGadd45β (supplementary Fig. S6). The high conservation of these regions suggests that they also mediate dimerization of Gadd45β. Yet, both appeared to be dispensable for Gadd45β-mediated binding and inactivation of MKK7 (Figs. 1–4). Consistent with this view, the dimerization surfaces of Gadd45β are well separated in space from those mediating contact with MKK7 (Fig. 7, B and C). Nevertheless, although monomeric Gadd45β retains strong inhibitory activity toward MKK7, *in vitro* (Fig. 4B, see 60–86), it is possible that interaction of Gadd45β with MKK7, *in vivo*, occurs in the context of a heterotetrameric complex of the MKK7·Gadd45β: Gadd45β·MKK7 type. Hence, whereas it might play a role in association of Gadd45 factors with certain proteins (e.g. PCNA) (19), dimerization appears to have no immediate relevance to Gadd45β-mediated blockade of MKK7.

Altogether, these findings provide important new insights into the functions of Gadd45 proteins and the basis for NF-κB-mediated control of JNK signaling (2). Previously, we identified the Gadd45β-MKK7 interaction as a crucial molecular link between the NF-κB and JNK pathways (2, 11), and importance of this link was validated *in vivo* through the use of knock-out models and cell-permeable peptides that selectively disrupt its activity (2, 11). This importance, as well as the relevance of our model for Gadd45β-afforded blockade of MKK7 to the control of JNK signaling, *in vivo*, is further underscored by the finding that the regions of Gadd45β mediating suppression of MKK7, *in vitro* (i.e. loops 1 and 2 and helix α3; Figs. 3 and 4), are also responsible for blunting TNFα-induced killing, *in vivo* (Fig. 5). A note of caution is, however, warranted as the proposed model for Gadd45β-MKK7 interaction suffers from lack of “genuine” structural data, and so awaits final validation through x-ray crystallography studies. Notwithstanding, this model depicts the three-dimensional view that most accurately explains the wealth of experimental data and the results of

the docking analyses presented in this study, affording a plausible detailed account of key molecular interactions between the two proteins.

These data further tighten the link between Gadd45β-mediated blockade of MKK7 and inhibition of PCD (2). Given the crucial role that the prosurvival activity of NF-κB plays in pathogenesis of widespread human diseases (see Refs. 1–3), these findings might help develop new therapies to treat these diseases.

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