Supporting Information

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SI Materials and Methods

Cloning. Mouse Gadd45γ cDNA was obtained from American Type Culture Collection (MGC-5695, IMAGE Clone ID 3493618). A construct containing amino acid residues 16–159 was subcloned into a pGEX vector derivative modified to contain a TEV cleavage site between the GST and the coding sequence for Gadd45γ. BL21(DE3) and B834 strains of *Escherichia coli* were transformed with the resulting plasmid. The protein resulting from TEV cleavage contains a vector-derived Gly-Ser dipeptide at the N terminus of the protein. Selenomethionine labeling of protein produced in B834 cells was confirmed by mass spectrometry. Point mutations were made using the QuikChange kit from Stratagene according to the manufacturer's instructions. Mutations were confirmed by sequencing the resulting clones and by mass spectrometry of the purified protein.

Expression and Purification. The transformed BL21(DE3) cells were cultured using Terrific Broth and B834 cells were grown in LeMaster medium supplemented with 100 μg/ml ampicillin. B834 cultures were also supplemented with 250 μM L-selenomethionine. Cells were grown at 30°C to an A_{600} of 0.6–0.8 and the temperature was reduced to 15°C and recombinant protein expression was induced by addition of IPTG to a final concentration of 1 mM. Cultures were allowed to grow 16–20 h after induction. Cells were harvested by centrifugation and the cell pellets were resuspended in PBS and lysed by sonication. The lysate was cleared by centrifugation at 37,000 × *g* for 1 h at 4°C. The supernatant was decanted and centrifuged at 250,000 × *g* for 1 h in a Beckman 60Ti rotor.

Recombinant protein in the high speed supernatant was purified by affinity chromatography on Glutathione Sepharose (GE Healthcare). After cleavage of the GST fusion with TEV protease, the recombinant protein was eluted with 5 column volumes of 20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. The eluted protein was loaded onto a Ceramic QHyperD anion exchange column (4.6 × 100 mm). After washing in 20 mM Hepes (pH 7.5), 200 mM NaCl, 1 mM DTT, and 1 mM EDTA until the A_{280} reached baseline, the protein was eluted in a 15 column volume gradient from 200–800 mM NaCl. typically eluting at about 500 mM NaCl. Wild type protein, the K48E mutant, and the T79E mutant typically eluted at ≈500 mM NaCl. The L80E mutant eluted from the anion exchange column at a NaCl concentration ≈40 mM lower than did the wild type and the other mutants. Gadd45γ containing fractions were pooled and concentrated on a YM10 membrane The protein was fractionated on Superdex 75 10/300 or 16/60 columns (GE Healthcare) in 20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. Gadd45γ-containing fractions were pooled and concentrated on a YM10 membrane to a final concentration of 5–10 mg/ml. Fifty-microliter aliquots were flash frozen in liquid nitrogen and stored at −80°C. Identical results were obtained using Tris buffers at pH 8.

Crystallization. Initial crystallization conditions were identified by sparse matrix screening using sitting drop vapor diffusion. The best conditions consisted of 0.9 M NaH_2PO4, 0.9 M KH_2PO_4 and 100 mM Hepes (pH 7.5) or 100 mM Tris (pH 8.5). Optimization was done using hanging drop vapor diffusion in 24-well plates with drops typically consisting of 2 μl of protein and 4 μl of reservoir solution. Crystallization was found to be independent of the cation and the final conditions consisted of 1.9 M NaH_2PO_4, 100 mM Hepes (pH 7.5). The crystals are orthorhombic and belong to space group $C222_1$ with unit cell dimensions a = 43.0, b = 122.5, and c = 105.4 Å. There are two molecules in the asymmetric unit.

Data Collection and Structure Solution. Crystals were transferred in two steps into 15% xylitol in 1.9M NaH_2PO_4 and the crystals were flash frozen in a nitrogen gas stream at −180°C. Diffraction data from native crystals were collected on a Q315 detector (ADSC) at beamline X25 at NSLS, Brookhaven National Lab. Data were processed using either HKL2000 (1) or D*trek (2). Phases were calculated using single-wavelength anomalous dispersion (SAD) methods from data collected at the peak of the Se absorption at beamline X8C on a Quantum 4 detector (ADSC). Heavy atom sites were located using BnP (3) followed by solvent flattening using RESOLVE (4). Most of the model was built using ARP/wARP (5) and refinement was done with Refmac5 (6, 7). Final adjustments to the model were made using O (8). The model has been refined to 1.7-Å resolution with R_{work} 22.4% and R_{free} 27.2%. Two loops in each monomer of the asymmetric unit are disordered and not included in the model. No interpretable density is observed for residues 106–115 or 127–130 of chain A nor for residues 107–115 or 126–130 of chain B. The N-terminal Gly-Ser dipeptide derived from the expression vector is also disordered and not included in the model. The quality of the model was evaluated using PROCHECK (9). Data collection, phasing and refinement statistics are shown in Table S2.

Size-Exclusion Chromatography (SEC). Analytical SEC analysis of wild type and mutant Gadd45γ was done on a Superdex 75 10/300 column (GE Healthcare). The column was calibrated using the low molecular weight kit from GE Healthcare. For one calibration aprotinin (Roche Diagnostics) was also included.

Dynamic Light Scattering. DLS analysis was done on a DynaPro Plate reader (Wyatt Technology). The protein concentrations ranged from 0.5–10 mg/ml and analysis was done using Dynamics 6.7 software provided by the manufacturer.

Circular Dichroism. Circular dichroism was measured on a Jasco (Easton, MD) J-815–150S spectrometer at 23°C. To reduce background counts caused by the buffer and salts, the buffer was exchanged by SEC to 20 mM Na,K PO_4 (pH 7.6), 200 mM NaF, and 1 mM DTT. Aliquots of the peak fractions were diluted ≈100-fold to 0.01 mg/ml in 10 mM Na,K PO_4 (pH 7.6), 100 mM NaF, and 0.5 mM DTT immediately before CD measurements. Two scans over the wavelength range of 350–190 nm were averaged for each sample and the contribution of buffer was subtracted. The melting point of each protein was measured by monitoring the ellipticity at 222 nm. The temperature was increased at a rate of 2°C per minute. The results are shown in Fig. 1.

Electrospray Mass Spectrometry. Molecular masses of wild type and mutant Gadd45γ were measured on an Agilent Technologies 1100 series LC/MS. Purified protein was dialyzed overnight into HPLC grade water. Five-microliter samples at 0.1 mg/ml were injected directly into the spectrometer at a flow rate of 0.3 ml/min in 20% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid. The scan covered a m/z range of 600-1900. The results are shown in Table 2.

Growth Inhibition Assay. Full-length mouse Gadd45γ wildtype and point mutants were subcloned into the BglII/EcoRI sites of the bicistronic mammalian expression vector pIRES2-DSred-Express (BD Biosciences). This vector contains a selectable marker that confers neomycin resistance. HepG2 cells were transfected using Lipofectamine (Gibco BRL). Transfection efficiency was monitored by DSRed expression and stably transfected HepG2 cells were selected using 1 mg/ml G418. Stably transfected HepG2 cells were grown in six-well plates in 2.5 ml DMEM-10 with changes of medium twice per week. After 3 weeks viable cells were quantitated following addition of 250 μl of the metabolic dye alamarBlue (Biosource) and continued incubation at 37°C. The fluorescence of the reduced product was measured after 1, 2 and 5 h of incubation at 37°C at an excitation wavelength of 530 nm and emission wavelength of 580 nm in a CytoFluor multiwell plate reader (Applied Biosystems). For staining of colonies with crystal violet, cells were washed twice with ice-cold PBS, fixed for 10 min in ice-cold methanol and stained for 15 min with 0.5% crystal violet. Plates were washed with distilled water and dried overnight. Colonies were counted manually.

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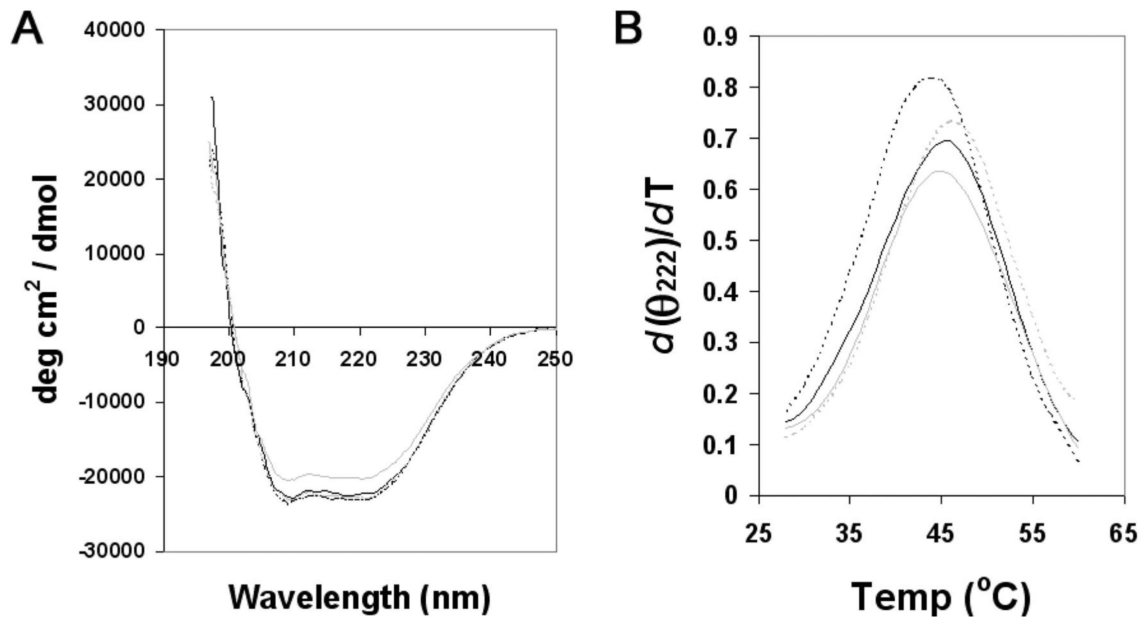

Fig. S1. CD spectra and melting point determinations of wild-type and mutant Gadd45γ. (*A*) The molar ellipticity is plotted. (*B*) The first derivative of the melting curve as determined by monitoring the molar ellipticity at 222 nm is plotted. In both *A* and *B* the curves for wild-type protein are shown by solid black lines, the L80E mutant is shown by dotted black lines, the T79E mutant is represented by dotted gray lines, and the K48E mutant is shown by solid gray lines.

Table S1. Data collection and refinement statistics

	Native		Se-Met	
Space group	$C222_1$		$C222_1$	
Unit cell				
a, Å	43.0		42.5	
b, Å	122.5		122.9	
c, Å	105.4		105.5	
$\alpha = \beta = \gamma$, °	90		90	
Molecules/asymmetric unit	2		2	
Data collection				
Wavelength, Å	1.4		0.9795	
Resolution range, Å	35.1–1.7	1.76–1.7	40.0–1.9	1.97–1.9
No. of observations	114,733	11,498	149,396	15,185
No. of unique reflections	30,674	3,061	41,294	4,250
Mean redundancy	3.74	3.73	3.6	3.6
R_{sym}, %	3.7	26.9	6.1	28.0
Completeness, %	99.8	99.9	98.1	99.9
Mean $I/\sigma I$	15.7	3.3	10.5	3.6
Phasing				
Se sites			2	
Phasing power			1.73	
FOM				
SAD phases			0.247	
Solvent flattened			0.61	
FC vs. FP				
R factor, %			30.5	
Correlation			0.74	
Refinement				
R_{work}, %/no. of reflections	22.4/27,545	35.3/2,174		
R_{free}, %/no. of reflections	27.2/1,547	41.8/103		
Geometry				
rms bonds, Å	0.015			
rms angles, °	1.45			
Final model	Chain A	Chain B		
Residues included	16–105, 116–126, 130–159	18–106,116–125, 131–159		
Ramachandran stats				
Most favored, no. of residues	105	102		
Allowed, no. of residues	9	10		
Mean B factors				
Protein, Å^2/no. of atoms	31.2/1,023	34.3/1,012		
Solvent, Å^2/no. of atoms	42.5/233			

Table S2. Mass spectrometry of wild-type and mutant Gadd45g

	Molecular mass		Mass difference from wild type	
	Calculated	Measured	Calculated	Measured
Wild type	15,788.6	15,783.7	—	—
L80E	15,804.6	15,800.2	16.0	16.5
T79E	15,816.6	15,811.9	28.0	28.2
K48E	15,789.6	15,784.1	1.0	0.4