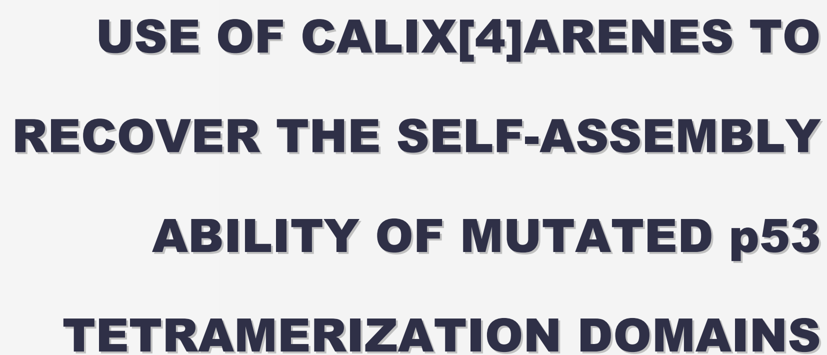


Susana Gordo Villoslada

2008

Memòria presentada per

Susana Gordo Villoslada

per optar al grau de doctor per la Universitat de Barcelona

Revisada per:

Prof. Ernest Giralt i Lledó

Universitat de Barcelona

Director

Programa de Química Orgànica

Bienni 2003-2005

Barcelona, abril de 2008

EXPERIMENTAL SECTION

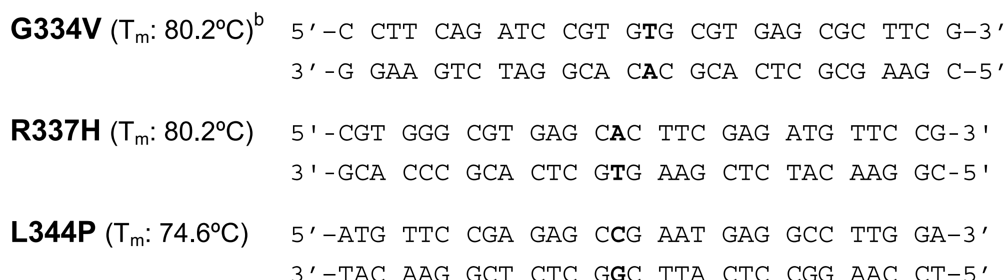
EXPERIMENTAL 1

Molecular biology for protein expression and purification

The clone for the recombinant production of the wild-type tetramerization domain of p53 (residues 311-367 inserted into the expression vector pET23b+, with resistance to ampicillin[a]) was a gift from Dr. M.G. Mateu.[1]

1.1. Site-directed mutagenesis

(a) Mutagenic primers:



G334V (T_m: 80.2ºC)[b] 5'-C CTT CAG ATC CGT G**T**G CGT GAG CGC TTC G-3'
3'-G GAA GTC TAG GCA C**A**C GCA CTC GCG AAG C-5'

R337H (T_m: 80.2ºC) 5'-CGT GGG CGT GAG C**A**C TTC GAG ATG TTC CG-3'
3'-GCA CCC GCA CTC G**T**G AAG CTC TAC AAG GC-5'

L344P (T_m: 74.6ºC) 5'-ATG TTC CGA GAG C**C**G AAT GAG GCC TTG GA-3'
3'-TAC AAG GCT CTC G**G**C TTA CTC CGG AAC CT-5'

(b) Plasmids mutagenesis:

The protocol described in the QuickChange® Site-Directed Mutagenesis Kit from Stratagene was modified as follows:

1. Mutagenesis reaction (in a sterile pre-chilled PCR tube):

- 39µL sterile milliQ water
- 5µL QuickChange® reaction buffer 10x (Stratagene)
- 1µL former plasmid p53wt (~1-5ng)
- 1.25µL sense mutagenic primer (~125ng)
- 1.25µL antisense mutagenic primer (~125ng)
- 1.5µL dNTPmix 100mM (Stratagene)
- 2µL *PfuTurbo* DNA polymerase (2.5u/µL) (Stratagene)

2. Thermal cycle for the polymerase reaction:

95ºC - 2min

95ºC - 1min
60ºC - 50s } x18
68ºC - 10min

4ºC - ∞

3. For the digestion of the methylated template plasmid, the mutagenesis reaction products were incubated with 2µL of *DpnI* endonuclease (10u/µL) for 1.5h at 37ºC.

[a] Instead of ampicillin, **carbenicillin** is recommended because it is less sensitive to the drop in pH of the grown medium that typically accompanies bacterial growth.

[b] $T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$

4. 4µL of *DpnI*-digested mixture was transformed into 50µL of XL1-Blue supercompetent cells (in a 14mL-falcon tube). Cells were plated in LB-agar (with carbenicillin) and incubated O/N at 37ºC.

Singles colonies were picked (10-20) and grown in 3mL LB medium (with carbenicillin) vigorously shaking at 37ºC. Plasmids were extracted from the cultures (QIAprep Spin Miniprep Kit) and analyzed in a 1% agarose gel. The coding region was sequenced using the T7 forward primer (BigDye® Terminator v3.1 Cycle Sequencing Kit) to check for the mutation.

1.2. Cloning L344P into vector pETM11[c]

The fragment corresponding to L344P (311-367) was amplified from the former pET23b+ clone using primers including *EcoRI* and *NcoI* sites, as well as a termination codon.

Forward (T_m: 58ºC): `5'- GGC GCC ATC GCG AAC ACC AGC TCC TCT CCC CAG -3'`
(GCC ATC GCG: *NcoI*; AAC ACC AGC TCC TCT CCC CAG: L344P (311-317))

Reverse (T_m: 62ºC): `3'- CCC TCG TCC CGA GTG AGG TCG ACT CTT AAG CTC G -5'`
(CCC TCG TCC CGA GTG AGG TCG: L344P (361-367); ACT: STOP; CTT AAG: *EcoRI*)

> PCR reaction composition: (200µL split in 4 pre-chilled PCR tubes)

- 154µL sterile milliQ water
- 20µL *PfuTurbo* reaction buffer 10x (Stratagene)
- 5µL template plasmid pET-23b L344P (~5ng)
- 5µL forward primer (~500ng)
- 5µL reverse primer (~500ng)
- 6µL dNTPmix 100mM (Stratagene)
- 8µL *PfuTurbo* DNA polymerase (2.5u/µL) (Stratagene)

> Touchdown thermal cycle for polymerase reaction:

95ºC - 2min

95ºC - 30s
T - 30s
68ºC - 1min
} xN → {63ºC x2; 62ºC x2; 61ºC x2; 59ºC x2; 58ºC x2; 56ºC x2; 54ºC x25}

68ºC - 10min

4ºC - ∞

PCR products were purified in a 1.2% agarose gel (QIAquick Gel Extraction Kit).

[c] Vector pETM11 from the EMBL Protein Expression and Purification Facility. Kanamicine resistance.

Copied insert and pETM11 (~1μg) were digested with *EcoRI* and *NcoI* in buffer H (Roche) for 2h at 37ºC, and then purified in a 1.2% agarose gel (QIAquick Gel Extraction Kit). Ligation was done with T4 DNA ligase O/N at 4ºC (for 20μL: 7μL digested vector + 9μL digested insert + 2μL T4 DNA ligase (~20u) + 2μL T4 reaction buffer 10x).

4μL of the ligation reaction mixture were transformed into 50μL of XL1-Blue supercompetent cells (in a 14mL-tube) and cells were plated in LB-agar (with kanaminicin) and incubated O/N at 37ºC.

Singles colonies were picked (10-20) and grown in 3mL LB medium (with kanamicine) vigorously shaking at 37ºC. Plasmids were extracted from the cultures (QIAprep Spin Miniprep Kit). The insertion was checked by digestion of the purified plasmid with *EcoRI* and *NcoI* for 2h at 37ºC and analysis of the digested product in a 1% agarose gel. For sequencing the coding region and the flanking sequences, T7 forward primer was used (BigDye® Terminator v3.1 Cycle Sequencing Kit).

1.3. Protein expression

1.3.1. General procedure for large scale protein expression

1-5ng of plasmid are transformed into 100μL of competent cells BL21(DE3) or BL21(DE3)-pLys (for ^{13}C and/or ^{15}N isotopic labeling) and cells are plated in LB-agar (with antibiotics) and incubated O/N at 37ºC. A single colony is inoculated into 10-50mL of sterile LB medium (containing antibiotics) and the culture is grown O/N at 37ºC with vigorous shaking. The large scale culture is set up by inoculating the O/N grown culture (1/100 dilution) and it is then incubated with vigorous shaking at the appropriate temperature for the required time, inducing expression if necessary (see next section). Cells are harvested by centrifugation in 1L-hermetic bottles at 3,500*xg* for 15min at 4ºC and the cell pellets are flash-frozen in liquid nitrogen and stored at -80ºC.

Protein L344P has to be produced at 16ºC (in auto-inducing media); the other three proteins can be expressed at any temperature.

1.3.2. Specific media and conditions for protein expression

(a) Standard LB medium with IPTG induction

5mL of the O/N grown culture are inoculated into 500mL of sterile LB medium with antibiotics in a 2L-flask (1L of medium for 3L-flask) and it is grown at 37ºC with vigorous shaking until OD_{600} reaches ~1.5.[d] Then, protein expression is induced by addition of IPTG to 300μM and the cultures are further incubated shaking for 4-6h at 37ºC (or O/N at 25º).

[d] OD_{600}: optical density at 600nm (determined by UV)

(b) M9 minimal medium with IPTG induction

Ideally, the O/N pre-culture should be also grown in M9 minimal medium, although it is not mandatory as far as only a 1% of the volume is inoculated. The procedure is the same than with LB medium, but expression is not induced until OD_{600} reaches ~1.6 with 500µM IPTG and cultures are then incubated for 5-6h at 37ºC (or O/N at 25º).

(c) Auto-inducing media (with or without ^{15}N labeling)

5mL of the O/N grown pre-culture (grown in LB or M9 media) are inoculated into 500mL of sterile auto-inducing medium with antibiotics in a 3L-flask (350-400mL of medium for 2L-flask). The culture is incubated with vigorous shaking at 37ºC for 1-2h and then the temperature is decreased progressively (over 2-4h): 37ºC – 30ºC – 25ºC – 20ºC (– 16ºC). Finally, the culture is grown at 20ºC for 36h (or at 16ºC for 48h).

F.W. Studier has published a thorough analysis of the mechanisms and the parameters influencing the auto-inducing protein expression.[2]

1.3.3. Media composition and stock solutions

LB medium (Luria-Bertani)

For 1L culture:
10g tryptone
5g yeast extract
10g NaCl
adjust pH to 7
> *heat-sterilize*

M9 minimal medium

For 1L culture:
6.8g Na_2HPO_4
3g KH_2PO_4
0.5g NaCl
780mL milliQ H_2O
> *heat-sterilize*
2mL $MgSO_4$ 1M
2mL solution Q
10mL vitamins mix*
20mL ^{13}C-D-glucose 0.2g/mL*
5mL $^{15}NH_4Cl$ 0.2g/mL*

(*sterilized by filtering through a 0.2µm membrane)

ZYM - 5052 auto-inducing medium[2]

For 500mL culture:
5 g tryptone
2.5g yeast extract
475mL milliQ water
> *heat-sterilize*
1mL $MgSO_4$ 1M
1mL solution Q
10mL 50x 5052
10mL 50x M

P-5052 ^{15}N-label auto-inducing medium[3]

For 500mL culture:
450mL milliQ water
> *heat-sterilize*
1mL $MgSO_4$ 1M
1mL solution Q
10mL 50x 5052
25mL 20x ^{15}N-NPS
5mL vitamins

50x 5052:

For 1L: 250g glycerol (99.9%)
25g α-glucose
100g α-lactose
> heat-sterilize

50x M:

For 1L: 177.5g Na_2HPO_4
170.0g KH_2PO_4
133.8g NH_4Cl
35.5g Na_2SO_4
> heat-sterilize

20x ^{15}N-NPS:

For 1L: 142.0g Na_2HPO_4
136.0g KH_2PO_4
50.0g $^{15}NH_4Cl$
14.2g Na_2SO_4
> heat-sterilize

1M $MgSO_4$:

For 100mL, 24.5g $MgSO_4$ x $7H_2O$
> heat-sterilize

1M IPTG

5g IPTG + 12mL milliQ water

> sterilize by 0.2µm filtration. Store at -20ºC

Solution Q (trace metals stock):

For 1L: 8mL HCl 5M
5g $FeCl_2$ x $4H_2O$
184mg $CaCl_2$ x $2H_2O$
64mg H_3BO_3
18mg $CoCl_2$ x $6H_2O$
4mg $CuCl_2$ x $2H_2O$
340mg $ZnCl_2$
605mg Na_2MoO_4 x $2H_2O$
40mg $MnCl_2$ x $4H_2O$
> heat-sterilize

Vitamins mix:

For 100mL: 50mg thiamine hydrochloride
10mg D-biotin
10mg choline chloride
10mg folic acid
10mg niacinamide
10mg D-pantothenic acid
10mg pyridoxal
1mg riboflavin
> sterilize through filtration at 0.2µm
> store at -20ºC protected from light

LB-agar plates

1L LB medium + 15g agar.
> heat-sterilize
Antibiotics are added when the solution is not hot.
20mL are plated per dish and stored at 4ºC.

Antibiotics

	stock	use
Carbenicillin (sodium)	100mg/mL (water)	100µg/mL
Chloramphenicol	34mg/mL (water: EtOH, 1:1)	34µg/mL
Kanamicin (sulfate)	30mg/mL (water)	30µg/mL

> sterilize by filtration through 0.2µm membrane and store at -20ºC

1.4. Protein purification

1.4.1. Purification of p53wt, G334V and R337H (from Mateu *et al.*[1])

Frozen cell pellet is resuspended with 40mM MES pH 6, preferably with some protease inhibitors. The volume of buffer required depends on the amount of pellet. As a rule of thumb, 40-50mL for cell harvests from 1L of non-auto-inducing media and 100-150mL for the auto-inducing harvests. The viscosity of the lysate depends on the expression conditions.

Cells are lysed by tip-sonication (0.7 power) in an ice-bath (in general, 10 cycles of 30s–sonication with 30s–pause are enough for a more fluid lysate, although longer sonication may be required for denser samples). Cell debris are pelleted by centrifugation at 40,000*xg* for 40min at 4ºC and the clarified supernatant is immediately filtered through a 0.2µm membrane, flash-frozen in liquid nitrogen and stored at -80ºC or -20ºC.

Cation exchange purification by FPLC at room temperature.

50-75mL of cell extracts are loaded into a 5mL HiTrap SP-Sepharose column at 2mL/min and extensively washed with 40mM MES pH 6 until A_{280nm}<0.1A.U. Protein is eluted with a 0-0.7M NaCl gradient (0-70% of 40mM MES pH 6, 1M NaCl) in 20cv at 2mL/min, collecting 3mL fractions. Protein elutes at ~200mM NaCl.

Size exclusion by FLPC at room temperature.

Protein fractions are concentrated by ultracentrifugation in 3.5kDa cut-off Amicon centricons (previously the membrane has to be rinsed to remove the glycerol) and fractions of 3-5mL are injected into a Superdex 75 preparative grade 16x80 home-packed column at 1mL/min 40mM MES pH 6, 200mM NaCl, collecting 3mL fractions. Protein elutes at ~100mL.

Desalting by FLPC.

Fractions of 8mL of protein sample are elute with water in a HiPrep Desalting 26/10 column at 6-8mL/min flow rate, collecting 6mL fractions.

Protein fractions are unified and lyophilized. Monomeric protein concentration is determined by UV spectrometry[4] in 25mM phosphate buffer at pH 7 (ε_{280nm}= $1280M^{-1}cm^{-1}$). Molecular weight is determined by MALDI-MS using freshly prepared ACH matrix (10mg/mL, H_2O:ACN, 1:1, 1%TFA). For storage and use, aliquots of 1µmol (monomer) in 1.5mL tubes are prepared and stored lyophilized at -20ºC.

1.4.2. Purification of L344P

Frozen cell pellet is resuspended with 50mM Tris-HCl pH 8, 500mM NaCl, 4M urea (100mL for cell harvests from 1L of culture) and tip-sonicated (0.7 power, 10 cycles of 30s–sonication with 30s–pause, in an ice-bath). Cell debris are pelleted by centrifugation at 40,000*xg* for 40min at 4ºC and the clarified supernatant is immediately filtered through a 0.2µm membrane.

His-tag purification by FPLC at room temperature.

50-75mL of cell extracts are loaded into a 5mL Ni^{2+}-HiTrap Chelating-Sepharose column at 2mL/min. Loaded column is extensively washed, first with ~50mL 50mM Tris-HCl pH 8, 500mM NaCl, 4M urea, and then with >100mL urea-free buffer. His-tagged proteins are eluted with urea-free buffer containing 500mM imidaloze.

TEV protease digestion.

5mL of the His-tag purified protein are diluted with 20mL of 50mM Tris-HCl pH 8 (therefore reducing NaCl to 100mM as well as the imidazole), and there are added 10µL EDTA 500mM, 20µL β-mercaptoethanol and 400µL of fresh TEV enzyme. Digestion is done at 4ºC O/N. Digested protein is analyzed by SDS-PAGE and western-blot developing the His-tag.

Dialysis and clearance.

TEV-digested samples are dialyzed against 50mM Tris-HCl pH 8, 500mM NaCl in 3.5kDa cutt-off membranes to remove imidazole, EDTA, β-mercaptoethanol and some cleaved His-tag fragment (3kDa). Crushed species are removed by centrifuging 5min at 3,000*xg* and filtering through a 0.2µm membrane.

His-tagged species removal by FPLC at room temperature.

The dialyzed and cleared sample is passed through a 1mL Ni^{2+}-HiTrap Chelating-Sepharose column at 1mL/min and the flow-through is collected. The column is further washed with 10mL of 50mM Tris-HCl pH 8, 500mM NaCl.

Desalting by FLPC.

Fractions of 10mL of protein sample are eluted with water in a HiPrep Desalting 26/10 column at 5mL/min flow rate, collecting 3.8mL fractions.

Protein fractions are unified and lyophilized. Monomeric protein concentration is determined by UV spectrometry[4] in 25mM phosphate buffer at pH 7 (ε_{280nm}= 1280$M^{-1}cm^{-1}$) and the molecular weight is checked by MALDI-MS.

1.5. General protocols for molecular biology

1.5.1. Plasmid transformation into competent cells

Competent cells (frozen at -80ºC) are thawed on ice and gently mixed by finger-flicking. 45-100µL of cells are transferred into a pre-chilled sterile 14mL tube (or 1.5mL tube) and ~1-5ng of plasmid (volume <10% than cells) is added and mixed gently by finger-flicking. Mixture is incubated on ice for 30min, pulse heated for 30-45s in a 42ºC water bath (without shaking) and cooled on ice for 2min. 500µL of fresh LB medium (better pre-heated at 37ºC) are added and cells are incubated at 37ºC for >1h shaking vigorously. Aliquots of 50-500µL are plated into LB-agar plates (pre-heated at 37ºC) containing antibiotic, and evenly spread with an sterile spreader until complete absorption.

Plates are incubated upside-down at 37ºC O/N. Plates with grown colonies are sealed with parafilm and stored upside-down at 4ºC (for no longer than a month).

1.5.2. Preparation of competent cells

An aliquot of the *E. coli* strain is inoculated into 3mL LB and incubated O/N at 37ºC (without antibiotics). 500mL LB medium are inoculated with 0.5mL of the grown pre-culture and incubated at 37ºC shaking until OD_{600nm}~0.5. The culture is then chilled for 10min on ice and centrifuged at 4,000*xg* for 10min at 4ºC (in a sterilized centrifuge bottle). Pelleted cells are gently resuspended into 100mL of pre-chilled sterile TB buffer and the cell suspension is incubated on ice for 10min. The culture is centrifuged at 3,000xg for 10min at 4ºC (in sterile 50mL falcon tubes). Pelleted cells are gently resuspended in 18.6mL of pre-chilled TB buffer. 1.4mL of sterile DMSO are added and the cell suspension is incubated on ice for at least 10min. Finally, cell suspension is aliquoted (300μL/tube) and aliquots are fast-frozen in liquid nitrogen and stored at -80ºC.

TB BUFFER: 10mM HEPES pH 6.7, 15mM $CaCl_2$, 55mM $MnCl_2$, 250mM KCl.
Mix all components but the $MnCl_2$ and adjust the pH to 6.7 with KOH. Add then the $MnCl_2$ and sterilize the mixture using 0.22μm filters.

1.5.3 Agarose gel electrophoresis for DNA analysis

Plasmidic DNA is usually analyzed in 1% (w/v) agarose gels in TAE buffer.

The agarose solution is boiled to complete solution, and cooled for a minute before adding 0.01% (v/v) ethidium bromide (for DNA staining). The gel is polymerized into the mold (for ~30min-1h) and then covered with TAE buffer before loading the DNA samples (which are solved into loading dye buffer). Electrophoresis is run at 80-100V for 1h. DNA bands are observed under UV light.

LOADING DYE BUFFER 6x:

3mL glycerol 99.9%
3mL 0.5M EDTA pH 8.0
3mg bromophenol blue
3mg xylene cyanol
4mL sterile milliQ water

TAE BUFFER 50x:

For 1L (pH 7.6): 242.2g TRIZMA® base
18.6g EDTA
57mL acetic acid

> *heat-sterilize*

1.5.4 SDS-PAGE (SDS - PolyAcrylamide Gel Electrophoresis)

For SDS-PAGE analysis of p53TD, 15% poly-acrylamide gels with 10% glycerol have been used, since they allow better detection and resolution of low molecular weight species.

Gel composition (for 2 gels of 0.75mm thickness):

	resolving	stacker
Acryl:bisacrylamide (37.5:1)	3.6 mL	0.9 mL
Tris-HCl 3M pH 8	3.0 mL	1.8 mL
water	1.2 mL	4.7 mL
SDS 20%	40 μL	75 μL
glycerol 87%	1.2 mL	-
APS 15% (w/V)	40 μL	30 μL
TEMED	6 μL	6 μL

Protein samples are mixed 1:1 with loading buffer 2x and denatured by heating 5min at 95ºC.

The gel is pre-run in electrophoresis buffer for 15min at 100V in order to remove stacker gel residues from the wells. Samples are then loaded and the gel is run at 120-140V for 2h.

Before Coomassie staining for 1h, proteins are fixed in 40% methanol + 10% acetic acid for 20min. Protein bands are made visible by distaining with 10% acetic acid. Other environmentally-safer stainers can be also used, but Coomassie blue is preferred because the brightness and clearness of the protein bands. Staining and distaining can be accelerated by heating in the microwave.

LOADING BUFFER 2x:

250μL Tris-HCl 0.5M pH 6.8
2mL glycerol 87%
250μL water
4mL SDS 10%
1mL bromophenol blue 0.4% (w/v)

RUNNING BUFFER 5x:

For 1L (pH 8.3-8.8): 15.14g TRIZMA® base
72.07g glycine
5g SDS

DYING:

For 1L: 0.25g Coomassie® blue G250
100mL AcOH

1.5.5 Western blot

A standard SDS-PAGE is initially run and the resolved gel is rinsed in blotting buffer. Proteins are transferred to the nitrocellulose membrane at 300mA for 1h in pre-chilled blotting buffer (proteins run from negative to positive). The nitrocellulose is rinsed with PBST (Ponceau red can be used to check for the transfection at this point) and then it is blocked with defatted powdered milk (10% w/v) for 1h. Before incubating with the primary antibody (against His-tag) for 1h, the membrane is

toughly rinsed with PBST (5×5min). And before incubating with the secondary antibody during 1h, the membrane is again toughly washed with PBST (5×5min). Finally, the secondary antibody is developed by luminescence with the Amersham ECL™ kit.

BLOTTING BUFFER:
For 1L (pH 8.3): 6.0g TRIZMA® base
28.8g glycine
400mL methanol

PBST:
For 1L (pH 7.4): 0.23g KH_2PO_4
0.74g Na_2HPO_4
8.7g NaCl
+ 0.5mL Tween 20

1.5.6. TEV-enzyme expression and purification

The His-tagged TEV enzyme is cloned into a plasmid with resistance to kanamicin (EMBL).
The TEV enzymes cleaves itself; hence, so for efficient protease activity the enzyme has to be fresh.
BL21(DE3)pLys *E. coli* are used for protein expression in auto-inducing media at 16ºC for 48h.
The purification procedure is the standard for a His-tagged protein in Ni^{2+}-chelating sepharose, using the following buffers:

- *lysis and loading buffer*: 50mM Tris pH 8, 500mM NaCl, 10% glycerol and protease inhibitors.

- *elution buffe*r: 50mM Tris pH 8, 100mM NaCl, 10% glycerol, 500mM imidazol

The elution buffer is exchanged to 50mM Tris pH 8, 200mM NaCl, 20mM DTT, 4mM EDTA, 10% glycerol (through a HiPrep Desalting 26/10 column). It is then diluted with glycerol up to 50% (of final glycerol), split in single-use aliquots, flash-frozen in liquid nitrogen and stored at -20ºC.

MATERIALS AND REAGENTS

KITS

QuickChange® Site-Directed Mutagenesis Kit	Stratagene
QIAprep Spin Miniprep/Maxiprep Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
Amersham ECL™ Western Blotting Detection	GE Healthcare*

CHEMICALS

> Salts and reagents (molecular biology grade)	Sigma-Aldrich
Acry-bisacrylamide (37.5:1)	Amresco
Agar	Conda Laboratories
Agarose (electrophoresis grade)	Roche
Antibiotics	Duchefa
Antibodies (western-blot)	GE Healthcare
APS (electrophoresis grade)	Sigma
Coomassie	BioRad
DMSO	Panreac
DNA molecular weight ladder	Roche
ethidium bromide (electrophoresis grade)	Sigma
glycerol 87%	Merck
Isotope labeled reagents	Cambridge Isotope Laboratories
LB premix	Conda Laboratories
Nitrocelulose membrane	Whatman
Oligonucleotide primers	MWG-Biotech AG
Protease inhibitors cocktail (without EDTA)	Roche
SDS-PAGE molecular weigh ladders	BioRad
TEMED (electrophoresis grade)	Sigma
Tween 20	Pharmacia Biotech
T4 DNA ligase	Roche
T7 primer	Promega

SOLVENTS

AcOH	SDS
EtOH	Panreac
HCl	Scharlau
MeOH	SDS
milliQ water (resistivity $>18 M\Omega \cdot cm^{-1}$)	MilliQ Plus filtration system (Millipore)

MATERIALS

Amicon centricones 3.5kDa	Millipore
Centrifuge polypropylene hermetic tubes	Beckman Coulter
HiPrep Desalting 26/10 column	Amersham Bioscience*
HiTrap Chelating-Sepharose column	GE Healthcare*
HiTrap SP-Sepharose column	GE Healthcare*
Quartz cells	Hellman
Slide-A-Lyzer® 3.5K 3-12mL Dialysis Cassettes	Pierce
Superdex 75 preparative grade	Amersham Bioscience*

INSTRUMENTATION

Electrophoresis cells	Mini-protean® BioRad (SDS-PAGE)
	BioRad (agarose)
Centrifuges	Beckman Coulter, rotors (J8-1000, J25-50)
	Eppendorf 5415R benchtop centrifuge
Sonicator	IKASONIC U200-S, IKA Labortechnik
FPLC	ÄKTA Explorer, Amersham Bioscience*
pH meter	Crison GLP21
Thermocycler	MiniCycler, MJ Research
UV-Vis spectrometer	Eppendorf UV Biophotometer

WEB-RESOURCES

Mutagenic primers designer	http://www.bioinformatics.org/primerx/
Primers melting temperature calculator	http://insilico.ehu.es/tm.php?formula=show
Sequence aligment:	http://clustalw.genome.jp/
	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
Protein physico-chemical parameters	http://www.expasy.org/tools/protparam.html
Protein Data Bank	http://www.rcsb.org/pdb/home/home.do
p53 main web-sites	http://www-p53.iarc.fr/index.html
	http://p53.bii.a-star.edu.sg/index.php

*GE Heathcare is the new brand for the formerly known Amersham Bioscience.

EXPERIMENTAL 2

Protein Chemical Synthesis

2.1. Solid Phase Peptide Synthesis

The synthesis of the p53 tetramerization domain (residues 320-356) and the mutant L344P (residues 311-367) was carried out automatically under the following conditions:

p53TD(320-356)	
Sequence	Ac-KKPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAG-NH_2
Chemistry	Fmoc / ^{t}Bu
Polymeric support	aminomethyl-ChemMatrix® (~35-200mesh, wet)
Functionalization	0.45 mmol/g
Linker	Rink-amide
Scale	0.09 mmols
Solvent	DMF
Amino acid excess	11eq Fmoc-AA (side-chain standard protecting groups)
Coupling reagents	11eq [HCTU + Cl-HOBt] + 22eq DIEA
Coupling solvent	NMP + DMF (70:30, v/v)
Coupling time	20min
Deprotection	piperidine (+0.3% Triton 100x)
Double coupling	none

L344P(311-367)	
Sequence	H-NTSSSPQPKKKPLDGEYFTLQIRGRERFEMFREPNEALELK DAQAGKEPGSRAHSS-OH
Chemistry	Fmoc / ^{t}Bu
Polymeric support	ChemMatrix® (~35-100mesh, wet)
Functionalization	0.38 mmol/g
Linker	PAL (already linked)
Scale	0.05 mmols
Solvent	DMF
Amino acid excess	11eq Fmoc-AA (side-chain standard protecting groups)
Coupling reagents	20eq [HCTU + Cl-HOBt] + 40eq DIEA
Coupling solvent	NMP + DMF (70:30, v/v)
Coupling time	20min
Deprotection	piperidine (+0.3% Triton 100x)
Double coupling	P24, F40, K49, S55 (numeration according to the coupling order)

All the experimental conditions detailed below only refer to p53TD(320-356). L344P synthesis was almost the same although it finally failed.

Technical synthetic notes:

- ChemMatrix® resin initial conditioning:

  For a proper performance of the ChemMatrix® polymeric support (100% PEG), it must be washed as follows:

1.	MeOH	5 × 1min
2.	DMF	5 × 1min
3.	DCM	5 × 1min
4.	DCM + 5% TFA	5 × 1min
5.	DCM + 5% DIEA	5 × 1min
6.	DCM	5 × 1min
7.	DMF	5 × 1min

- Rink-amide linker was coupled by hand, with 10eq of Fmoc-Rink-amide linker, 10eq PyBOP + 10eq HOBt and 20eq DIEA, in DMF, shaking vigorously for 1h. After washing with DMF (5×1min) and DCM (5×1min), the ninhydrin test[5] was performed and it resulted positive. Non-reacted aminomethyl groups were capped with 50eq Ac_2O and 50eq DIEA for 25min (resin swelled in DMF). Ninhidrine tests after the capping reaction was still slightly positive (ninhydrin solutions were not fine, though).

- Before transfer the resin to the synthesizer vessel, the Fmoc-protecting group of the Rink linker was partially removed by washing twice for 5min with piperidine:DMF (20:80, v/v). Once in the reactor, the automatic synthesis was started by washing with DCM, DMF and deprotecting with piperidine.

- Synthesizer stock solutions:

  2M DIEA in NMP (freshly prepared)

  Piperidine

  0.45M HCTU + 0.45M Cl-HOBt in NMP:DMF, 2:1, v/v (freshly prepared and light protected)

- Final Fmoc-deprotection of the peptidyl-resin was done by hand (DBU:piperidine:toluene:DMF, 5:5:20:70, v/v, 1x1min, 2x10min) and free N-termini were capped with 50eq Ac_2O and 50eq DIEA for 25min (the final ninhydrin test was negative).

2.2 Peptide cleavage and side-chain deprotection

(a) Cleavage mini-tests

An aliquot of peptidyl-resin (*i.e.* some beads) was taken into a 1.5mL tube and treated with 1mL of TFA-scavengers cocktail. Two were checked: reagent K and TFA:H_2O:TIS:EDT (94:2.5:2.5:1, v/v), for times ranging from 2 to 4h, shaking vigorously, at room temperature. Resin beads were removed by filtering the acidic sample trough glass wool and TFA was then evaporated under nitrogen flow. The cleaved products were extracted with chilled ether (3×1mL diethyl eter, centrifuging at 10,000*xg* for 3min at 4ºC to pellet the peptide). Final ether-insoluble products

were dried under nitrogen, solved in H_2O:ACN (~1:1) and lyophilized before analysis by HPLC and MALDI-MS.

The best cleavage and deprotection conditions resulted from a 2.5h treatment with reagent K.

(b) Large scale cleavage

For precaution, only one half of the peptidyl-resin was treated with reagent K (freshly prepared, TFA:H_2O: thioanisole:ethanedithiol:phenol, 85:5:5:2.5:2.5) for 2.5h, shaking at 280rpm. Because ChemMatrix® resin swells extremely well in TFA, a large volume of reagent K had to be used (10mL for ~500mg of peptidyl-resin). Beads were removed from the cleaved products by filtration and then rinsed with AcOH (2×2min). Acids were evaporated under a nitrogen flow and the remaining products were extracted with chilled ether (40mL+30mL+20mL diethyl eter, centrifuging at 4,000*xg* for 10min at 4ºC after each extraction). The final peptide pellet was dried under nitrogen and products were solved in H_2O:ACN:AcOH (~6:3:1); sonication helped to completely solubilized the crushed peptide. ACN and AcOH were partially removed in the rotavapor and crude was lyophilized.

The synthesis crude was analyzed by HPLC (in C18 and C4 columns) and the molecular weight was confirmed by both HPLC-MS and MALDI-MS (in freshly prepared ACH matrix, 10mg/mL, H_2O:ACN, 1:1, 1%TFA).

2.3. Peptide purification

The purification was carried out by semi-preparative HPLC, in a reverse-phase C8 column. Peptide crude was solved in H_2O:ACN:AcOH (~7:2:1) and injected in fractions of 4-7mL (~10mg). Working at a flow rate of 15mL/min, the gradient used was:

time (min)	0	5	35	40	45
%ACN	0	10	28	30	100

Protein peak appeared at ~34min, and fractions were collected manually.

The purity of the final product, determined by analytical RP-HPLC (C4 column), was >98%.

Once lyophilized the peptide was quantified by UV (ε_{280nm}= 1280$M^{-1}cm^{-1}$ in 25mM phosphate buffer pH 7.0). The total yield of the synthesis could not be calculated because not all the crude was purified. Approximately, from ~120mg of raw wet crude, ~20mg of pure product were obtained.

MATERIALS AND REAGENTS

PEPTIDE SYNTHESIS

ChemMatrix® resin	Matrix Innovation Inc.
Fmoc-AA	IRIS Biothech
PyBOP	IRIS Biothech
HOBt	IRIS Biothech
HCTU	Lonza
Cl-HOBt	Lonza
Fmoc-Rink-amide linker	IRIS Biothech

SOLVENTS AND GENERAL CHEMICALS

Ac_2O	Aldrich
ACH	Fluka
ACN	SDS
AcOH	SDS
DBU	Fluka
DCM	SDS
DIEA	Merk
diethyl ether	SDS
DMF	SDS
EDT	Fluka
HCl	Scharlau
MeOH	sds
milliQ water (>18MΩ·cm^{-1})	MilliQ Plus filtration (Millipore)
NMP	IRIS Biothech
phenol	Fluka
Piperidine	SDS
TFA (HPLC grade)	Fluorochem
TFA (synthesis grade)	Fluorochem
TIS	Fluka
others	Merk, Sigma or Fluka

INSTRUMENTATION

Automatic peptide synthesizer*	AB433A Applied Biosystems
Analytic HPLC-PDA	WATERS Alliance 2695 photodiode array 996 UV/Vis detector automatic sampler solvents: H_2O 0.045% TFA ACN 0.036%TFA
Analytic HPLC-Breeze	WATERS binary pump 1525 dual 2487 UV detector 717 Plus autosampler solvents: H_2O + 0.045% TFA ACN + 0.036%TFA
Analytic HPLC-MS	WATERS AllianceHT 2795, dual 2487 UV detector and Micromass ZQ detector automatic sampler solvents: H_2O + 0.1% formic acid ACN + 0.07% formic acid
Semipreparative HPLC	WATERS Delta 600 dual 2487 UV detector sample Manager 2700
MALDI-TOF/TOF**	Applied Biosystems 4700, proteomics analyzer
Centrifuges	Beckman Coulter Allegra 21R Eppendorf 5415R benchtop centrifuge
Spectrometer	Eppendorf UV Biophotometer

** Unitat de Síntesi de Pèptids, Severis Científico-Tècnics de la Universitat de Barcelona, PCB*

*** Unitat de Proteòmica, Severis Científico-Tècnics de la Universitat de Barcelona, PCB*

EXPERIMENTAL

3

Biophysics

3.1. Nuclear Magnetic Resonance

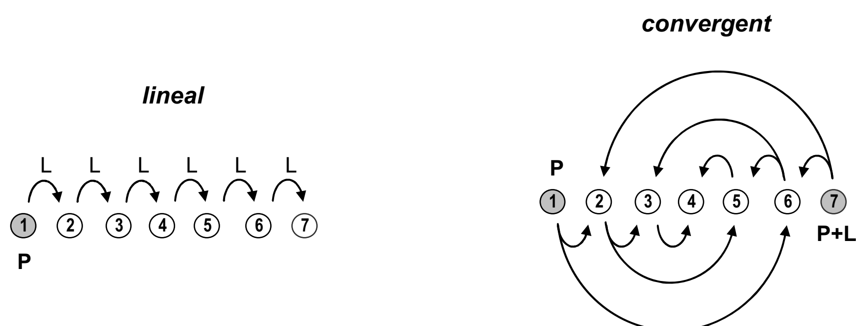
3.1.1. ^1H-^{15}N-HSQC

Samples of 100-125μM (tetramer) ^{15}N-protein were prepared in H_2O:D_2O (9:1) adjusting pH with micro-volumes of 0.1M-0.1mM NaOH and HCl.

Two different strategies of titration were followed (depending on the equipment probe):

i. *Additive lineal titration*: the ligand to be added at each point was lyophilized and the protein sample itself was used to solve it. pH was readjusted at each point (with volumes <5μL). For 5mm tubes, 600μL of sample were prepared, whereas for 3mm only 200μL.

ii. *Convergent titration*: the first and the last samples of the titration were prepared and intermediate points resulted from mixing the former ones. In this way, the protein concentration was kept totally constant along the titration and the ligand excess was perfectly known. pH was also preserved when mixing the samples. Despite the many advantages of this strategy, it is only feasible and affordable for small amounts of labeled protein and/or precious ligand (*i.e.* if 3mm tubes are used, where 180μL are enough).



In both cases, final samples were recovered, flash-frozen in liquid nitrogen and store at -20ºC. **Table 1** summarizes the titrations followed by HSQC presented in the manuscript.

All the HSQC experiments were recorded at 298K in a Bruker Digital Advance 600MHz spectrometer equipped either with a triple resonance TXI 5mm probe with gradients on X, Y and Z or with a triple resonance TCI cryoprobe, acquiring 2048x256 complex points with a total of 8 scans. Both HSQC-sensitivity enhancement (HSQC-SE) and fast-HSQC (fHSQC) pulse sequences were used.

NMRPipe–NMRDraw package software[6] (for Linux) was used for processing HSQC data and NMRViewJ (for Windows) for spectra analysis.
Mathematical adjustment of the titration curves was performed by minimum least squares using Origin 7.0.

Table 1. ^{1}H-^{15}N-HSQC titrations

ligand	$[^{15}N\text{-protein}]_{TET}$	titration points (eq to tetramer)	strategy	experiment
calix4bridge	125µM p53wt	1 - 2 - 3 - 4 - 6 - 8 - 12 - 16 - 20 - 28	linial ©	fHSQC
	125µM R337H	1 - 2 - 3 - 4 - 6 - 8 - 12 - 16 - 20 - 28	linial ©	fHSQC
	125µM G334V	1 - 2 - 4 - 6 - 8 - 12 - 16 - 24	converg ©	fHSQC
	125µM L344P	8 - 16	linial ©	HSQC-SE
NH_2-calix4bridge	125µM p53wt	0.4 - 0.8 - 2 - 3 - 4 - 5 - 6 - 8 - 12 - 16 - 24	linial	fHSQC
	125µM R337H	0.4 - 0.8 - 2 - 3 - 4 - 5 - 6 - 8 - 12 - 16 - 24	linial	fHSQC
	100µM G334V	4 - 8 - 12 - 16	converg ©	fHSQC
calix4prop	125µM p53wt	0.2 - 0.4 - 0.8 - 1.6 - 2.8 - 4 - 8 - 8.8 - 10	linial	fHSQC
	125µM R337H	0.2 - 0.4 - 0.8 - 1.5 - 2.7 - 4.5 - 5.3 - 6.3 - 8	linial	fHSQC
	125µM R337H (pH 5)	0.4 - 0.8 - 1.6 - 2.4 - 3.2 - 4 - 4.8 - 6.8 - 8 - 10	linial	HSQC-SE
	125µM R337H (pH 9)	0.2 - 0.4 - 0.8 - 1.6 - 2.4 - 3.2 - 4 - 4.8 - 6	linial	HSQC-SE
	125µM G334V	0.2 - 0.4 - 0.8 - 1.6 - 2.8 - 4 - 5.2 - 6.4 - 8	converg ©	fHSQC
	125µM L344P	4 - 8	linial ©	HSQC-SE
NH_2-calix4bridge	100µM p53wt	0.8 - 1.6 - 2.8 - 4 - 5.2 - 6.4 - 8 - 10 - 12 - 14 - 16	linial	fHSQC
	100µM R337H	0.8 - 1.6 - 2.8 - 4 - 5.2 - 6.4 - 8 - 10 - 12 - 14 - 16	linial	fHSQC
	100µM G334V	0.8 - 1.6 - 2.8 - 4 - 6 - 8 - 10 - 12 - 14 - 16	converg ©	fHSQC
4G4Pr-cone	100µM p53wt	4 - 8 - 16	linial	fHSQC
	100µM R337H	4 - 8 - 16	linial	fHSQC

© *cryoprobe*

3.1.2. ^{1}H-STD

All the ^{1}H-STD experiments were recorded in a Bruker Digital Advance 600MHz spectrometer equipped with a triple resonance TXI 5mm probe and gradients on X, Y and Z, using the pulse sequence *sdtdiff.3* (from the manufacturer) with 2048 scans. Protein was selectively irradiated by a 50ms Gaussian shaped pulsed at 0.72ppm (off-resonance set to *ca.* 30ppm) and a 20ms spin-lock pulse was used to eliminate residual protein resonances. No water-suppression sequences were applied and therefore the HDO band should be minimal.

Samples were solved in large volumes of D_2O and lyophilized (at least) twice before recoding the spectra. Once extensively lyophilized, they were stored under dry atmosphere and not prepared in "100" D_2O until the very same moment of doing the experiment. The 5mm NMR tube was additionally purged with nitrogen (before and after) and sealed with parafilm.

One-dimensional spectra were processed and analyzed with MestReC software. Mathematical adjustment of the experimental data was done by minimum least squares in Origin 7.0.

(a) STD titration:

Ligand titration was performed in the convergent manner. Initial samples contained:

> ~4µM (tetramer) p53wt + 73µM calix4bridge

> ~4µM (tetramer) p53wt + 1.5mM calix4bridge

And they were mixed as follow:

	[calix4bridge] (µM)							
	73	**147**	**292**	**876**	**1167**	**1485**	**[L] (µM)**	**L/P ratio**
composition (µL)							73	20
	947					53	147	40
		730			120		292	80
			450	150			437	120
			300	300			584	160
		170			430		876	240
	147					553	1167	320
							1485	400

Titration was carried out at once, recording spectra at 283K with 3s saturation time.

(b) STD build-up curves

For obtaining the STD build-up curves, ^{1}H-STD spectra were recorded from a single sample at varying saturation times (randomly ordered):

> 1mM calix4bridge + 12.5µM tetramer p53wt (at 288K): 3, 2, 1.5, 1, 0.7, 0.5, 0.3 and 0.2s.

> 1mM NH_2-calix4bridge + 16µM tetramer p53wt (at 283K): 3, 2, 1.5, 1, 0.6 and 0.3s.

3.1.3. Longitudinal relaxation time measurement

T1 for each proton of the ligand was determined by the inversion recovery experiment, recording a ^{1}H spectrum (16k points, 8 scans) with 16 different randomly ordered relaxation delays (10, 0.01, 5, 0.2, 2.5, 0.1, 4, 0.25, 3, 0.5, 2, 0.7, 1, 0.4, 1.5 and 10s).

The samples for these experiments were prepared in "100" D_2O and contained:

> 1mM sample of free ligand (at 288K)

> 1mM calix4bridge + 12.5µM tetramer p53wt (at 288K)

> 1mM NH_2-calix4bridge + 16µM tetramer p53wt (at 283K)

Data processing was performed with XWINNMR software.

3.1.4. NOESY & ROESY

NOESY and ROESY experiments were recorded in a Bruker DMX 500MHz spectrometer equipped with a triple resonance TXI 5mm probe and gradients on X, Y and Z, acquiring a total of 2048x512 complex points with 32 transients per increment. For free ligand samples, the mixing time was set to 500ms while for ligand-protein samples it was set to 100ms. Pulse sequence without water suppression were used (*noesy-tppi* and *roesy-tppi*), thus samples had to be prepared in "100" D_2O (as described for the STDs).

Data processing, analysis and visualization were performed with MestReC software.

3.1.5. Protein backbone assignation

R337H:

Assignation experiments were carried out in a Bruker Digital Avance 800MHz spectrometer equipped with a triple resonance TXI 5mm probe and gradients on X, Y and Z, at 308K.
Sample: 1.5mM ^{13}C-^{15}N-R337H in 25mM sodium phosphate buffer pH 5, with 10% D_2O, 0.02% NaN_3 and 0.2mM DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt, standard for 0ppm).

G334V:

Assignation experiments were carried out in a Bruker Digital Avance 800MHz spectrometer equipped with a triple resonance TCI cryoprobe, at 298K.
Sample: 1.1mM ^{13}C-^{15}N-G334V in 25mM phosphate buffer pH 7, with 10% D_2O, 0.02% NaN_3 and 0.2mM DSS.

Table 2 summarizes the performed experiments. NMRPipe–NMRDraw software[6] (Linux) was used for processing the multi-dimensional data and NMRViewJ (Windows) for spectra analysis.

Table 2. Assignation experiments

experiment	pulse sequence	NS	$^{1}H \times {}^{15}N \times {}^{13}C$	R337H	G334V
^{1}H-^{15}N-HSQC	fhsqcf3gpph	32	2048 x 256	✓	✓
HNCO	hncogpwg3d	8	2048 x 80 x 84	✓	
CBCA(CO)NH	cbcaconhgpwg3d	32	2048 x 80 x 104	✓	✓
CBCANH	cbcanhgpwg3d	32	2048 x 80 x 104	✓	✓
HNCA	hncagpwg3d	16	2048 x 80 x 100	✓	
HN(CO)CA	hncocagpwg3d	16	2048 x 80 x 100	✓	

3.2. Differential Scanning Calorimetry

DSC experiments were recorded in a Microcal VP-DSC unit, scanning from 10ºC to 120ºC, at 30ºC$\cdot h^{-1}$ and a constant pressure of 2atm. Samples were degassed under vacuum at 18ºC for 30min before filling the calorimeter cell; reference cell was filled with the corresponding degassed sample containing everything but the protein. Samples were rescanned to assess reversibility. Between protein samples, cells were thoroughly washed (with a vacuum pump) and a buffer blank was recorded before loading the sample (thus assessing the stability of the baseline along time).

A volume of 1mL was prepared to easily fill the microcalorimeter cell (~0.55mL).

pH titration experiments:

Sample: R337H at 172μM and 50μM (monomer) in 25mM phosphate buffer pH 5-9.
Reference: phosphate buffer (pH 5-9).

Ligand titrations:

Sample: protein at 100μM (monomer) and the corresponding ligand concentration, in water at pH 7.0 (adjusted with HCl or NaOH). Samples with the highest ligand excess were repeated to prove reproducibility.
Reference: samples with the corresponding ligand concentration, in water at pH 7.0.

Despite that for good quality data (and reproducibility) it is essential the perfect matching of buffers between the sample and the reference (and thus samples are usually dialyzed to ensure the perfect sameness), in the samples with ligand it was impossible to satisfy this condition.

The only "successful" DSC thermogram for mutant G334V was recorded in a Microcal MCS-DSC calorimeter, at a concentration of 50μM (monomer) in 25mM phosphate buffer pH 7.0, scanning from 20ºC to 95ºC at 90ºC$\cdot h^{-1}$. Complete removal of crushed protein in the sample cell was achieved by washing at 60ºC with 0.5% SDS (~250mL), followed by extensive rinsing with water (~1L) and a water blank scan.

Experimental data were processed and analyzed with Microcal-DSC Origin7.0 software. Heat capacity was normalized to the concentration of monomer[4] (although for the mathematical adjustments, it was used the tetramer concentration). The buffer baseline was subtracted to the recorded thermogram. The excess heat capacity function for the unfolding transition, $\langle C_P^{tr} \rangle$, was obtained by subtracting a progressive baseline traced between the native and the unfolded states. The area under the $\langle C_P^{tr} \rangle$ curve is the experimental unfolding enthalpy, ΔH_m. The area under the $\Delta H_m/T$ curve (T in Kelvin) provides the experimental unfolding entropy, ΔS_m

3.3. Isothermal Titration Calorimetry

Isothermal titrations were performed in a Microcal MSC-ITC unit equilibrated at 25ºC (room temperature set at 20ºC). Samples of protein and ligand (Tables 3 and 4) were prepared in water at pH 7.0 (adjusted with micro-volumes of HCl and NaOH). A total of 2.1mL of protein was prepared to easily fill the ~1.5mL cell. The ligand was prepared in a large volume stock (counting ~350µL per titration). Ideally, titrated and titrating solutions should be dialyzed towards the same buffer for perfect matching; unfortunately, it was impossible due to the the low molecular weight of the ligand (and the water media).

Injections were done at 400s intervals on a sample stirred at 270rpm and heat data were recorded with an offset of 15% at 2s filtering. The initial 1µL injection was discarded for all the experiments.

Data were analyzed by Microcal-ITC Origin7.0 software. The baseline for the integration of the injections was traced manually (repeated several times to mean errors) and heat data was normalized considering tetrameric protein concentration. The ligand dilution heat (recorded from a titration over water) was subtracted

Table 3. ITC with calix4bridge

$[protein]_{TET}$	[calix4bridge]	injections
50µM p53wt	5mM (batch #1)	1µL - 10x4µL - 16x15µL
100µM R337H	5mM (batch #1)	1µL - 10x4µL - 16x15µL
125µM G334V	6.5mM (batch #2)	1µL - 9x4µL - 4x8µL - 15x15µL
100µM L344P	6.5mM (batch #2)	1µL - 9x4µL - 4x8µL - 15x15µL

Table 4. ITC with calix4prop

$[protein]_{TET}$	[calix4prop]	injections
50µM p53wt	5mM (batch #1)	1µL - 10x4µL - 16x15µL
50µM R337H	5mM (batch #1)	1µL - 21x4µL - 7x15µL
50µM G334V	5mM (batch #2)	1µL - 5x4µL - 4x6µL - 5x8µL - 2x15µL - 4x10µL - 8µL - 2x10µL - 7x15µL
50µM L344P	5mM (batch #2)	1µL - 5x4µL - 4x6µL - 5x8µL - 2x15µL - 4x10µL - 8µL - 2x10µL - 4x15µL

3.4. Circular Dichroism

All circular dichroism experiments were recorded in a Jasco J-810 spectropolarimeter, equipped with a Jasco-CDF-426S Peltier thermostatted cell holder and a Julabo external bath.

(a) CD spectra

In general, far UV-CD spectra were the average of 3 scans recorded at a scanning rate of $10nm\cdot min^{-1}$, with 4s response time, 1nm bandwidth and 0.1nm data pitch. For samples with low ellipticity signal more scans were accumulated (at faster scanning rate). Square quartz cells of either 10mm (~450µL) or 1mm (~300µL) path length were used, keeping the HT voltage below 700mV. Black spectra were also recorded (with everything but the protein).
The spectra were processed with the software provided by the manufacturer (Spectra Manager). The smoothed blank baseline was subtracted to the raw spectrum and CD ellipticity was normalized to the mean residue concentration (monomer concentration$\times$number of amino acids), θ_{MR}. Spectra were then smoothed by Savitsly-Golay algorithm (25 points window), carefully checking the goodness by comparison with the raw data after each smoothing cycle.

(b) Proteins time stability by CD spectra

A 2mL sample containing 10µM of protein (monomer) –in the corresponding buffer– was prepared and split into 300µL aliquots that were incubated at 37ºC in an aluminum block. For each measurement a single aliquot was taken (and then it was not recovered). Once every day samples were gently shake in order to recover the water condensed in the cap of the tube.

(c) CD unfolding curves

CD unfolding curves were recorded measuring the CD ellipticity at 220nm while heating from 15ºC to 95ºC at $1.5ºC\ min^{-1}$ with 4s response time, 1nm bandwidth and 0.1ºC data pitch. Square quartz cells of either 10mm (~650µL) or 1mm (~400µL) path length were used, completely filled with sample and cap sealed.
Recorded data were processed in Spectra Manager software, normalizing concentration and smoothing by the binomial method. For some samples, the initial and final baseline slopes were also corrected by subtraction of an sloped straight line. Data were then exported to Origin 7.0 software and transformed into the normalized unfolded fraction curve (assuming a two-state unfolding model).

(d) Cell cleaning

In general, for measurements at room temperature, cell was rinsed with water and methanol, and then dried with a vacuum pump. For thorough cleaning, Hellmanex®II was used. In those samples of G334V where the protein aggregates got stuck into the walls, a 10min treatment with chromic mixture was required for a complete cleaning. For the later cleaning conditions, the cell was then extensively washed with water followed by some methanol.

3.5. Chemical cross-linking

Chemical cross-linking reactions were carried out by incubating a sample of 100µM (monomer) protein for 20min at 37ºC with either 0.1% glutaraldehyde or [20mM EDC + 5mM NHS], in the presence of 30% of glycerol. The reactions were stopped by adding SDS-PAGE loading buffer, and cross-linked products (10µL) were analyzed in a 15% acrylamide - 10% glycerol gel (see section 1.5.3). Gels were scanned and bands intensity was analyzed with the ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Preparation for protein-calixarene samples (20µL volume):
2µL of protein 1mM (monomer) and 9.8µL of the calixarene (in water) were incubated 10min at room temperature. Then, 6.2µL of glycerol 87% were added and gently mixed, and the samples were incubated 10min more. Finally, 2µL of cross-linker 10x (*i.e.* 1% gluteraldehyde or 200mM EDC + 50mM NHS) were mixed and reactions were carried out for 20min at 37ºC. The reaction was stopped by adding 20µL SDS-PAGE loading buffer 2x.

3.6. EMSA

DNA binding reactions were performed in a total volume of 15µL containing 10µL of 20mM Tris-HCl pH8, 2µL of plasmid (~1µg pEGFP-CI) and 3µL of calixarene at different concentrations. Samples were incubated for 1h at room temperature. Before loading the 1% agarose gel, 5µL of glycerol 87% and 2µL of blue loading buffer 6x were added to the DNA sample. Instead of TAE, the buffer used for preparing and running the gel was TA (40mM Tris-acetate). EDTA was omitted because the possible competition for the DNA.[7] For the same reason, ethidium bromide was minimized to 0.005% (v/v).
See section 1.5.5 for the agarose gel electrophoresis protocol.

3.7. ElectroSpray Ionization – Mass Spectrometry

Protein samples were extensively ultracentrifuged in Microcon YM-3 devices (3kDa cut-off, 0.5mL volume, rinsed previously to remove the glycerol) with >3 volumes of milliQ water MS-grade in order to minimize the presence of salts (although proteins were purified in water). Little aliquots of 5nmols were lyophilized in 1.5mL Eppendorf® tubes[e], sealed and stored at -20ºC. Thus, for each experiment a fresh sample would be used.
Conversely, calixarene ligands could not be desalted. They were solved in milliQ water MS-grade, aliquoted and stored at -20ºC.

ESI-MS measurements were kindly performed by Dr. Marta Vilaseca from the Mass Spectrometry Core Facility at the IRB-Barcelona (PCB), in a Synapt HDMS mass spectrometer (Waters) equipped with a NanoMate automated nanoelectrospry sample dispenser (Advion BioSciences)

Proteins were solved in 10mM ammonium acetate pH 7.0 (a volatile salt) at a final concentration of 50µM (monomer). The concentrate stocks of calixarene ligands were also prepared in 10mM ammonium acetate. Protein-ligand samples were preincubated for at least 5min before performing the MS experiment.

Samples were introduced into the mass spectrometer by the NanoMate, which sequentially aspirated the samples from a 384-well plate with disposable conductive pipette tips and infused the samples through the ESI Chip, consistent of 400 nozzles in a 20 x20 array. Spray voltage was set to 1.7 kV and delivery pressure at 0.3psi.

The mass detection was carried out in positive mode at the source, at 80ºC. Specific parameters for the detection were the following:

Sampling cone: 70
Source temperature: 80 ºC
Trap Collision Energy: 10
Transfer Collision Energy: 10
Trap Gas Flow: 1.5 ml/min
IMS Gas Flow: 32 ml/min
IMS Wave Velocity: 300
IMS variable Wave Height: Start 8V-End 10V
Ion cooling: 5.69e0 mbar (Backing)
m/z range: 900 to 5000

[e] they must be from Eppendorf™; low-quality cheap tubes could contaminate samples with plastic components

3.8. X-ray crystallography

Crystallization experiments were carried out in the laboratory of Prof. Ignasi Fita at the IRB Barcelona (PCB).

Protein crystals were obtained by handing-drop vapor diffusion crystallization using two different conditions reported in the literature:

i. 3M sodium formate, 0.5M ammonium sulfate, 50mM Tris-HCl pH 8-9 (Jeffrey *et al.*[8])

ii. 1M sodium citrate, 100mM HEPES pH 8.5 (Mittl *et al.*[9])

The drop was formed by 1µL of buffer + 1µL of synthetic p53TD at 10mg/mL (solved in water) and then suspended from a glass coverslip over the reservoir solution containing 1mL of buffer. Protein was aliquoted and lyophilized in fractions of 20µg, which were solved in water at room temperature just before seeding the drop. Protein solution could not be cooled, otherwise it did not crystallize.
Crystals were readily obtained after overnight incubation at 20ºC (whatever the buffer) and they were stable over time (at least for more than 6 months). Morphology and shape was rather random.
Initial crystal X-ray diffractions were performed in a Rigaku 007 X-ray generator (Plataforma Automatitzada de Cristal·lografía, PCB) and in the synchrotron from Grenoble.

Crystal storage

Protein crystals were fished with loops of 0.1mm (or 0.3mm for the biggest ones). Those formed in formate buffer were rinsed twice in fresh buffer and flash-frozen in liquid nitrogen (the buffer itself acted as cryobuffer). Those from the citrate buffer were soaked first in buffer containing 10% glycerol and then in buffer containing 20% glycerol before flash-frozen. Crystals were stored in liquid nitrogen.

Crystal soaking

Protein crystals were fished and transferred to a 2µL fresh drop (at pH 7.5 for the Tris buffer and pH 7 for the HEPES buffer) and a 0.25uL drop of calix4arene 12mM was then added. Soaked crystals were fished after 3h and 24h and stored in liquid nitrogen.

Protein-ligand co-crystallization

Either citrate or formate buffers were used, following two strategies:

i. protein and ligand were pre-incubated at room temperature (10mg/mL protein + 2.4mM ligand) and then 1µL of the complex was added to the 1µL drop of buffer (not *vice versa*).

ii. 0.25µL of ligand 12mM was added to a drop formed by 1µL buffer + 1µL protein

Additionally, a blank control drop without protein was also seed in the same coverslip.

BIOPHYSICAL INSTRUMENTATION

NMR

Unitat de RMN d'alt camp, Serveis Cientificotècnics de la Universitat de Barcelona, PCB

spectrometers	Bruker DMX 500MHz
	Bruker Digital Advance 600MHz
	Bruker Digital Avance 800MHz
probes	Triple resonance TXI 5mm probe with gradients on X, Y and Z
	Triple resonance TCI cryoprobe

Microcalorimetry

Unitat de Química Fina, Serveis Cientifico-Tècnics de la Universitat de Barcelona, PCB

Microcal VP-DSC microcalorimeter

Laboratory of Dr. Margarita Menéndez, Instituto Química-Física Rocasolano – CSIC, Madrid

Microcal MCS-DSC microcalorimeter
Microcal MSC-ITC microcalorimeter

Circular Dichroism

Unitat d'Espestroscopia Molecular, Serveis Cientificotècnics de la Universitat de Barcelona

Jasco J-810 spectropolarimeter
Jasco-CDF-426S Peltier thermostatted cell holder
Julabo external bath

Mass Spectrometry (ESI)

Platform of Mass Spectrometry, IRB Barcelona

Synapt HDMS (Waters, Manchester, UK)
NanoMate automated nanoelectrospray (Advion BioSciences, Ithaca, NY, USA)

X-Ray

Plataforma Automatitzada de Cristal·lografía, PCB

Rigaku 007 X-ray generator

MATERIALS AND REAGENTS

CHEMICALS AND SOLVENTS

Salts and reagents (molecular biology grade)	Sigma-Aldrich
acetic acid	SDS
Ammonium acetate (MS grade)	Sigma
Chromic mixture for cleaning of glassware	AppliChem
D_2O “100” (99.99 atom %)	Sigma
D_2O 99.98% (standard)	Cambridge Isotope Laboratories
DSS	Merk
EDC (400mM)	Biacore
glutaraldehyde solution 25%, grade I	Sigma
glycerol 87%	Merck
Hellmanex®II cleaning concentrate	Hellman
milliQ water (resistivity >18MΩ·cm^{-1})	MilliQ Plus filtration system (Millipore)
milliQ water (MS grade)	Riedel-de-Häen
NaN_3	Sigma
NHS (100mM)	Biacore

MATERIALS

Microcon YM-3 (3kDa, 0.5mL)	Millipore
High field quality tubes, Ø 5mm, 528-PP	Wilmad
High field quality tubes, Ø 3mm, 100mm length	Norell
NMR Pipettes (9 inchs length)	Aldrich
Quartz cells	Hellman
Syringes (for microcalorimetry)	Hamilton
Crystallography (plates, loops, coverslips…)	Hampton Research

HANDY INSTRUMENTATION

Electrophoresis cells	Mini-protean® BioRad (SDS-PAGE) BioRad (agarose)
pH meter	Crison GLP21
Microelectrode	Mettler-Toledo

EXPERIMENTAL 4

Cell cultures and transient transfection*

* HeLa cultures and experiments were designed and performed thanks to the support of S. Pujals

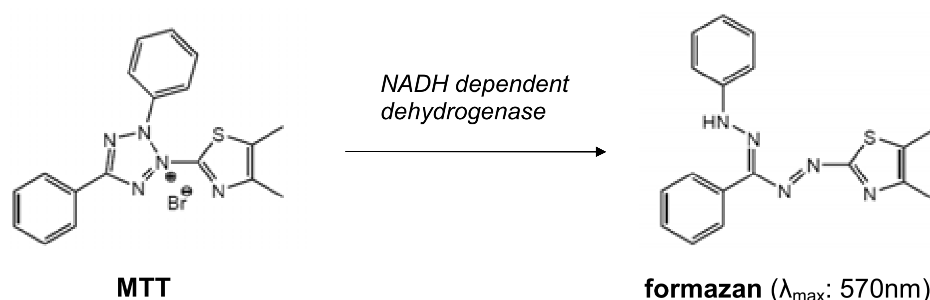
4.1. HeLa cllular cultures

Human cervical adrenocarcinoma epithelial cell line HeLa were maintained as a monolayer in D-MEM grown medium incubated at 37ºC in a humidified atmosphere with 5% CO_2. Culture medium was changed every 1-3 day and cells were sub-cultured to a fresh culture vessel when growth was over 70% of confluence. Healthy cells double population every 24h.

For sub-culturing, cells were detached by incubation with trypsin-EDTA for 5min (at 37ºC, 5% CO_2), previous removal of the grown media and PBS washing of the bottle surface (otherwise trypsin would be deactivated). Trypsinated cells were centrifuged at 1,000rpm for 4min at 22ºC, and gently resuspended into 10mL of fresh medium preheated at 37ºC. Cellular density was determined in a Neubauer counting plate[f] and the appropriate volume was inoculated into a fresh culture vessel. After 24h incubation, cells are fully attached to the surface.

4.2. MTT viability assay

The so-called MTT assay is a colorimetric test for measuring cellular growth and therefore it can be used to determine compounds cytotoxicity. It is based on the transformation of yellow MTT into blue formazan catalyzed by NADH dependent dehydrogenases; the reaction only takes place when the mitochondrial enzyme is active and consequently, blue intensity of the culture is directly proportional to the number of living cells.



The assay was performed in 96-well plates, with 3,150 cells in 100µL per well, seeded 24h before performing the experiment.

Toxicity was evaluated after 4h and 24h incubation with the calixarenes in both D-MEM and OPTI-MEM culture media (at 37ºC in a humidified atmosphere with 5% CO_2). MTT was added (to a final concentration of 0.5mg/mL) 2h before completing the treatment. At the end of the incubation time, the medium was removed and 200µL of isopropanol were added to solve the formazan crystals. Samples were protected from light and rocked for 30min at 100rpm. Absorbance at 570nm was measured in a UV-plate reader.

---

[f] Neubauer counting plate: for 10µL, [4 sub-squares mean]x10^4 cells/mL

Viability was expressed considering 100% for the cells blank. Hexaplicates were done for each sample.

4.3. HeLa transient transfection assays

Transfection assays were performed in 8.8cm^2 plates, with 20,000 cells in 1.5mL per plate, seeded 24h before performing the experiment.

2ng of pEGFP plasmid were incubated with calix4prop (at the appropriate concentration) for 30min at room temperature in a total volume of 15µL of water. It was then diluted with 1mL of D-MEM medium and chloroquine was added to a concentration of 10µM. Following the aspiration of the culture medium, the 1mL transfection solution was carefully added to the cells and they were incubated at 37ºC in a humidified atmosphere with 5% CO_2 for 4h. The transfection solution was then removed, 1.5mL of fresh D-MEM pre-heated at 37ºC were added to each plate and cells were left to incubate for 24h-48h.

FuGENE® (Roche) was used as positive transfection control (samples prepared according to the manufacturer instructions), and beside a blank control of untreated cells, a pEGFP black control was also performed.

4.4. Flow cytometry

Cells incubated for 24h-48h after the transfection treatment were detached from the plate surface with 200µL of trypsin-EDTA (5min at 37ºC, 5% CO_2), previous removal of the grown media and PBS washing. From now on, reagents and samples were kept at 4ºC on an ice bath. Detached cells were resuspended with 0.5mL of pre-chilled fresh D-MEM and transferred to a sterile pre-chilled 14mL falcon tube. The plate was further washed with another 0.5mL of fresh medium. Samples were centrifuged at 1,000rpm for 4min at 22ºC and pellet cells were resuspend in 0.5mL of fresh pre-chilled D-MEM. At that point, 5µL of propidium iodate 1mg/mL were added and gently mixed (it would label dead cells). Finally, samples were transferred into pre-chilled cytometer tubes.

Analyses were done in a Beckman MCL flow cytometer with an argon laser measuring EGFP fluorescence at 525nm and propidium iodate at 620nm. The blank-fluorescence levels were set with the blank cells sample.

4.5. Confocal laser scanner microscopy

For CLSM samples, cells were fixed on coverslips. That was achieved by initially placing some sterile glass coverslips on the surface of the plastic plate before adding the culture. 48h after the transfection assay, coverslpis were taken from the culture with sterile twizer and washed extensively with [PBS, 1.1mM $CaCl_2$ and 1.3mM $MgCl_2$]. Attached cells were fixed onto the coverslip by a 15min treatment with 3% *p*-formaldehyde and 60mM sacarose in PBS, and after washing thoroughly with PBS, the air dried coverslips were mounted onto glass slides using 8µL Mowiol-Dabco medium (on the cell-containing surface). Samples were dryed for 2h at room temperature and stored at 4ºC, always protected from light.

Images were taken in a confocal laser scanning microscope OLYMPUS Fluoview 500 with an oil immersion objective 60X/1.4 NA, Paplo 60x0, exciting with an argon laser (8% off-set) at 488nm and detecting emission in the 515-530nm range. Pictures of intermediate cells sections were taken at a resolution of 1024x1024, for both fluorescence emission and transmitted light.

4.6. Media and stocks

D-MEM (Dulbecco's Modified Eagle's Medium)

10% fetal calf serum

1g/L glucose (culture medium low glucose)

2mM L-glutamine

110mg/L sodim pyruvate

50µg/mL penicillin

50mg/mL streptomycin

OPTI-MEM® (reduced serum medium)

HEPES buffer

2.4mg/L sodium bicarbonate

hypoxantine

thymidine

L-glutamine

sodim pyruvate

trace elements

growth factors

phenol red reduced to 1.1mg/L

> 0.25% trypsin -1% EDTA

> MTT 5mg/mL

> Chloroquine 10mM

> Propidium iodate 1mg/mL

MATERIALS AND REAGENTS

CHEMICALS

> HeLa cell line	ATCC
> Salts and reagents (molecular biology grade)	Sigma-Aldrich
Chloroquine	Sigma
D-MEM	Biological industries
DMSO	Panreac
EtOH	Panreac
FuGENE®	Roche
isopropanol	Panreac
milliQ water (resistivity >18MΩ·cm^{-1})	MilliQ Plus filtration system (Millipore)
Mowiol	Calbiochem
MTT	Sigma
OPTI-MEM®	GIBCO – Invitrogen
penicillin	Gibco
p-formaldehyde 16%	Sigma
Propidium iodate	Sigma
streptomicin	Gibco
trypsin -EDTA	Biological industries

MATERIALS

Culture vessels and plates	Nunc
Neubauer plaque (0.100mmx0.0025mm^2)	Neubauer, Marienfeld

INSTRUMENTATION

Confocal microscope*	Olympus Flouview V. 4.3.57
Flow cytometer*	Beckman Coulter Epics XL MCL
Microscope*	Nikon Eclipse TS100
Plate spectrofotometer	Bio-Tek Power Wave X

**From the Unitat de Microscopia Confocal i Manipulació cel·lular and the Unitat de Citometria de Flux dels Serveis Científico-Tècnics de la Universitat de Barcelona, PCB*

Bibliography

1. Mateu, M. G. & Fersht, A. R. Nine hydrophobic side chains are key determinants of the thermodynamic stability and oligomerization status of tumour suppressor p53 tetramerization domain. *EMBO J.* **17**, 2748-2758 (1998).
2. Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* **41**, 207-234 (2005).
3. Tyler, R. C. *et al.* Auto-induction medium for the production of [U-15N]- and [U-13C, U-15N]-labeled proteins for NMR screening and structure determination. *Protein Expr. Purif.* **40**, 268-278 (2005).
4. Johnson, C. R., Morin, P. E., Arrowsmith, C. H. & Freire, E. Thermodynamic analysis of the structural stability of the tetrameric oligomerization domain of p53 tumor suppressor. *Biochemistry* **34**, 5309-5316 (1995).
5. Kaiser, E., Colescott, R. L., Bossinger, C. D. & Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **34**, 595-598 (1970).
6. Delaglio, F. *et al.* NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277-293 (1995).
7. Dudic, M. *et al.* A general synthesis of water soluble upper rim calix[n]arene guanidinium derivatives which bind to plasmid DNA. *Tetrahedron* **60**, 11613-11618 (2004).
8. Jeffrey, P. D., Gorina, S. & Pavletich, N. P. Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. *Science* **267**, 1498-1502 (1995).
9. Mittl, P. R., Chene, P. & Grutter, M. G. Crystallization and structure solution of p53 (residues 326-356) by molecular replacement using an NMR model as template. *Acta Crystallogr. D. Biol. Crystallogr.* **54**, 86-89 (1998).