The structure of p53 tumour suppressor protein reveals the basis for its functional plasticity

Andrei L Okorokov^{1,2,*}, Michael B Sherman^{3,8}, Celia Plisson⁴, Vera Grinkevich⁵, Kristmundur Sigmundsson⁶, Galina Selivanova⁵, Jo Milner⁷ and Elena V Orlova^{4,*}

¹Department of Pathology, Royal Free and University College Medical School, University College London, London, UK, ²Wolfson Institute for Biomedical Research, University College London, London, UK, ³Department of Biological Sciences, Purdue University, West Lafayette, IN, USA, ⁴School of Crystallography, Birkbeck College, London, UK, ⁵Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden, ⁶Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden and ⁷YCR p53 Laboratory, Department of Biology, University of York, York, UK

p53 major tumour suppressor protein has presented a challenge for structural biology for two decades. The intact and complete p53 molecule has eluded previous attempts to obtain its structure, largely due to the intrinsic flexibility of the protein. Using ATP-stabilised p53, we have employed cryoelectron microscopy and single particle analysis to solve the first three-dimensional structure of the full-length p53 tetramer (resolution 13.7 Å). The p53 molecule is a D2 tetramer, resembling a hollow skewed cube with node-like vertices of two sizes. Four larger nodes accommodate central core domains, as was demonstrated by fitting of its X-ray structure. The p53 monomers are connected via their juxtaposed N- and C-termini within smaller N/C nodes to form dimers. The dimers form tetramers through the contacts between core nodes and N/C nodes. This structure revolutionises existing concepts of p53's molecular organisation and resolves conflicting data relating to its biochemical properties. This architecture of p53 *in toto* suggests novel mechanisms for structural plasticity, which enables the protein to bind variably spaced DNA target sequences, essential for p53 transactivation and tumour suppressor functions.

The EMBO Journal (2006) 25, 5191–5200. doi:10.1038/sj.emboj.7601382; Published online 19 October 2006

Subject Categories: structural biology

Keywords: cryoelectron microscopy; p53; single particle analysis; structure; tumour suppressor

Introduction

The p53 tumour suppressor protein is a classic gatekeeper of cellular fate (Ko and Prives, 1996; Vogelstein *et al*, 2000; 25 Years of P53 research, 2005). In its normal latent form, p53 has low affinity for its specific DNA sequences, but acquires high-affinity binding in response to genotoxic stress, thus adopting the active form. Once activated, p53 initiates cell cycle arrest, senescence or apoptosis via pathways involving transactivation of p53 target genes (Ko and Prives, 1996; Vogelstein *et al*, 2000; see for review 25 Years of P53 research, 2005). In addition, a number of reports link p53 to DNA repair processes (Sengupta and Harris, 2005). However, the exact molecular mechanism(s) of p53 activation as a transcription factor and/or participant in DNA repair pathways remains unknown.

p53 protein is a polypeptide of ~400 aa residues in length (393 aa for human p53). p53 forms tetramers in solution, which are thought to be the predominant assembly (Friedman *et al*, 1993; Wang *et al*, 1994). p53 domains include the transcription activation domain (1–67), proline-rich region (67–98), core domain (98–303), nuclear localisation signal-containing region (303–323), oligomerisation domain (323–363) and C-terminal basic domain (363–393) (Figure 1A). The core domain is responsible for binding to sequence-specific DNA elements located close to promoters of the p53 target genes (Cho *et al*, 1994). Two decameric half-site sequences, 5'-RRRC(A/T)(T/A)GYYY-3' (where R = purine and Y = pyrimidine), have been shown to form the consensus recognition site of p53 (Kern *et al*, 1991; el-Deiry *et al*, 1992; Tokino *et al*, 1994). Unlike other transcription factors, p53 has a second DNA-binding domain that maps to its C-terminus (residues 323–393), which forms stable complexes with non-sequence-specific DNA, including mismatched DNA, double-strand breaks and single-stranded DNA (Bakalkin *et al*, 1995; Lee *et al*, 1995). Notably, the same part of the C-terminus influences the efficiency of p53 to act as a transcription factor. Its post-translational modifications and interactions with other proteins were reported to modulate the stability of p53-specific DNA complexes (Ko and Prives, 1996; Vogelstein *et al*, 2000). This led to the hypothesis that the regulatory interplay between the core and the C-terminal domains of p53 may involve global rearrangements within the p53 molecule when it becomes activated (Hupp and Lane, 1994). An alternative steric hindrance model suggests that core and C-terminal domains of p53 compete for DNA binding, without any global conformational change being involved, which is supported by biochemical and NMR data (Bayle *et al*, 1995; Anderson *et al*, 1997; Ayed *et al*, 2001). However, neither of these models, based on experiments with short oligonucleotides, could explain why deletion of the last C-terminal 30 aa impairs the stability of p53 complexes with long stretches of DNA (Espinosa and Emerson, 2001; McKinney *et al*, 2004) and p53's efficiency as a transcription factor (Cain *et al*, 2000).

*Corresponding authors. EV Orlova, Department of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, UK.

Tel.: +44 20 7631 6845; Fax: +44 20 7631 6803;

E-mail: e.orlova@mail.cryst.bbk.ac.uk or

AL Okorokov, Department of Pathology, Royal Free and University College Medical School, University College London, London WC1E 6JJ, UK. Tel.: +44 20 7679 0959; Fax: +44 20 7388 4408;

E-mail: a.okorokov@ucl.ac.uk

⁸Present address: Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555-1055, USA

Figure 1 EM of p53. **(A)** Schematic representation of p53 major domains. TA, transcription activation domain; Pro, proline-rich domain; core, core domain; NLS, nuclear localisation sequence; 4x, oligomerisation domain; Ct, C-terminus. **(B)** p53 protein sample used for EM. Lane 1, molecular weight markers (kDa); lane 2, Coomassie-stained protein before tag removal; lane 3, silver-stained final p53 sample after tag removal and gel filtration; lanes 4–6, the immunoblot with PAb248 (anti-Nt), PAb240 (anti-core) and PAb421 (anti-Ct) antibodies. **(C)** Part of a micrograph of p53 in ice. Arrows point at encircled p53 particles. The bar is 300 Å. **(D)** Image analysis of p53. (i) Representative class averages of p53 particles. (ii) Reprojections of p53 structure in the orientations found for the class averages in (i).

It has been suggested that the C-terminus of p53 provides additional anchorage to specific DNA sites via nonspecific flanking interactions, thus stabilising the whole complex (Cain *et al*, 2000; Espinosa and Emerson, 2001; McKinney and Prives, 2002; McKinney *et al*, 2004).

The p53 protein has an intrinsically flexible structure (Milner and Medcalf, 1991) that serves its multifunctional activity. Although the flexibility complicates structural analysis, crystallography and NMR have succeeded in the analysis of two separate domains of p53: the core domain in complex with DNA and co-interacting proteins (Cho *et al*, 1994; Gorina and Pavletich, 1996; Zhao *et al*, 2001; Derbyshire *et al*, 2002; Huyen *et al*, 2004) and the C-terminal domain, which is required for p53's oligomerisation (Clore *et al*, 1995; Jeffrey *et al*, 1995). Crystal structures of small peptide regions from p53 N- and C-termini were obtained in complex with important p53-binding proteins (Kussie *et al*, 1996; Avalos *et al*, 2002; Bochkareva *et al*, 2005).

Based on biochemical and structural data, two hypothetical models of p53 organisation were proposed. One suggested that core domains make pairs with C2 symmetry, linked by the oligomerisation domain that has D2 symmetry (Cho *et al*, 1994; Jeffrey *et al*, 1995). This model has overall C2 symmetry where the oligomerisation domain is located on the symmetry axis on one side of the structure. Another putative model arranges the monomers in D2 symmetry with the oligomerisation domain in the centre of the assembly and the cores positioned around it (Goodsell, 2003).

In this study, we have exploited cryoelectron microscopy (cryoEM) to determine the first three-dimensional (3D) structure of the full-length tetrameric p53. The analysis of the structure provides the first insight into the architecture of the p53 molecule *in toto* and suggests mechanisms for p53's DNA binding and activation.

Results

3D structure of p53

A 3D structure of p53 was examined using cryoEM with single particle analysis. Purified ATP-stabilised murine p53 protein (Figure 1B) showed a homogeneous population of particles (Figure 1C). Structural analysis was performed using several starting models to take into account that four identical sub-units may form three possible symmetric arrangements in space: C2, C4 and D2 (222). Seven thousand single molecular images were selected from micrographs and subjected to an alignment procedure followed by statistical analysis. For the initial steps of structural analysis, we used the lowest symmetry C2. However, the consistency between the original classes and reprojections of the reconstruction obtained with the symmetry C2 was quite poor. The reconstruction with C4 symmetry failed to produce projections corresponding to the characteristic molecular views. In contrast, D2 symmetry reconstruction gave a good agreement between classes and reprojections (Figure 1D; see Materials and methods). The final p53 3D map with 13.7 Å resolution was obtained from the

best 250 classes (0.5 threshold of Fourier Shell Correlation function) (Supplementary Figure 1).

The overall shape of the p53 tetramer resembles a hollow skewed cube of $66 \times 82 \times 85$ Å in size (Figure 2). The molecule has eight vertices/nodes of two types connected by linkers. Four nodes are bigger in size and denser. There are large openings (~ 26 Å in diameter) in the centres of each facet (Figure 2A–D). The volume of the whole molecule is ~ 220 nm³ at 1σ threshold and can accommodate ~ 185 kDa of molecular mass of the protein. This agrees well with the predicted 174 kDa molecular mass of the p53 tetramer. Each of the four larger nodes has room for ~ 25 kDa of protein, which correspond to about 200–220 aa. The four smaller nodes can host ~ 170 aa each. The remaining amino-acid residues could be distributed between linker densities with about 15–20 aa in each. The 3D map of p53 has two layers of density, each composed of four nodes (Figure 2E).

The unveiled architecture of p53 tetramer explains the discrepancy previously reported in the mass determined by size-exclusion chromatography (400–450 kDa) compared with ultracentrifugation (160–180 kDa) (Friedman *et al*, 1993; Wang *et al*, 1994). The overall volume of the p53 tetramer including the hollow space inside is equivalent to 470 nm³, corresponding to a globular protein with a molecular weight of ~ 400 kDa. This agrees with p53's behaviour on gel-filtration columns.

Docking of the core domain atomic structure into p53 EM map

We used the atomic structures of the core domain determined for both human and murine p53 (1tup, Cho *et al*, 1994; 1hu8,

Zhao *et al*, 2001) to identify the location of this domain within the EM structure. An automated search for the location of the core domains was performed by URO software (Navaza *et al*, 2002). As a result, the core domain was allocated to the larger node's density (Figure 3). The overall density distribution of this node corresponds well to the shape of β-sheets of the core domain even at the threshold level of 5σ (Figure 3A and B). We therefore assigned the large nodes (hereafter termed core nodes) to the core domains (cores) of p53. The small α-helix (H1, 1tup) that is involved in Zn binding protrudes from the molecular envelope of the EM map, indicating that this section of p53 has a different location in the EM structure. This discrepancy may be due to alternative intermolecular contacts present in the crystal lattice and in the native tetrameric p53 structure. The fit resulted in four cores positioned so that the DNA-binding residues and Zn-fingers were on the surface of the molecule.

The docking of the core domain left room for neither the C- nor the N-terminal domains of p53 within the large nodes of the map. To analyse the positions of N- and C-termini in more detail, we used the atomic structures of the oligomerisation domain of p53 (1c26; Jeffrey *et al*, 1995) and two α-helices (1t4f, Kussie *et al*, 1996; 2b3g, Bochkareva *et al*, 2005) of p53 representing two homologous transactivation subdomains TAI and TAIL from the p53's N-terminal part (Figure 3C). The automated fitting unambiguously placed the C-terminal fragment into the small node. The remaining space within this small node (hereafter the N/C node) was able to accommodate two small N-terminal helices with some room to spare. The resulting fit of three helices (the oligomerisation domain and two helices from the N-terminus)

Figure 2 3D reconstruction of p53. The skewed-cube-shaped p53 molecule viewed at different angles. Side views (A, C, D) and the top view (B). The surface rendering is shown at 1σ density threshold. LN and SN indicate positions of the large and small vertices/nodes, respectively. (E) Stereo view of the p53 molecule.

Figure 3 Identification of the core domain position within the p53 map. (A) A representation of core domains occupying all four core nodes (surfaces are shown at 1 σ in blue and 5 σ in green). (B) The large node with the core structure fitted into the EM map. Zn atoms are represented as cyan spheres. (C) Atomic coordinates of the oligomerisation domain and two α -helices representing N-terminus were fitted into the N/C node of the 3D map (3 σ). Ct (yellow) and N1 and N2 (red) indicate positions of the C- and N-terminal helices, respectively.

within the 3D map shows that they form a putative α -helical bundle (Figure 3C). The overall fitting results for core, N-terminus and C-terminus of p53 are in good agreement with cryoEM map with cross-correlation of 0.6.

The organisation of the p53 tetramer

The top view of the p53 molecule shows that the upper four nodes are located in one layer and form the vertices of a parallelogram, whereas the other layer of four nodes is rotated by $\sim 70^\circ$ (Figures 2B, 4A and C). Two vertices (core nodes) of each parallelogram are linked to the smaller nodes, suggesting that they may belong to one dimer (Figure 4B and D). The N-terminus of one monomer appears to abut the C-terminus of the partner in the dimer, forming 'joints' within the less dense N/C nodes of the 3D map. The overall volume of the N/C node can accommodate about 170 aa of protein

sequence, which is consistent with the length of both N- and C-termini of p53 and the results of the fitting.

The dimer can be envisaged as twins facing each other and holding hands. Thus, the left hand of one twin would hold the right hand of the other and vice versa, with the left hand representing the C-terminal domain and the right hand the N-terminal domain (Figure 4A–D, core pairs are coloured green/orange and red/yellow). The N/C nodes are apparently formed by the first ~ 70 –80 aa residues from the N-terminus of p53 and the last 70–80 aa of the C-terminal part of p53, thus encompassing the 323–363 region essential for p53 oligomerisation.

The organisation of the p53 tetramer is shown in Figure 4E and F. Two core domains of each dimer—coloured green/orange and red/yellow—occupy the larger nodes of the 3D map. The dimers are linked through the interaction between the N/C nodes of one dimer and core nodes of the second dimer (Figure 4E and F). The linkers between core domains and their N- and C- termini (Figure 4F, tubes coloured blue and magenta) are large enough to accommodate segments of about 20–25 aa residues each, which may comprise the proline-rich region of the N-terminus and the sequence of p53 encompassing the nuclear localisation signal. The interface between N/C node and the core node involves three conservative loops on the side opposite to the sequence-specific DNA-binding surface of p53 (Supplementary Figure 2).

Our structure predicts that the p53 N- and C-terminal domains interact to form a dimer. To test this putative interaction, the binding between the p53 N- and C-terminal domains was examined using three independent approaches: two *in vitro* methods (GST-pull-down assay and surface plasmon resonance, that is, BIAcore technology) and an *in vivo* method (co-immunoprecipitations).

Both *in vitro* tests confirmed the existence of the interaction between the isolated N-terminal domain (residues 1–100 or 1–63) and C-terminal part (aa 323–393) (Figure 5A, left panel; Supplementary Figure 3). Importantly, substitution of amino-acid residues F341, L344, L348 and A355 (with K, E, E and K, respectively) in the p53 C-terminus, previously shown to negate p53 oligomerisation (Sturzbecher *et al*, 1992; Tarunina and Jenkins, 1993), resulted in loss of C-terminal binding to the N-terminal domain (Figure 5A, right panel).

To test the interaction of N- and C-termini *in vivo*, His-tagged N-terminal and FLAG-tagged C-terminal fragments of p53 were expressed in HCT116 p53^{−/−} cells. The cell lysates were analysed by co-immunoprecipitation followed by Western blotting with appropriate antibody. We readily observed the interaction between the p53 N- and C-terminal fragments (Figure 5B). The biochemical data thus support the structural organisation described here and implicate the intramolecular interaction between the N- and C-terminal domains within the p53 oligomer.

Labelling of N/C node with PAb421 antibody

According to the X-ray-based model with C2 symmetry, all four p53 C-termini interact with each other and are located on one side of the molecule. However, results of the fitting suggest that the C-termini are located on the opposite sides of the p53 molecule, that is, two C-termini take part in the formation of an upper dimer, whereas the other two belong to the lower dimer (Figure 4). In order to confirm the positioning of C-termini within the p53 tetramer, cryoEM was used to

Figure 4 Structural organisation of p53. (A) The upper density layer of the p53 3D map at threshold 1σ , with fitted structures of two core domains shown in green and orange. N and C represent positions of the N- (blue) and C-termini (magenta), respectively. (B) Schematic representations of the corresponding monomer interactions. N/C nodes are represented as blue/magenta joints and the linkers representing N-termini are in blue and those for C-termini are in magenta. The core nodes are shown as spheres coloured similar to their corresponding core domains. Cut away view (C) and schematic representation (D) of the lower density layer with the second pair of core domains (coloured red and yellow) fitted in. (E) Domain structures are fitted into the corresponding nodes of the tetramer. (F) A schematic model of p53's quaternary organisation. The core nodes are coloured similar to their corresponding core domains. Grey linkers represent core node to N/C node contacts.

analyse the complex formed between p53 and monoclonal antibody PAb421, which binds the C-terminus. Several types of p53/PAb421 complexes were observed, with one, two (Figure 5C), and in some cases three or four antibodies attached to one p53 tetramer. The cryoEM images of p53 complexed with the antibodies clearly demonstrate that two immunoglobulin molecules are attached by both variable chains to opposite sides of one p53 tetramer (Figure 5C, row iii). It is difficult at the available resolution to distinguish whether each of the doubly bound antibodies is bridging the C-termini from one dimer or from two different dimers. However, the arrangement of the antibodies around the tetramer is consistent with the arrangement of the N/C nodes in our cubic model and stands in stark contrast with the previous model of p53 organisation, which presupposes that the C-termini are localised in a single region.

To verify and interpret the resulting images, we have modelled p53 complexes with one or two antibodies and calculated projections of those complexes (Figure 5C, columns 3 and 4; see Materials and methods). The comparison of the class averages with projections of the models confirms that C-termini are located on the opposite facets of the p53 molecule. The best correlation between the class averages and model reprojections was achieved when antibodies were rotated by $\sim 70^\circ$, which is dictated by the C-termini positions according to the fitting (Figures 4 and 5C, iii).

Discussion

p53 structure in toto

The intact and complete p53 molecule has eluded previous attempts to obtain its structure, largely due to the intrinsic flexibility of the protein. Such flexibility enables diverse p53 functions including recognition of variably spaced target sequences on DNA. When analysed in isolation, the residues 323–363 at the C-terminus of p53 are able to oligomerise and form a tetrameric structure (Sturzbecher *et al*, 1992; Iwabuchi *et al*, 1993; Clore *et al*, 1995; Jeffrey *et al*, 1995). However, in the intact protein, the interaction between residues 323 and 363 appears to be suppressed and is replaced by N/C interactions with further complexing via core–N/C interactions to form the tetrameric p53 molecule (see Results). Thus, oligomerisation is dependent on α -helical interaction between monomers as previously hypothesised but with interactions occurring between N- and C-terminal α -helices, as opposed to just α -helices in the C-termini. The departure of the current model of p53 organisation from previously proposed models of the oligomerisation domain can readily be explained by the fact that previous X-ray and NMR structures were performed using the isolated C-terminal domain. In contrast, our work is based on full-length p53 protein, which we believe represents the *in vivo* nature of p53 oligomerisation. The structure described here is consistent with known biochemical

information on p53 and is in keeping with the p53's DNA-binding properties.

Each monomer has three regions of contacts with the other subunits of the tetramer (Figure 4E and F). Two of these contacts are provided via its N- and C-termini and the third one is between the core node from one dimer and the N/C node of the second dimer, thus providing a basis for tetramerisation. The results described here confirm reports on the cooperativity of N- and C-termini, which contribute equally to p53's stability, activity and conformation (Hansen *et al*, 1998; Cain *et al*, 2000). It has also been reported that N-terminal regions, comprising amino-acid residues 80–98 and the vicinity of Thr55, act synergistically with the C-terminus of p53 to negatively regulate DNA binding by the core domain (Muller-Tiemann *et al*, 1998). The 3D structure of p53 also corroborates the fact that monoclonal antibodies against both N- and C-terminal domains of p53 have been reported to modulate its DNA-binding activity (Hupp *et al*, 1992; Hansen *et al*, 1998; Cain *et al*, 2000) and that some proteins can interact with both N- and C-termini of p53 simultaneously (Horikoshi *et al*, 1995; Lin *et al*, 1996).

p53 and DNA interaction

The previously reported crystal structure of the p53 core domain in complex with DNA suggested a model where four core domains could bind one p53 DNA recognition element simultaneously (Cho *et al*, 1994). This has been further supported by the recent high-resolution structure of the p53 core domains in complex with the DNA half-site (Ho *et al*, 2006; Kitayner *et al*, 2006). According to the model, the core domains of p53 are arranged on DNA with C2 symmetry, and formation of this configuration is dependent on flexibility of linkers between C-termini and core domains. The model, however, was based on the composition of separate structures of isolated p53 domains and not of the full-length protein.

The EM structure of the full-length p53, which we report here, provides an alternative explanation both for its flexibility and functionality and suggests a model for the p53/DNA interaction. Analysis of the potential p53–DNA complex architecture was undertaken by fitting the structures of core domains each bound to DNA (chain B and DNA, 1tup; Cho *et al*, 1994) into the 3D map of p53. Four cores in the D2 arrangement form two DNA-binding surfaces on opposite sides of the tetramer, that is, one binding site is formed by each dimer. The two DNA molecules bound to the independent core domains from one dimer line up accurately (Figure 6A–C). Interestingly, the results of the fitting have shown that the distance between two half-sites (if two aligned DNA molecules are considered as one continuous chain) is 6 bp (Figure 6 and Supplementary Figure 4). It is possible that this particular distance is a consequence of p53's stabilisation by ATP- γ -S in its latent state with low DNA-binding affinity (Okorokov and Milner, 1999), but nonetheless is well within the range of 0–13 bp size DNA spacer reported in the literature (Tokino *et al*, 1994). It follows then

Figure 5 Biochemical and structural characterisation of the N/C nodes. **(A)** GST or GST-p53 proteins N and dN, spanning residues 1–100 and 1–63, respectively, were immobilised on glutathione-Sepharose and incubated with the His-tagged C-terminal fragment 323 (left panel) or oligomerisation-defective C-terminal mutant KEEK (right panel). N-terminus-bound fractions were analysed by Western blot using PAb421 antibody, and after C-terminal fragments were detected with this antibody, the membrane was stripped and re-blotted using anti-GST antibody to visualise GST and GST-fusion p53 proteins (lower panels). **(B)** His-tagged N-terminal and FLAG-tagged C-terminal fragments of p53 (1–186 and 187–393, respectively) were coexpressed in p53-negative HCT116 cells and their binding *in vivo* was analysed using co-immunoprecipitations. The panels on the left show the presence of the expressed p53 fragments in total cell lysates. The C-terminal p53 fragment was found to interact with the N-terminal fragment, as it co-immunoprecipitated with the anti-N-terminal antibody DO1 (IP with DO1; upper right panel) and, accordingly, the N-terminus was co-immunoprecipitated with the C-terminal fragment (IP with FLAG; lower right panel). The immunoprecipitates were identified by Western blotting using PAb122 (upper right panel) or DO1 (lower right panel) antibodies. **(C)** Complexes of p53 and PAb421 (anti-C-terminus) were studied by cryoEM and single particle analysis. Particles of antibody alone (i1), p53 in complex with one antibody (ii1) and p53 in complex with two antibodies (iii1) were selected into separate groups for statistical analysis. The subsets of images were aligned and those with the highest correlation were averaged (shown as i2, ii2 and iii2). To verify and interpret the resulting averages, p53 complexes with one or two antibodies were modelled (ii3 and iii3) and projections of those complexes were calculated (iii4 and iii4). i3 and i4 are the model and projection of the antibody molecule (PDB: 1igt). Bar is 150 Å.

Figure 6 Model of p53–DNA complex formation. Views of p53 bound to DNA on one side via one dimer (**A**, **B**) and on both sides with the whole p53 molecule involved in specific DNA binding (**C**). N and C represent positions of the N- and C-termini, respectively. (**D**) A schematic model for p53 re-adjustment during specific DNA binding. The semi-transparent spheres depict the p53 molecule bound to one half-site and the solid spheres represent re-adjusted p53 bound to both half-sites. The arrow indicates the general direction of the corresponding core node movement.

that the p53 structure must re-adjust every time it encounters variously spaced sequence-specific DNA response elements, in order to bind two half-sites simultaneously. Such re-adjustments would be facilitated by the plasticity of the D2 organisation of p53. The parallelogram formation of each dimer is perfectly suitable to allow flexibility of its conformation without requiring changes in the linker sizes between domains. This could be achieved by changing the corresponding positions of the core and N/C nodes using linkers between them as levers to adjust the distances between the nodes (Figure 6D). Consequently, two core domains of each dimer may move closer or further away from each other without breaking links. Correspondingly, the N/C nodes may move further away or closer to each other. Such coordinated plasticity would provide a universal mechanism for p53 to bind different recognition sequences that have various spacer lengths between the half-sites. Moreover, binding DNA on one side of the molecule by one dimer will automatically bring the second dimer into the high-affinity DNA-binding conformation (Figure 6C).

This mechanism would provide a basis for the DNA looping shown to be essential for synergistic transcription activation of promoters with distant p53 regulatory elements and observed in earlier electron and atomic force microscopy studies (Stenger *et al*, 1994; Jackson *et al*, 1998; Jiao *et al*, 2001). Interestingly, similar D2 organisation has been reported for another class of DNA-binding proteins, namely the type IIF restriction enzymes, such as SfiI, Cfr10I and NgoMIV that bind two recognition sites simultaneously and loop DNA (Wentzell and Halford, 1998; Siksnys *et al*, 1999; Deibert *et al*, 2000).

The arrangement of core domains within our structure suggests that there is a possibility of two p53 tetramers binding one response DNA element simultaneously. A sandwich of DNA between two p53 tetramers would allow p53

binding to all four quarter sites; such a complex is consistent with the previously reported ‘stacks’ of p53 on specific DNA sites (Stenger *et al*, 1994). Remarkably, it is also consistent with the biochemical data that specific DNA stimulates p53 tetramers to assemble into octameric (or double tetrameric) DNA-bound complexes (Wang *et al*, 1995). Such arrangement implies that to bind a second p53 tetramer to DNA with organisation of core domains described previously (Cho *et al*, 1994; Ho *et al*, 2006; Kitayner *et al*, 2006), the core domains of the second p53 molecule would have to rotate $\sim 60^\circ$ with respect to the first molecule. This is in agreement with the $\sim 70^\circ$ rotation reported for NMR conditions (Veprintsev *et al*, 2006).

The interplay between core and C-terminal domains of p53 in DNA binding has been the subject of many debates and hypotheses (Hupp and Lane, 1994; Anderson *et al*, 1997; Ahn and Prives, 2001; Yakovleva *et al*, 2002; Kim and Deppert, 2005). Our structure indicates that the C-terminal domains are in close proximity to the DNA-binding surfaces of the core domains (Figures 4 and 6). Such positioning allows synergistic DNA binding by both core and C-terminal domains, with C-termini providing an additional anchorage to DNA (Figure 6). In general, C-terminal domains on opposite sides of the tetramer could serve as nonspecific DNA contacts allowing p53 to slide along continuous double-stranded DNA, or to transfer from one DNA molecule to another by switching the nonspecific interaction from one dimer to another (Jiao *et al*, 2001; McKinney *et al*, 2004).

Conclusions

The structure of full-length p53 is one of the smallest proteins resolved by single particle cryoEM. This novel structure provides a model for the coordinated conformational re-adjustment of the p53 DNA-binding domains. The structural plasticity required for p53’s activation and activity is provided by the D2 symmetry of p53 tetramer. The structure suggests a stepwise mechanism in which the initial contact with DNA is made by a pair of C-terminal domains that (together with the core domains) are involved in DNA scanning via a linear diffusion mechanism (McKinney *et al*, 2004). Whenever the sequence-specific DNA element is encountered, a coordinated re-adjustment of DNA-binding domains occurs, placing them in a favourable position for a stable complex with the sequence-specific DNA. It also follows that post-translational modifications within the N- and/or C-termini may fine-tune the plasticity of the p53 molecule and the efficiency of p53–protein and p53–DNA interaction. The new 3D structure of the complete p53 tetramer is consistent with existing biochemical and physiological data, provides a comprehensive model of p53 architecture and opens the way for better understanding of this crucial tumour suppressor protein.

Materials and methods

Recombinant proteins

Recombinant murine p53 was expressed in the baculoviral system as described (Okorokov *et al*, 1997; Okorokov and Milner, 1999). Sf9 cells infected with recombinant baculovirus were lysed for 30 min on ice in 10 ml of lysis buffer (150 mM NaCl, 25 mM Tris–HCl, pH 9.0, 0.5% NP-40, 10% glycerol) supplemented with EDTA-free protease inhibitors cocktail (Roche) and 2 mM DTT. The lysate was centrifuged for 30 min at 20 000 r.p.m. in a Sorwall

SS-34 rotor at 4°C and recombinant p53 was purified first by Ni-chelating affinity chromatography on 5 ml His-trap column (Amersham Biosciences). The protein was eluted with 250 mM imidazole in the buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.0 and 5 mM MgCl₂) and was untagged by factor Xa (Qiagen). Protease was removed using Xa removal resin (Qiagen). Untagged protein was desalted and applied to a Source 30Q column (Amersham Biosciences). p53 was eluted using a gradient from 35 mM NaCl 25 mM Tris-HCl pH 8.0 to 1 M NaCl 25 mM Tris-HCl pH 8.0. Fractions containing p53 (broad peak between 0.4 and 0.6 M NaCl) were collected, concentrated, desalted into 150 mM NaCl, 5 mM MgCl₂ and 25 mM Tris-HCl pH 7.5 buffer and applied to a Superose 6 gel filtration column (Amersham Biosciences).

p53 protein sample was homogeneous and migrated as a peak with V_e corresponding to $M_w \sim 450$ kDa (tetrameric p53). The peak fractions were collected, concentrated using the Vivascience cartridge (cutoff 30 kDa) and tested by reactivity with PAb248- and PAb421-specific monoclonal antibodies directed against the N- and C-termini of p53, respectively. The purity of the protein sample was routinely verified by SDS-PAGE, followed by silver staining (Figure 1B). p53 protein sample for cryoEM analysis was typically concentrated to 0.5 mg/ml. The starting EM sample of p53 was prepared using 250 ng of purified p53 in 50 μ l volume of a sample buffer (150 mM NaCl, 5 mM MgCl₂, 25 mM Tris-HCl pH 7.5 and 25 μ M ATP- γ -S) and used for subsequent dilutions when preparing the EM probe. Inclusion of ATP in the sample was found to improve its quality by preventing aggregation and stabilising p53 in latent conformation (Supplementary Figure 5).

GST-p53dN(1–63) and His-tagged C-terminal constructs encoding residues 323–393, either wild type or KEEK mutant (Sturzbecher *et al*, 1992), were generated by cloning the corresponding PCR fragments amplified from human wild-type p53 cDNA or oligomerisation-defective KEEK mutant of p53 into pGEX-2T (Amersham Biosciences) and pQE-30 (Qiagen) vectors, respectively, and confirmed by DNA sequencing. DNA plasmids encoding GST-Np53 (1–100) and GST-C-terminus (320–393) were described previously (Selivanova *et al*, 1999). cDNA encoding KEEK mutant, carrying substitutions of residues 341, 344, 348 and 355, was kindly provided by Professor J Jenkins. Expression vectors encoding His-tagged N-terminus (1–186) and FLAG-tagged C-terminus (187–393) were a gift from Professor G Melino. The expression and purification of GST-p53 and His₆-p53 proteins as well as pull-down assays were performed as described before (Selivanova *et al*, 1999).

CryoEM

All p53 specimens were stored at 4°C and plunge-frozen in liquid ethane on holey carbon films (R2/2, QuantifoilMicro Tools GmbH, Jena, Germany). The cryo-grids were maintained at –174°C in a Gatan 626 cryo-holder. p53 micrographs were recorded on Kodak SO-163 film at 300 keV with a dose of ~ 20 electrons/Å² in an FEI CM300-FEG at $\times 47\,000$ nominal magnification. A range of defocus settings (1.7–3.2 μ m) was used to record the micrographs. For preparation of the p53/PAb421 complexes, the proteins were incubated at a molecular ratio of 4:1 (p53/antibody) at room temperature for 30 min in p53 buffer. Samples (3.5 μ l) of complexes were frozen as above and images were recorded in a Tecnai F20 at 200 kV under low-dose conditions at $\times 50\,000$ magnification using a defocus range between 2 and 6 μ m.

Image processing and computer molecular modelling

Micrographs were digitised on a Zeiss SCAI microdensitometer (Z/I Imaging) with a step size of 7 μ m corresponding to a pixel size of 1.5 Å at specimen level for p53 and 1.4 Å for the complex with antibody. The contrast transfer function of the microscope was estimated from incoherently averaged Fourier transforms of randomly selected patches of each micrograph. Particle images of p53 (~ 7000) were selected manually.

Images were normalised to the same standard deviation and band-pass filtered: the low-resolution cutoff was ~ 100 Å to remove uneven background in particle images; the high-resolution cutoff was ~ 7 Å. Alignment and classification of images was performed as described (van Heel *et al*, 2000) and yielded classes representing characteristic views of the molecule. Angular orientations of class averages were determined by angular reconstitution (AR) (van Heel, 1987). 3D maps were calculated using the exact-filter back projection algorithm (Harauz and van Heel, 1986; Radermacher, 1988).

Primary structural analysis was performed using either an *ab initio* or a starting model-based approaches. For the *ab initio* reconstruction, the orientations of the best 10–15 classes were determined by AR using symmetry constraints. Symmetries C1, C2 and C4 resulted in diffuse density distributions. Reprojections of the reconstructions obtained using these symmetries did not resemble the original characteristic views of the molecule. In contrast, D2 symmetry provided a well-defined 3D reconstruction with correlation between reprojections and input classes above 0.5. The D2 symmetry map revealed a structure with eight nodes of density around a hollow centre.

In parallel, a model was built from four uniform spheres of radius 20 Å positioned with D2 symmetry and ~ 60 Å separations corresponding to the distances between density maxima on classes. The vertical separation of the spheres was kept small, 30 Å, resulting in a model that was almost flat, thus allowing detection of either C4 or D2 symmetries. The four-sphere model was used to assign angles for the best classes using AR. The best result was obtained for D2 symmetry and, importantly, the structure was similar to that obtained by *ab initio* approach. This first model was used for the next round of alignment and classification of images. The structure of p53 was refined by an iterative procedure, with the number of classes gradually increasing to 400. The classes were sorted according to the error level between images used for the reconstruction and their reprojections obtained from this reconstruction; classes with an error above the average were excluded from the final reconstruction. The final reconstruction was calculated from the best 250 classes, each containing 8–15 images. Resolution of the map was assessed using the 0.5 threshold of Fourier Shell Correlation function (Saxton and Baumeister, 1986; van Heel *et al*, 2000), which corresponds to 13.7 Å.

To analyse the p53/antibody complex, ~ 800 images of p53 complexed with one or two antibodies were selected manually and subjected to the standard procedure of centring and classification. The best characteristic views of p53 with one or two antibodies bound were selected from 100 classes and images constituting these classes were extracted into separate groups. The subsets of images were aligned and those with the highest correlation were averaged. To verify and interpret the resulting averages, models of p53 complexed with one or two antibodies were generated and projections of these complexes were calculated (see Figure 5C). Image analysis was performed using IMAGIC-5 (van Heel *et al*, 1996).

Domain fitting into the 3D map of p53 was performed automatically using URO software (Navaza *et al*, 2002). Fitting quality was estimated by cross-correlation coefficient (CC) between the fitted atomic structures and the EM reconstruction. The automated fitting of the core domain had CC of 0.6. The CC for alternative positions was 0.47.

Illustrations were generated using PyMOL (<http://pymol.sourceforge.net/>). Surface representations (unless stated otherwise) are displayed at a threshold level of 1σ corresponding to $\sim 100\%$ of the expected mass. This threshold was determined using the criteria that the mass of the complex is 180 kDa and the specific density is $0.84 \text{ kDa}/\text{\AA}^3$.

Accession codes

The EM map of p53 protein and the atomic coordinates of the model have been deposited in the macromolecular structure database (EBI) under accession codes EMD-1141 and EBI-25101, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Professors Helen Saibil, John Jenkins, Gerry Melino, Björn Öbrink and Christine Slingsby for their invaluable support and discussions, Dr David Houldershaw for computer support and Mr Tim Hoe and Andrias O'Reilly for help with manuscript preparation. JM is supported by Yorkshire Cancer Research. CP is supported by the Human Frontier Science Program. GS is supported by the Swedish Research Council and Swedish Cancer Society.

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