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Structural evolution of C-terminal domains in the p53 family

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The tetrameric state of p53, p63, and p73 has been considered one of the hallmarks of this protein family. While the DNA binding domain (DBD) is highly conserved among vertebrates and invertebrates, sequences C-terminal to the DBD are highly divergent. In particular, the oligomerization domain (OD) of the p53 forms of the model organisms *Caenorhabditis elegans* and *Drosophila* cannot be identified by sequence analysis. Here, we present the solution structures of their ODs and show that they both differ significantly from each other as well as from human p53. CEP-1 contains a composite domain of an OD and a sterile alpha motif (SAM) domain, and forms dimers instead of tetramers. The Dmp53 structure is characterized by an additional N-terminal β-strand and a C-terminal helix. Truncation analysis in both domains reveals that the additional structural elements are necessary to stabilize the structure of the OD, suggesting a new function for the SAM domain. Furthermore, these structures show a potential path of evolution from an ancestral dimeric form over a tetrameric form, with additional stabilization elements, to the tetramerization domain of mammalian p53.

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Introduction

Since its discovery in 1979, the role of p53 within cells has been intensely investigated by many research groups. Its importance in cancer biology is highlighted by the fact that more than 50% of tumors have mutations in p53, which makes it one of the most important proteins with respect to human disease. p53 is a tetrameric transcription factor that suppresses tumor formation by activating a set of genes that induce either cell cycle arrest or apoptosis (Kastan *et al*, 1992; el-Deiry *et al*, 1993; Symonds *et al*, 1994). Which one of the two pathways is chosen depends on the nature of the cellular stress that triggers the p53 response, such as DNA damage by UV or γ-irradiation, hypoxia, or oncogene activation (Levine *et al*, 2006). However, the question how p53 distinguishes these diverse cellular stress signals and determines which set of genes to be turned on is still not well understood, although recent reports on the role of ASPP (Samuels-Lev *et al*, 2001) and acetylation of K120 in the DNA binding domain (DBD) of p53 by Tip60 suggest a mechanism in which the apoptotic pathway is preferred over the cell cycle arrest pathway (Sykes *et al*, 2006; Tang *et al*, 2006).

During the past 10 years, p53 has expanded into a protein family with complexity and diversity, exemplified by the discovery of different splice variants of p53 and two mammalian paralogues, p63 and p73 (Yang and McKeon, 2000; Lu and Abrams, 2006). Sequence analysis of the two mammalian paralogues has revealed major differences to p53. Structurally, p63 and p73 have additional domains that are not found in p53, and exist in various isoforms created by a combination of C-terminal splicing and different N-terminal promoters (Kaghad *et al*, 1997; De Laurenzi *et al*, 1998; Yang *et al*, 1998). While p53 has an N-terminal transactivation domain (TA) for recruitment of core transcriptional factors, a central DBD for recognition of promoter sequences, an oligomerization domain (OD) for tetramerization, and a short basic stretch of 30 amino acids for regulation of transcriptional activity, the C-terminus of p63 and p73 contains (depending on the splice form) a sterile alpha motif (SAM) domain and a transcriptional inhibitory domain (TID). SAM domains are small protein–protein interaction modules that are found in a wide variety of different proteins, ranging from kinases and transcriptional regulators to cell surface receptors (Schultz *et al*, 1997). The TID, an unstructured region C-terminal to the SAM domain, was shown to inhibit the transcriptional activity of p63 by interacting with the TA domain (Serber *et al*, 2002). Functional analyses through knockout mice studies have shown that both proteins do not seem to be tumor suppressors like p53, instead they play important roles in development and maintenance of epithelial tissue (p63) or maintenance of certain neurons (p73) (Mills *et al*, 1999; Yang *et al*, 1999, 2000). The identification of mammalian p53, p63, and p73 allowed rapid classification of p53 homologues in other species. Vertebrate species like *Xenopus*, zebrafish, and chicken possess all three paralogues, while invertebrate species such as squids, clams, and molluscs have p53 homologues that are more closely related to the mammalian p63 and p73 than to mammalian p53. Interestingly, sequences from other invertebrate species like nematodes, fruit flies, and beetles cannot be classified as either p53 or p63/p73 like, and show similarity only within the DBD.

According to the current hypothesis on the evolution of the p53 protein family, p63/p73 resemble the ancestral form, while p53 evolved later (Lu and Abrams, 2006). The

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identification of many new p53 protein family members in invertebrates could further elucidate the evolutionary development of this important protein family. The structural differences between p53 and p63/p73 also reflect their different functions in surveillance of the genetic integrity of a cell and in tissue development, respectively. Since some of the invertebrate p53 forms show significant differences to both p53 and p63/p73, studying these family members might shed some new light on the evolution of function of p53. Particularly, the recent discovery of p53 homologues in the two important model organisms *Drosophila melanogaster* (Dmp53) and *Caenorhabditis elegans* (CEP-1) provides new genetic tools for the investigation of their function (Brodsky *et al*, 2000; Ollmann *et al*, 2000; Derry *et al*, 2001). Both proteins show only a low degree of sequence homology to p53 in the DBD, yet they exhibit very similar DNA binding specificity (Brodsky *et al*, 2000; Huyen *et al*, 2004). Studies have shown that both proteins are functionally distinct from mammalian p53. While the hallmark of p53 is its dual function in inducing either cell cycle arrest or apoptosis, both CEP-1 and Dmp53 can only induce apoptosis and not cell cycle arrest upon irradiation. These observations led to the idea that induction of cell cycle arrest was a later evolutionary development, since *Drosophila* and *C. elegans* have long diverged from the vertebrates.

The lack of any recognizable domain C-terminal to the DBD, including the highly conserved OD and the distinct functions of CEP-1 and Dmp53, prompted us to investigate the domain organization of the C-terminus in both proteins. We have determined the NMR structure of the C-terminus of CEP-1 and Dmp53. The CEP-1 C-terminus is composed of two sub-domains, an OD followed by a SAM domain that closely interacts with the OD. Surprisingly, the OD domain of CEP-1 forms a dimer instead of the usual tetramer observed in human p53. Equally surprising, the Dmp53 C-terminus reveals a unique oligomerization fold not found in other p53 proteins, in which an extra β-strand and an extra helix complement the canonical p53 oligomerization fold. Furthermore, deletion studies and NMR data show that protein domains C-terminal to the OD are necessary to maintain the tertiary fold. The findings from this study suggest that the ancestral p53 form had a similar domain organization as p63/p73, and that the OD of vertebrate p53 that is conformationally stable without additional structural elements evolved later.

Results

Identification of a conserved domain in the C-terminus of CEP-1

Based on the protein sequence length, CEP-1 with 644 amino acids seems more closely related to p63 and p73; thus, we suspected the existence of additional domains C-terminal to the DBD in CEP-1. However, sequence analysis failed to reveal significant homology to domains frequently found in p63/p73, such as the SAM domain or even the OD identified so far in all members of the p53 family (Figure 1A).

Sequence comparison of CEP-1 from two Nematode species, *C. elegans* and *Caenorhabditis briggsae*, identified a conserved region C-terminal to the DBD (after residues 510, *C. elegans* numbering) (Supplementary Figure S1). After several rounds of optimization, we identified a core domain between residues 528–644 in the C-terminus of CEP-1, with a molecular weight of 13.6 kDa. A melting curve monitored by circular dichroism spectroscopy revealed a sharp transition point at 41°C, an indicator of a folded domain with a two-state unfolding transition (Supplementary Figure S2). Since this identified domain is C-terminal to the DBD, it should contain the OD responsible for tetramerization, a hallmark of the p53 protein family (Lee *et al*, 1994). In contrast to p53, however, the protein elutes off a size-exclusion column as a dimer (Figure 2A). This dimeric state was further confirmed by velocity sedimentation measurements, in which the data fit the expected molecular weight of a dimer (27.2 kDa) (Table I). Since neither a classical OD nor any other domains could be identified based on sequence comparison, we used standard heteronuclear NMR spectroscopy to investigate the structure of the C-terminus.

Overall structure of the C-terminus of CEP-1

The three-dimensional structure of the C-terminus of CEP-1 reveals that it consists of two interacting domains. Despite the lack of a significant sequence homology, one of these two domains is an OD, as found in all members of the p53 protein family, and the second domain is a SAM domain (Figures 1B and C). The dimerization interface of the OD consists mostly of interactions between the β-strand and the α-helix of one OD packing against the other OD in an antiparallel manner, as observed in the human p53 OD structure (Lee *et al*, 1994). The superimposed structures of the OD in human p53 dimer and CEP-1 have an r.m.s.d. of 2.0 Å, with the α-helix in CEP-1 being two turns shorter than the corresponding helix in human p53 (Supplementary Figure S3). The SAM domain is composed of five helices that adopt the same topology as other SAM domain structures, for example, in EphB2 and p73 (Chi *et al*, 1999; Thanos *et al*, 1999). However, the SAM domain in CEP-1 resembles the EphB2 structure more closely than the p73 SAM domain, as indicated by its smaller r.m.s.d. values: 1.45 Å between EphB2 and CEP-1, and 1.83 Å between p73 and CEP-1 (Supplementary Figure S3). The OD and the SAM domain are connected by a 16-amino-acid linker, of which some resonances are either missing or could not be assigned due to significant overlap in the spectra. This linker region contains a short helix in its C-terminus that makes contacts with the first helix of the SAM domain. Direct interactions between the OD and the SAM domain are observed between the α-helix of the OD and the last α-helix of the SAM domain within the same monomer, and the β sheet of the OD from the other monomer. The relative angle between the helix of the OD and the last helix of the SAM domain is 125°.

The OD of CEP-1

The OD is a well-conserved domain among vertebrate p53 forms, due to its necessity in tetramer formation (Chene, 2001). In contrast, the OD of CEP-1 shows a poor sequence homology, including key residues that are strictly conserved in all vertebrate p53 sequences (Figure 3A). The signature glycine residue that permits a sharp turn between the β-strand and the α-helix in the structure of the OD of p53 is replaced by a serine, threonine dipeptide in CEP-1 (Figures 3A and B). This substitution forms the angle between the α-helix and the β-strand within a monomer to widen from 26°, as seen in the p53 OD, to 50° in CEP-1. Further substitutions

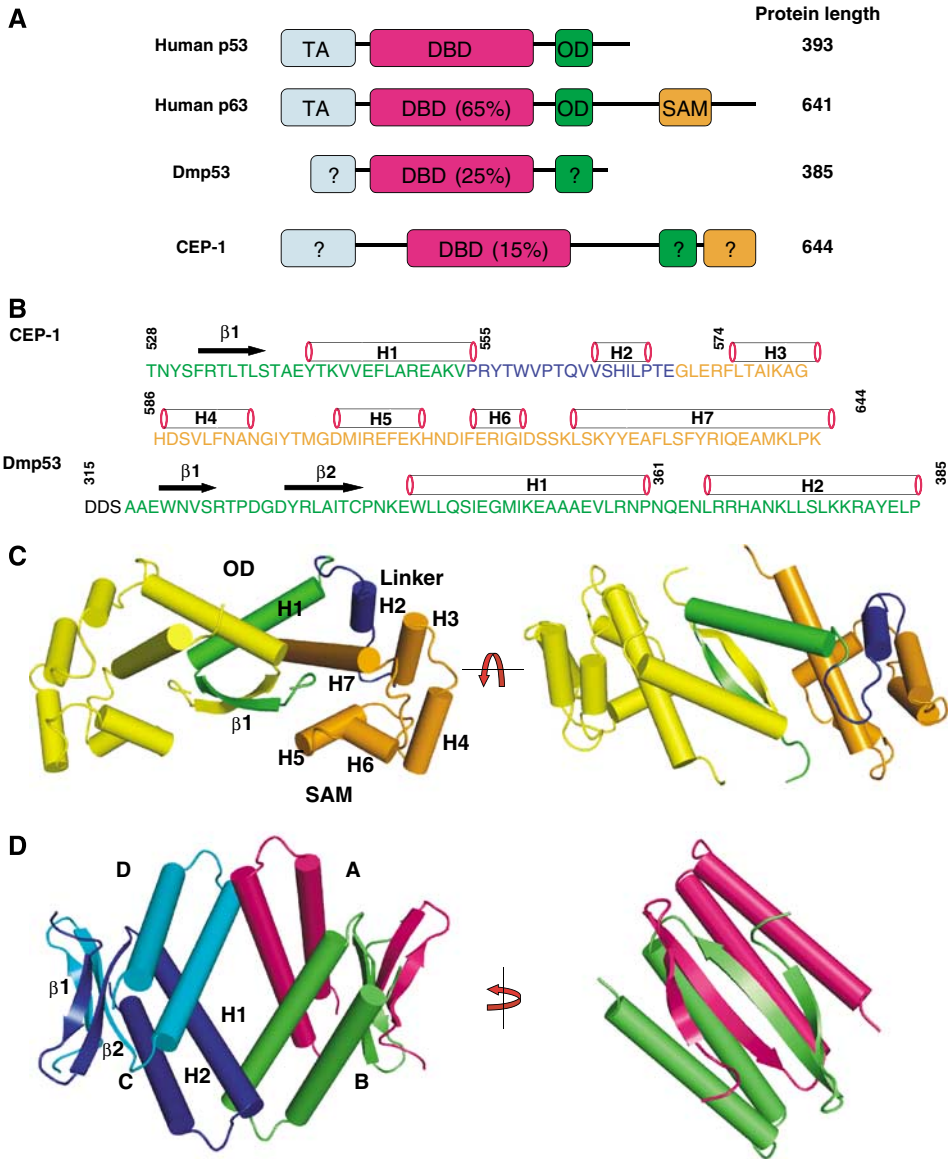

Figure 1 Domain architecture of the p53 protein family. (**A**) The p53 protein family can be categorized into two classes by the number of individual domains: p53-like proteins have three domains, and p63-like protein has four domains. TA, transactivation domain; DBD, DNA binding domain; OD, oligomerization domain; SAM, sterile alpha motif domain. Question marks indicate domains with low sequence homology to known domains that however retain the same fold. Numbers inside the DBD box represent sequence identity in comparison with human p53. (**B**) Secondary structure elements of the C-terminal domains of CEP-1 and Dmp53. The colored letters correspond to the domain color designation in panel A. (**C**) The overall structure of the C-terminus of CEP-1 reveals its dimeric structure, containing an OD (green), a SAM domain (orange), and a linker region (blue). The second monomer is shown in yellow. (**D**) The overall structure of the C-terminal domain of Dmp53. The monomers are colored in magenta (chain A), green (chain B), blue (chain C), cyan (chain D).

Table I Velocity sedimentation of the C-terminal domain of CEP-1 and Dmp53

Protein	Variants	Theoretical molecular weight (Dalton)	Sedimentation coefficient (S)	Experimental molecular weight (Dalton)	Oligomeric state
CEP-1					
C-terminus	528–644	14 004	2.39	28 000	Dimer
	552–644	11 210	1.67	15 200	Monomer
	561–644	10 079	1.45	9900	Monomer
	Triple mutants	13 948	3.75	62 600	Tetramer
Dmp53					
C-terminus	315–385	8560	3.07	32 000	Tetramer
	315–361	5422	1.33	12 500	Dimer

Triple mutants: K544M, R551L, E552L.

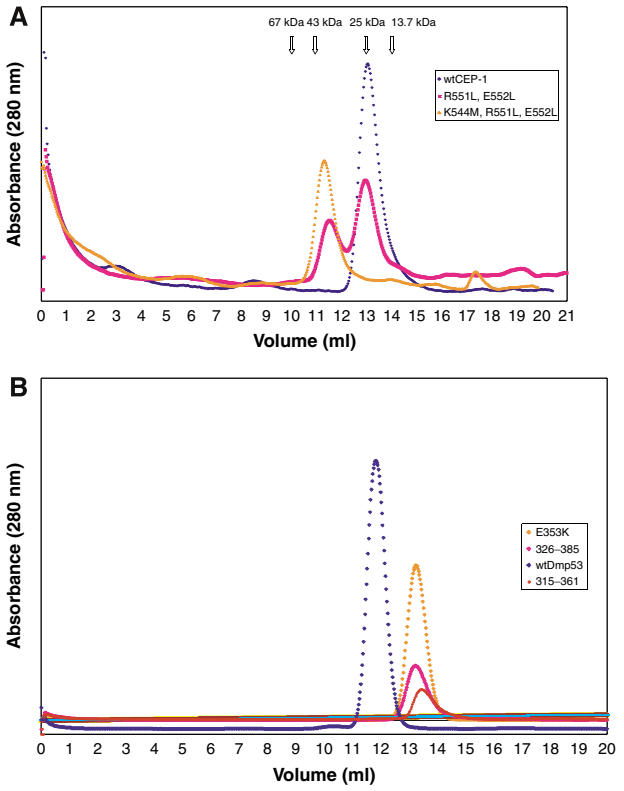

Figure 2 Gel filtration curves of the C-terminus of CEP-1 and Dmp53. (**A**) Gel filtration curve of the C-terminus of CEP-1 and two mutant proteins. The R551L, E552L mutant displays an equilibrium between dimer and tetramer. In the K544M, R551L, E552L triple mutant, this equilibrium is strongly shifted toward the tetramer. (**B**) Gel filtration experiments with the C-terminus of Dmp53 and different mutants. Mutants with deletion of strand β1 (326–385) or helix H2 (315–361), or carrying the point mutation E353K all convert the tetramer into a dimer.

involve the dimer interface. In p53, the dimer interface is stabilized by hydrophobic interactions between F338 from the α-helix of one monomer with F328 of the β-strand of the other monomer. In CEP-1, this dimer interface is maintained by interaction between Y542 and the aliphatic part of the side chain of R533, confirmed by NOEs between the β, γ, and δ protons of R533 and the δ and ε protons of Y542.

The most significant difference between CEP-1 and other members of the p53 protein family is its oligomerization property. While all other p53 protein family members form a tetramer through the OD, CEP-1 forms a dimer (Figure 2A; Table I). The typical OD of p53 family members is a dimer of dimers, involving two separate and distinct interfaces (Jeffrey *et al*, 1995) (Figure 4A). The first dimerization interface consists of an antiparallel β-sheet formed by the β-strand of each monomer, with additional contacts to the α-helix. This dimerization interface is, as described above, conserved in CEP-1. The second dimerization interface in p53 is formed by interaction of the two helices of each dimer with the corresponding helices of the second dimer, thus creating a four-helix bundle. Key residues of the hydrophobic core of this tetramerization interface in p53 are M340, L344, A347, L348, and L350 from each helix (Chene, 2001) (Figure 4A). A structural alignment of the CEP-1 OD with other members of the p53 protein family shows that M340, A347, and L348 are replaced by K544, R551, and E552 in CEP-1 (Figures 3A and B). This substitution forms a ring of charged residues that encircles F548 from each monomer in the center of the ring (Figure 4B). If the OD of CEP-1 formed a tetramer with similar packing arrangement as the human p53 OD, then this electrostatic charged ring would form unfavorable interactions in the tetrameric interface due to charge repulsion. Thus, the lack of charge complementarity for residues K544, R551, and E552 explains the inability of the OD in CEP-1 to form a tetramer.

To test this hypothesis, we conducted two series of mutations toward converting the OD of CEP-1 from a dimer into a tetramer. Upon mutation of R551 and E552 to leucine, we observed an equilibrium of two oligomeric forms, with roughly 33% of CEP-1 in a tetrameric and 67% in a dimeric form (Figure 2A). Additional mutation of K544 to methionine transforms the OD of CEP-1 completely into a tetrameric state, as indicated by gel filtration and confirmed by analytical ultracentrifugation (Figure 2A; Table I). It is also worth noting that our constructs contained the whole C-terminus of CEP-1, which includes the SAM domain. Thus, it excludes the possibility that the SAM domain could contribute to tetramerization, since tetramerization can be achieved solely by mutations in the OD of CEP-1.

The SAM domain of CEP-1

Another significant difference between the OD of CEP-1 and that of other p53 family members is its close interaction with the C-terminal SAM domain. SAM domains are found in many different proteins, including ephrin receptors, transcriptional repressors, as well as the p53 homologues p63 and p73. They are composed of four short helices followed by a long helix that completes its hydrophobic core. SAM domains are protein–protein interaction modules that have the ability to homo- and hetero-dimerize, as well as oligomerize. In the ephrin receptor, the SAM domain forms a dimer in the crystal structure involving the last helix and loop 3 (between helices 3 and 4) (Thanos *et al*, 1999). In CEP-1, the equivalent loop 3 region is exposed to the solvent, and the C-terminus of the last helix interacts with the helix in the OD domain; thus, it is unlikely that this site constitutes a dimerization surface with another SAM domain. In the p63/p73 protein family, the SAM domain is a monomer in isolation and, to date, no homo-dimerization tendency has been identified (Chi *et al*, 1999). Similarly, the SAM domain of CEP-1 exists as a monomer in isolation (Table I), and no contacts are made between the SAM domains in the full-length construct of the C-terminus of CEP-1. Recently, SAM domains in the Smaug family, a translational repressor, have also been found as part of RNA binding modules that recognizes RNA hairpins with the loop sequence CUGGC (Aviv *et al*, 2003; Green *et al*, 2003). The RNA interaction surface on the SAM domain is composed of the N-terminus of helix 5 and loop 1 (between helices 1 and 2), which has a positively charged surface. In CEP-1, this region does not have a highly positive charged surface, so it is unlikely that the SAM domain in CEP-1 interacts with RNA as in Smaug.

Characterization of C-terminus of Dmp53

In addition to CEP-1, the *Drosophila* homologue of p53, Dmp53, shows low sequence homology to other p53 protein family members, again mainly confined to the DBD. The

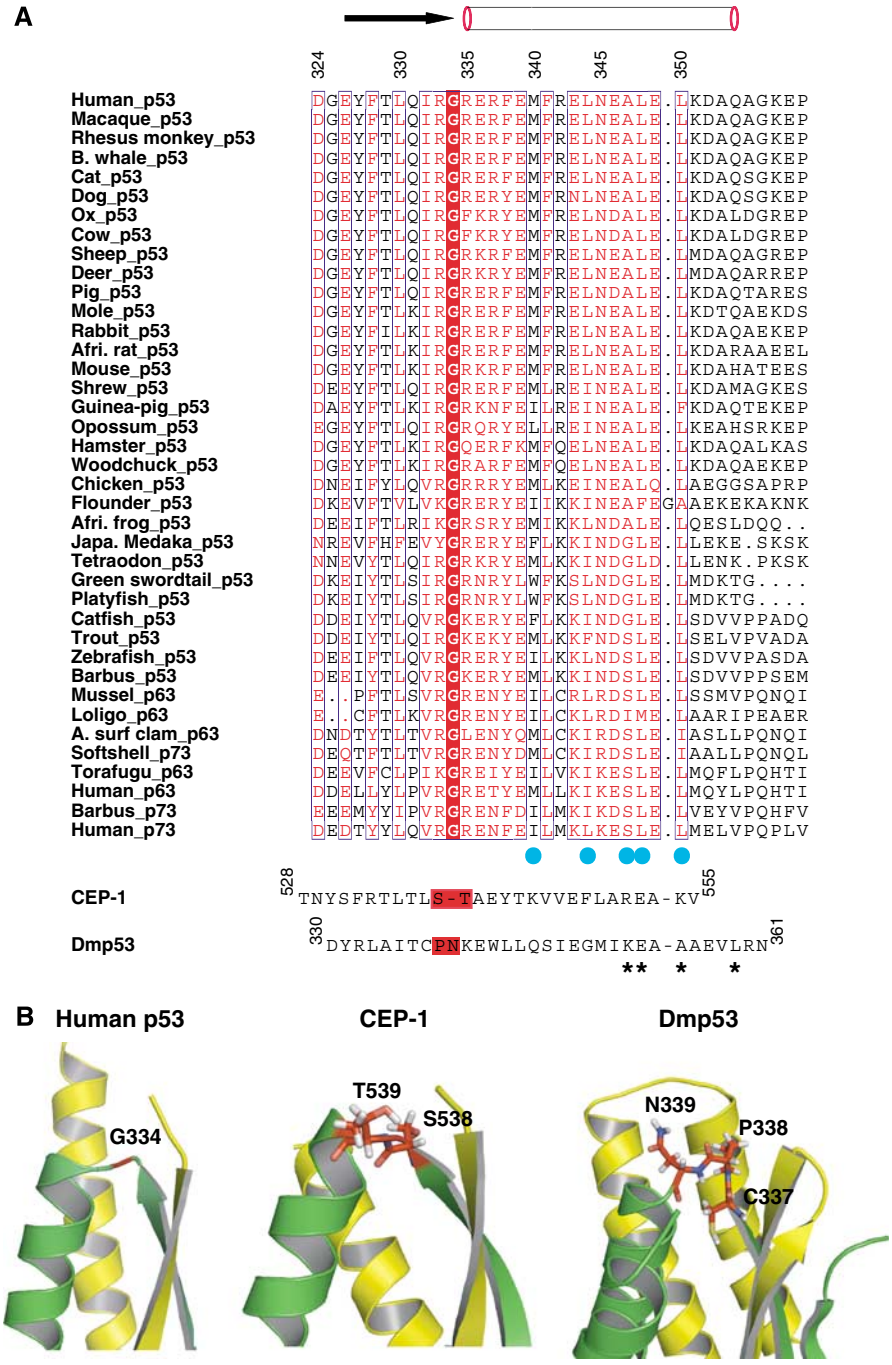

Figure 3 Sequence divergence of the OD in CEP-1 and Dmp53 relative to other p53 protein family members. (**A**) The signature hinge residue G334 (human p53 numbering) is conserved across many different species, as well as the five residues, marked by cyan dots, that are involved in the tetrameric interface. In both CEP-1 and Dmp53, the sequence similarity is very low in comparison to other members of the p53 protein family, especially at the tetrameric interface. The dipeptides in CEP-1 and Dmp53 that replaced the glycine residue are shaded in red. Residues marked by an asterisk in the Dmp53 sequence are important for the tetramerization interface. (**B**) The highly conserved glycine residue (colored in red) of the p53 OD is replaced with the dipeptides, S538/T539 in CEP-1 and P338/N339 in Dmp53.

protein sequence length suggests it is more related to p53. Dmp53 induces apoptosis through the transcription of genes, such as *hid*, *reaper*, and *sickle* (Brodsky *et al*, 2004). The functional similarity of Dmp53 and CEP-1 and their low sequence homology to other p53 protein family members prompted us to investigate the oligomeric state of Dmp53 as well. We have purified the OD of Dmp53 (315–361, Dmp53 numbering in Ollmann *et al*, 2000), and shown by gel filtration as well as analytical ultracentrifugation that it forms a dimer (Figure 2B; Table I). NMR investigations of this dimeric form, however, have revealed that it exhibits conformational heterogeneity characterized by broad peaks in the NMR spectrum (Figure 5). Similar to human p53, Dmp53 also contains a stretch of highly basic residues C-terminal to the OD. Others have shown that this basic region in human p53 serves as a regulatory tail that controls DNA

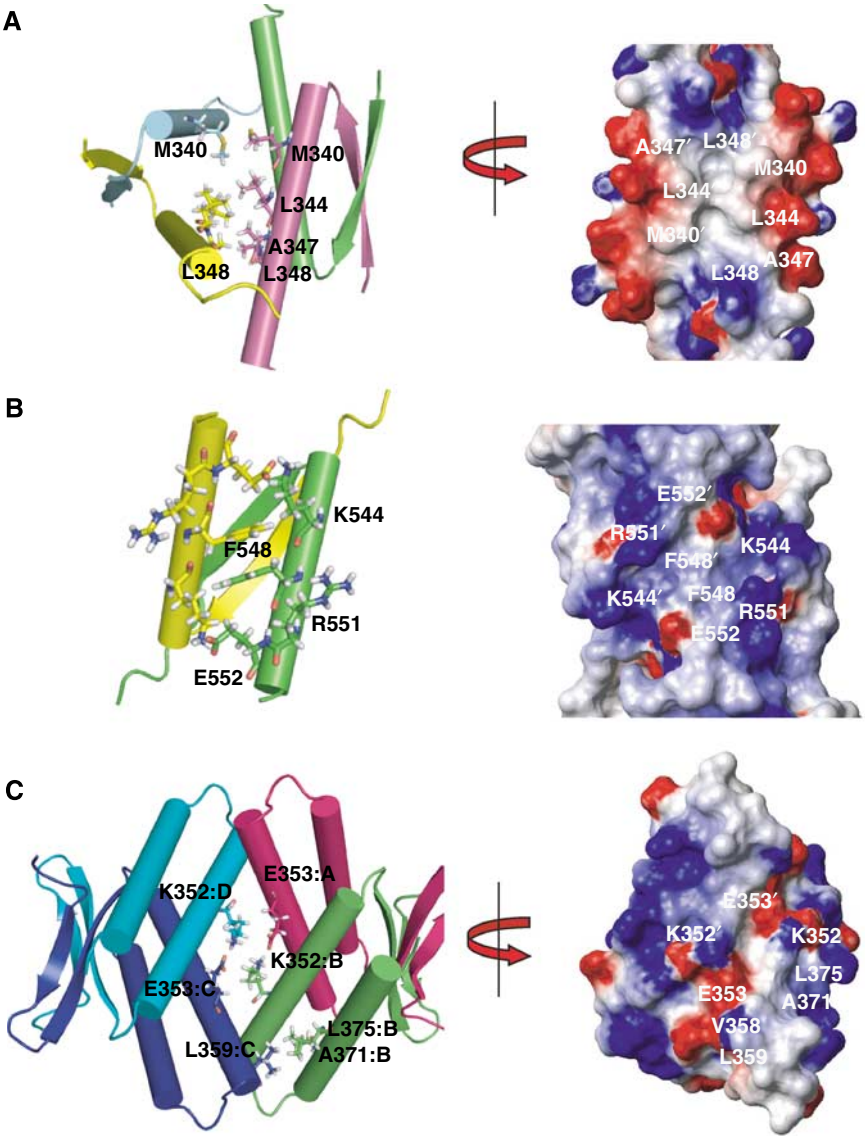

Figure 4 The tetrameric interface in the OD of human p53, CEP-1, and Dmp53. (**A**) In human p53, M340, L344, A347, L348, L350 constitute the tetrameric interface. The electrostatic map shows that the tetrameric interface is mostly hydrophobic. Positively charged surface is colored blue, negatively charged surface is colored red, and non-polar surface is colored white. The second dimer subunit is removed for clarity. (**B**) The electrostatic map of the potential tetramerization interface of the CEP-1 OD shows the charged residues (K544, R551, E552) surrounding F548 from each monomer. (**C**) The tetrameric interface in Dmp53 consists of a charged cluster in the center, and hydrophobic contacts on the outer edge of the interface. K352 and E353 from one monomer form salt bridges with K352 and E353 across the tetrameric interface. At the outer edge, L359 from helix H1 contacts A371 and L375 in helix H2 across the tetrameric interface.

binding affinity (Ahn and Prives, 2001). To test whether this region has any structural function in Dmp53, and in particular, if it stabilizes the dimer, we purified a construct that includes the OD and this C-terminal region (additional 24 residues). Surprisingly, this extended OD forms a tetramer and its NMR spectra indicate the existence of a well folded single conformation (Figure 5; Table I). Structure determination by NMR spectroscopy shows that the C-terminal 24 amino acids form an α-helix that packs against the α-helix that forms the canonical OD (Figures 1B and D). In addition, the N-terminus is extended relative to the p53 OD and contains an additional β-strand that forms together with the canonical β-strand and the corresponding elements of another monomer an antiparallel four-stranded β-sheet. Thus the Dmp53 OD domain contains both an N-terminal extension (the β1-strand) as well as a C-terminal extension (the H2 α-helix) relative to human p53 and can best be described as a four-helical hairpin bundle sandwiched between two four-stranded β-sheets. In Dmp53, the conserved glycine residue in the canonical OD is replaced by proline and asparagine (Figures 3A and B). The tetramerization interface is built by the inner four helices that pack against each other similar to the four helices of human p53 (Figure 4C). In contrast to human p53, the tetramerization interface of Dmp53 contains a central cluster of charged residues made up of lysine 352 (K352) and glutamic acid 353 (E353) from each helix, which can form four salt bridges that constitute the core of the tetramerization interface. Mutating E353 of this cluster to lysine disrupts the electrostatic pairings and destroys the tetramerization interface, thus forming a stable dimer (Figure 2B). The core of the tetramerization interface is shifted by one helical turn toward the C-terminus compared

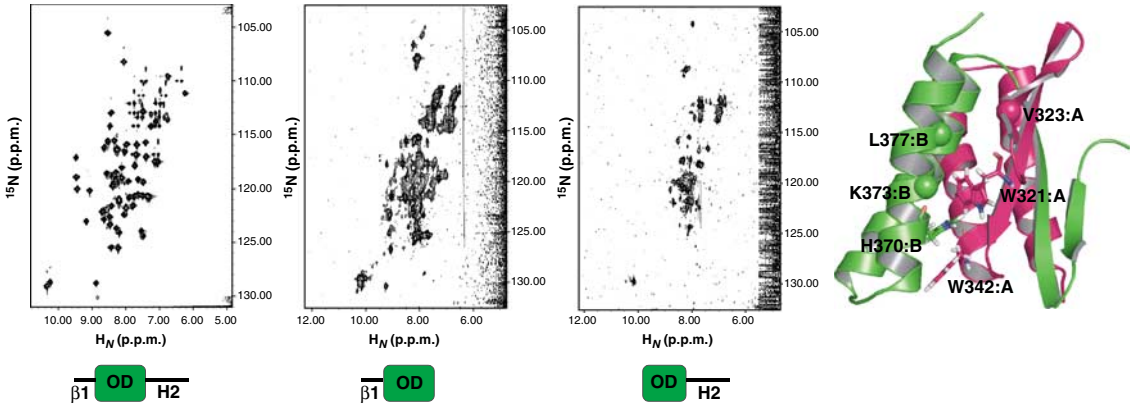

Figure 5 OD of CEP-1 and Dmp53 are stabilized by additional structural elements. In Dmp53, extensive contacts between the additional structural elements (strand β1 and helix H2) and the canonical OD of p53 are present with 1902 Å^2 of buried solvent accessible area. Deletion of either structural element destabilizes the OD and results in multiple conformations, as shown by broad peaks in the NMR spectra.

to human p53 (Figure 3A). In addition, the inner helix of each monomer (H1:C) also packs across the tetrameric interface to the C-terminal helix (H2:B) of another monomer, with contacts between L359 from the inner helix and A371 and L375 of the C-terminal helix (Figure 4C).

Further contacts of the C-terminal helix (H2:B) that contribute to the dimer interface involve a surface formed by the inner helix (H1:B) of the same monomer and the N-terminal β-strand (β1:A) of another monomer within a dimeric unit. In addition, this structural arrangement of the dimeric unit is capped by interaction of H370 (H2:B) with two tryptophans from the β-strand (W321, β1:A) and the inner helix (W342, H1:A) of the other monomer (Figure 5). The involvement of the C-terminal helix in both the dimeric as well as the tetrameric interface explains why its deletion destabilizes the structure and results in a conformationally unstable dimer. It also predicts that removing the first β-strand should have a similar destabilizing effect as deleting the C-terminal helix. Indeed, the loss of strand β1 forms a dimer that shows broad peaks in NMR spectra as well as a non-sigmoidal transition in CD-melting experiments, suggesting that it is conformationally unstable (Figures 2B and 5; Supplementary Figure S4). These results demonstrate that the minimal OD, consisting of one β-strand followed by one helix, as identified in human p53, does not form a stable tetrameric structure in Dmp53. The additional N-terminal (β-strand) and C-terminal (α-helix) extensions play a key role in stabilizing Dmp53.

The SAM domain stabilizes the OD in CEP-1

Based on these observations with Dmp53, we asked if the SAM domain in CEP-1 might have a similar stabilizing function as the C-terminal helix in Dmp53. The absence of the SAM domain in the C-terminus of CEP-1 (528–555) indeed forms a less stable structure, as indicated by the lack of a sharp sigmoidal transition in the temperature denaturation curve as well as the absence of an alpha helical pattern (two minima at 208 and 222 nm) in the wavelength scan, as observed by CD spectroscopy (Supplementary Figure S2). Since the expression level of the isolated OD decreased significantly relative to the full-length C-terminus of CEP-1, we were not able to record an NMR spectrum.

Evolution of the p53 protein family

The structural analysis of the C-termini of CEP-1 and Dmp53 provides an opportunity to trace the evolution of the p53 protein family, based on the presence of the SAM and structure of the OD domains in the C-terminus. While the positioning of nematodes in a phylogenetic tree remains controversial (ecdysozoa versus coelomata) (Aguinaldo *et al*, 1997), we placed the currently available sequences of the p53 protein family members from the NCBI library into a phylogenetic tree constructed from the ecdysozoa perspective, which is the currently most favored view. As shown in Figure 6, the SAM domain has been integrated early into the p53 protein family, since it can be identified in the protostome branch represented by nematodes and molluscs, as well as in the early deuterostomes such as echinoderm and urochordate. In phylum Urochordata, a gene duplication seems to have occurred that results in a p53 form without a SAM domain, which resembles vertebrate p53 in addition to a p63/p73-like form. The loss of the SAM domain occurred also within the protostome branch in the Arthropoda phylum represented by Dmp53. However, in arthropods, a C-terminal helix replaces the SAM domain and serves a similar functional role in stabilizing the OD. While the functional role of the SAM domain in human p63 and p73 remains elusive, the existence of the SAM domain in p53-like molecules in the protostome phylum underscores its important role within the p53 protein family, such as its contribution in stabilizing the OD. The current study provides additional evidence that the vertebrate p53 is a recent evolutionary development, and p63 and p73 are the more ancestral family members.

However, this ecdysozoa-based phylogenetic tree does not permit a direct correlation of the oligomeric state and the evolutionary development of p53. Instead this tree would either suggest that the dimeric CEP-1 has de-evolved from an ancestral tetrameric p53, or that tetramerization has evolved twice independently. Given the high sequence identity between the oligomierzation domains of vertebrates and molluscs, this last hypothesis seems less likely. Alternatively, a phylogenetic tree that is based on a coelomata view places nematodes in an earlier phylogenetic branch than both *Drosophila* and vertebrates (Figure 6B). This hypothesis supports the view that p53 evolved from a dimeric molecule

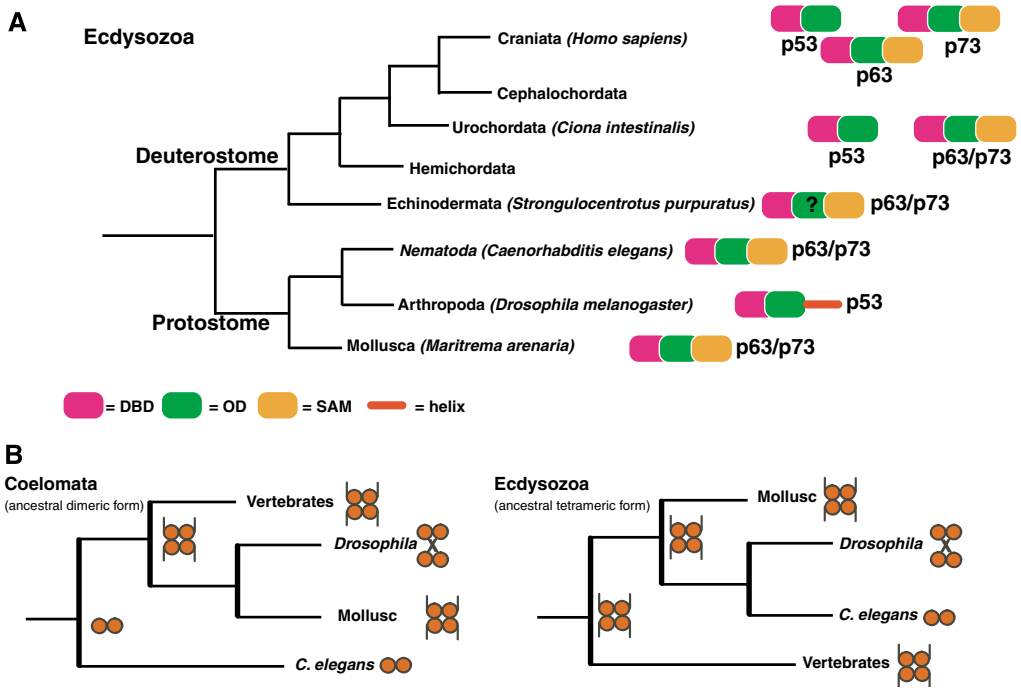

Figure 6 Different hypothesis on the evolution of the p53 protein family. (**A**) A phylogenetic tree of the p53 protein families based on the ecdysozoa topology shows that the SAM domain has appeared in all protostomes identified to date that have p53-like molecule, except in the arthropoda phyla, in which a helix has replaced the SAM domain in stabilizing the OD. In the deuterostome branch of the urochordate phylum, the p53 protein first appeared in two isoforms: one form with the SAM domain, and another form without the SAM domain. (p63/p73) denotes that the protein could be classified as either p63 or p73. Question mark indicates no significant sequence homology by sequence alignment. (**B**) A comparison of a phylogenetic tree based on the ecdysozoa topology and the coelomata topology predicts different oligomeric states for the ancestral p53 form. Each red circle represents a monomer and each bar symbolizes the C-terminal tail. In the coelomata topology, a dimeric form of p53 represents the ancestral form, which evolved into the current vertebrate and mollusc p53 tetrameric form, as well as into the *Drosophila* form that uses a different mode of tetramerization. In the ecdysozoa topology, the ancestral form was either a tetramer, and the dimeric CEP-1 form evolved from this tetrameric state, or the ancestral form was a dimer, and tetramerization in the vertebrate branch and in the mollusc branch has evolved independently twice.

into a tetrameric form with further divergence between molluscs and arthropods. A variation of this phylogenetic tree places the Arthropoda phylum before the divergence of molluscs and vertebrates, and would be most consistent with our structural data (Sidow and Thomas, 1994; Hedges, 2002).

Discussion

The low homology of the C-terminus of CEP-1 and Dmp53 with other p53/p63/p73 proteins suggests that significant differences in both structure and function might exist in comparison with other p53 protein family members (Lu and Abrams, 2006). Indeed, this study shows that the C-terminus of CEP-1 is a dimeric molecule, making the *C. elegans* form of p53 the first native dimer of the entire protein family. The existence of a natural dimeric form of p53 reaffirms previous identification of residues that are essential for tetramerization (Mateu and Fersht, 1998, 1999), since the C-terminus of CEP-1 can be converted into a tetrameric form by mutations of residues at the tetrameric interface (K544, R551, E552) into hydrophobic residues.

The structure of the OD of CEP-1 illustrates a conserved strategy in the p53 protein family to utilize the electrostatic nature of the tetrameric interface of the OD to control the oligomeric state of the protein. McCoy *et al* (1997) had reported that mutating three key residues of the tetrameric interface of the OD in human p53 (M340K, F341I, L344Y) results in a dimeric molecule, with the orientation of the helices switched from antiparallel to parallel. Interestingly, in CEP-1, the corresponding residues (K544, V545, F548) show greater similarity to the p53 mutant sequence than to the p53 wild-type sequence, yet the structure of the CEP-1 OD resembles the structure of the p53 wild-type OD more closely, by adopting an antiparallel packing of helices (angle between the helices of one dimer unit: p53 156°; CEP-1 125°; mutant 78°). Mapping CEP-1 residues onto the structure of the p53 mutant shows that CEP-1 retains the antiparallel orientation due to unfavorable charge repulsion by the two lysines (K554) located at the C-terminus of the helix. In the mutant p53 molecule, these lysines are replaced by two leucines (L350) that form favorable hydrophobic packing, thus enabling a parallel orientation of the helices. The structural and mutational analyses of CEP-1 show that controlling the electrostatic nature of the interface allows the formation of both dimers as well as tetramers, without changing the antiparallel topology of the OD.

The presence of the SAM domain at the C-terminus of CEP-1 is a surprise discovery, since sequence alignment does not reveal its existence. The SAM domain of CEP-1 is C-terminal to the OD and makes contacts with the OD through the last helix, but not to the SAM domain from the other monomer. In other SAM domain containing proteins, the SAM domain functions as a homo- or hetero-dimerization domain, for example, in the ephrin receptor B2 and the SAM domains of Ste11 and Ste50 in *Saccharomyces cerevisiae* (Thanos *et al*, 1999; Kwan *et al*, 2006). Based on our structure, however, the

SAM domain of CEP-1 does not seem to play a direct role in oligomerization. This result also has implications for p63 and p73, which both contain SAM domains at their C-terminus. The structure of the C-terminus of CEP-1 as well as the incapability of the isolated CEP-1/p63/p73 SAM domains to oligomerize suggests that SAM domains in the p53 protein family also do not form homo dimers or oligomers in the context of the full-length protein.

Despite the observation that CEP-1 is a dimer in solution, the possibility exists that two CEP-1 dimers can form a functional tetramer through cooperative binding to their promoter sites. Analyzing the promoter sequences of known targets of CEP-1 and Dmp53 should reflect the difference in the oligomeric state of both proteins *in vivo*. The promoter region of *egl-1* (Hofmann *et al*, 2002), a CEP-1 inducible gene, contains only a half site (AAACAAGCTT), which satisfied the p53 consensus sequence motif for a dimeric p53 molecule (CEP-1) (el-Deiry *et al*, 1992). In contrast, the *Drosophila* p53 responsive gene, *reaper*, has two half sites (TGACATGTTT/GAACAAGTCG) (Brodsky *et al*, 2000), which allows binding of a tetrameric p53 molecule (Dmp53).

The hypothesis that a dimeric p53 is the ancestral form is further supported by a study on the formation of human p53 in rabbit reticulate lysate (Nicholls *et al*, 2002). This study showed that human p53 first forms a dimer co-translationally, and that tetramers are only formed at a later stage post-translationally. It was further shown that when mutant and wild-type p53 are coexpressed, there is only one form of heterotetramer, which consists of a dimer of wild type and a dimer of mutants. Thus, even after millions of years of evolution, the mammalian p53 still retains a dimeric building block as its basic unit.

Dmp53 and perhaps the entire arthropod phylum have adopted a different tetramerization mode by utilizing an additional helix C-terminal to the canonical mammalian p53 OD, and an additional β-strand before the OD. By sequence alignment with CEP-1, one would conclude that Dmp53 would be a dimer due to the presence of the charged resides K352 and E353 in Dmp53. However, the helix and the additional β-strand transform Dmp53 into a tetramer. The additional β-strand provides a stable dimeric unit, which positions helix 2 of Dmp53 in the correct orientation to interact with the helix 1 of another dimeric unit, and concomitantly allows attractive electrostatic interactions along the tetrameric interface between the two charged residues. The composition of this unique mode is necessary for tetramerization, since a deletion of either additional element results in incorrect topology of helices, thus reverting to the dimeric state as in CEP-1.

It is interesting to note that only the C-terminus of the p53 protein family has undergone significant evolutionary changes (dimer or tetramer, presence or absence of the SAM domain), while the DNA specificity and the structure of the DBD have remained basically unchanged. The X-ray crystal structure of the DBD of CEP-1 was recently solved (Huyen *et al*, 2004). It demonstrates that despite a low sequence homology of only 15%, the core structure is very similar, and the DNA binding sequence specificity of CEP-1 is virtually identical to human p53, even though loop L1, a part of the DNA binding interface in the DBD of human p53, adopts a different conformation in CEP-1. In Dmp53, the DBD is 25% identical to human p53, which is higher than in the case of CEP-1. This higher sequence identity in Dmp53 and the high conservation of the structure and function of the DBD of CEP-1 predict that the structure of the Dmp53 DBD and its DNA binding specificity are also highly conserved. Despite this high conservation in the DBD, the biological function of the individual members of the p53 protein family is distinct, and sequences C-terminal to the DBD show very significant divergence, thus suggesting that these C-termini play an important role in specifying the biological function of the individual family members.

The availability of structural and biochemical data for CEP-1, Dmp53, and human p53 in combination with p53 sequence data from many species sheds some light on the ancestral form of p53 and its evolutionary development. It was estimated that *C. elegans* and *Drosophila* have diverged from vertebrates 550 millions years ago, thus CEP-1 and Dmp53 could resemble the ancestral form of p53. Despite the lack of a SAM domain in Dmp53, the additional structural elements (the first β-strand and the last α-helix) serve a similar stabilizing function as the SAM domain in CEP-1. Oligomerization in the ancestral p53 forms most likely required either the fusion of two domains (like in CEP-1) or additional structural elements (like in Dmp53). The minimal OD found in vertebrate p53 that can form stable tetramers by itself is probably a later evolutionary result, while the C-terminal tail that is a necessary structural element in Dmp53 became an important regulatory region including many sites for posttranslational modifications.

In summary, we have determined the structure of the C-terminal domain of CEP-1 and Dmp53, and show nature's multifaceted means to achieve oligomerization in p53, besides the canonical form found in mammalian p53. Furthermore, we have shown that additional structural elements identified in CEP-1 and Dmp53 that are not present in human p53 are necessary for the integrity of the OD. A loss of these elements results in conformationally unstable structures, and in Dmp53, leads to a change in the oligomeric property. The structural investigations described here suggest an evolutionary path from an ancestral dimeric form over tetrameric forms that need additional stabilization elements to the minimal tetramerization domain known from mammalian p53.

Materials and methods

Protein expression and molecular clonings

All CEP-1 and Dmp53 constructs used for structural studies were cloned into plasmid pGEX-6P-2 (Amersham Bioscience) or pBH4 (gift from Wendell Lim laboratory) using *Bam*HI and *Xho*I sites. Mutagenesis constructs of both CEP-1 and Dmp53 were prepared by the QuickChange protocol from Stratagene. BL21 cells were grown to an OD_{600} of 0.8 and induced with 500 μM IPTG at 25°C for 8 h. The proteins were purified as described in the manufacturer's protocol, cleaved by precision protease for the pGEX plasmid or by TEV protease for the pBH4 plasmid, and further purified on a Superdex-75 gel filtration column. Protein samples were stored in a buffer containing 20 mM sodium phosphate (pH 7.0), 100 mM sodium chloride, and 0.03% sodium azide. For the expression of ^{15}N- and ^{15}N/^{13}C-labeled proteins, bacteria were first grown in LB media to an OD_{600} of 0.8, then transferred to M9 minimal media with the appropriate isotopic components, and induced under the same condition as described above. Protein samples used to obtain inter-monomer NOEs consisted of an equal ratio of ^{15}N- and ^{13}C-labeled proteins. For CEP-1, 2 weeks of equilibration time

was needed to obtain signals in the experiment. For Dmp53, equal molar ratio of proteins were mixed, denatured in 6 M guanidium hydrochloride, and then refolded in the buffer described above.

NMR experiments and structure calculations

Backbone residues of CEP-1 and Dmp53 were assigned using the TROSY version of HNCA and HNCOCA. For CEP-1, specific labeling of lysine, tyrosine, and leucine were used to confirm assignments. Distance constraints were derived from ^{15}N-NOESY-HSQC, and ^{13}C-NOESY-HSQC. Aromatic protons were assigned based on 2D-D_2O-NOESY, 2D-D_2O-TOCSY, and non-constant time 3D-^{13}C-NOESY. Inter-monomer NOEs were obtained through a 4D constant time J-Resolved NOESY (Melacini, 2000), measured with a 1:1 mixture of ^{12}C- and ^{13}C-labeled proteins in both cases. In the case of Dmp53, the protein had to be denatured first with guanidium hydrochloride before mixing of the ^{12}C- and ^{13}C-labeled proteins, and subsequent refolding by dialysis. For CEP-1, 44 unambiguous inter-monomer distance constraints, all located in the β-sheet, were identified from the 4D J-Resolved NOESY. Overall, 114 inter-monomer NOEs were assigned. In the case of Dmp53, 184 NOEs obtained from the 4D J-Resolved NOESY and an overall of 240 inter-monomeric NOEs were used. Of these inter-monomeric NOEs, 46 are located in the β-sheet, 130 between a β-strand of one monomer and an α-helix of the other monomer within one dimeric unit, and 31 between helices of different monomers within a dimer were observed. In addition, 33 NOEs across the tetrameric interface were identified. Dihedral angle constraints were derived from TALOS based on chemical shifts of N, CA, HA, and CB (Cornilescu *et al*, 1999). Hydrogen bond constraints of secondary structure elements were based on TALOS calculations and confirmed by characteristic NOE patterns for α-helices and β-sheets, as well as deuterium hydrogen exchange measurements. Structure calculations were carried out with Aria 1.2, with modified protocols that imposed a C_2 symmetry for CEP-1 and a D_2 symmetry for Dmp53 throughout every stage of calculation (Linge *et al*, 2001). For the structure calculation of CEP-1, unambiguous inter-monomer constraints obtained from the 4D J-Resolved NOESY were included in every iteration of the calculation. For Dmp53, unambiguous NOEs between the β2-strands of two monomers established the existence of an antiparallel inter-monomeric β-sheet. Using these unambiguous NOEs as a starting point, we first calculated dimer structures. Based on these dimer structures, the other distance constraints obtained from the 4D J-Resolved NOESY were evaluated for their consistency with the dimer structure. Several distance constraints that could not be satisfied within the dimer were assumed to be constraints across the tetrameric interface, and were used as those in the following structure calculations. Twenty structures were calculated in seven iterations, and 100 structures were calculated in the last iteration. Initial assigned peaks were separated into unambiguous and ambiguous peaks, and ambiguous peaks with multiple assignments and violated restraints were manually inspected. The new ambiguous peak list included peaks with multiple assignments and inter-monomer assignments (not obtained from the 4D constant time J-Resolved NOESY). After multiple iterations of peak inspections and structure calculations, 100 structures were calculated and the best 20 structures were used for water refinements and analysis. The structural statistics of CEP-1 and Dmp53 are listed in Supplementary Tables 2 and 3. Images were prepared with Pymol (Delano, 2002). MOLMOL was used for structural alignment of NMR-derived models and generation of the electrostatic map of the molecule (Koradi *et al*, 1996). LSQMAN was used for structural alignment of different proteins (Kleywegt, 1996) and the structure validation program of the PDB server for further structural analysis.

Circular dichroism experiments

Temperature scans (20–90°C for CEP-1, 20–100°C for Dmp53) were measured in a Jasco 810 CD spectrometer. For CEP-1 OD alone construct (528–555), it contains additional his-tags and TEV protease cleavage site. Due to the unusual circular dichroism spectra in Dmp53, the observed wavelength for temperature scan was chosen based on signal intensity. Thus, for wild-type Dmp53, CD was observed at 234 nm, Dmp53 (326–385) at 228 nm, and Dmp53 (315–361) at 220 nm. For temperature denaturation scans, ellipticity of each protein sample was converted into fractional ellipticity with respect to the signal at 100% denatured state, in order to normalize the data for all samples. Protein concentration of each construct ranged from 50 to 300 μM, in 20 mM sodium phosphate (pH 7.0) and 100 mM NaCl buffer.

Analytical ultracentrifugation experiments

Analytical ultracentrifugation runs were conducted on an Optima XL-A centrifuge (Beckman Coulter Instruments, CA). The data were collected at a wavelength of 280 nm.

Sedimentation velocity. Sedimentation velocity (SV) experiments were conducted with 200–300 μl samples in 20 mM sodium phosphate (pH 7.0), 100 mM NaCl at protein concentrations of 0.5–1 mg/ml. Absorbance data were acquired at rotor speeds of 35 000–40 000 r.p.m. and at a temperature of 20°C. The buffer density of 1.005 g/ml and viscosity of 1.031 cPoise and the protein partial-specific volumes were calculated using the software SEDNTERP, kindly provided by Dr J Philo. Data were analyzed using the c(s) continuous distribution of Lamm equation solutions with the software SEDFIT (Schuck, 2000; Schuck *et al*, 2002).

Sedimentation equilibrium. Sedimentation equilibrium experiments were conducted at 20°C at rotor speeds of 15 000 r.p.m. at an optical density of 0.283. Global non-linear regression of the experimental absorbance profiles was performed using the software SEDPHAT, kindly provided by Dr P Schuck.

Supplementary data

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

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