The EMBO Journal Vol.18 No.16 pp.4438–4445, 1999

Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain

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***p73* and *p63* are two recently cloned genes with homology to the tumor suppressor *p53*, whose protein product is a key transcriptional regulator of genes involved in cell cycle arrest and apoptosis. While all three proteins share conserved transcriptional activation, DNA-binding and oligomerization domains, p73 and p63 have an additional conserved C-terminal region. We have determined the three-dimensional solution structure of this conserved C-terminal domain of human p73. The structure reveals a small five-helix bundle with striking similarity to the SAM (sterile alpha motif) domains of two ephrin receptor tyrosine kinases. The SAM domain is a putative protein–protein interaction domain found in a variety of cytoplasmic signaling proteins and has been shown to form both homo- and hetero-oligomers. However, the SAM-like C-terminal domains of p73 and p63 are monomeric and do not interact with one another, suggesting that this domain may interact with additional, as yet uncharacterized proteins in a signaling and/or regulatory role.**

Keywords: NMR spectroscopy/p53/SAM domain/tumor suppressor

Introduction

p53 is a tumor suppressor gene and the most frequent site of genetic alterations found in human cancers (Hollstein *et al*., 1991, 1996). The p53 protein is a transcription factor that regulates the expression of a wide variety of genes involved in cell cycle arrest and apoptosis (reviewed in Donehower and Bradley, 1993; Ko and Prives, 1996; Levine, 1997). The p53 pathway is activated in response to several types of cellular stress such as DNA damage (Kastan *et al*., 1991, 1992), hypoxia (Graeber *et al*., 1996), mitotic spindle defects (Cross *et al*., 1995) and viral or oncogene activation (Vousden, 1993; Wu, 1993). Both humans and mice with germline p53 mutations develop normally, but have a greater susceptibility to a variety of cancers (Malkin *et al*., 1990; Donehower *et al*., 1992), indicating that p53 is not essential for normal development, but rather exerts a crucial checkpoint function preventing deleterious genomic damage from being propagated (Lane, 1992).

The importance of p53's activity as a DNA-binding transcription factor is underscored by the fact that the vast majority of p53 mutations found in human cancers result in a protein that is either partially or completely inactive for DNA binding (Pavletich *et al*., 1993; Di Como and Prives, 1998). Like many transcription factors, p53 is a modular molecule with a conserved transcriptional activation domain, DNA-binding domain and an oligomerization domain that mediates tetramerization (Pavletich *et al*., 1993). The products of two recently cloned genes, *p73* (Jost *et al*., 1997; Kaghad *et al*., 1997) and *p63* (also called KET, p51 and p40; Schmale and Bamberger, 1997; Osada *et al*., 1998; Trink *et al*., 1998; Yang *et al*., 1998), share strong sequence homology with the transactivation, DNA-binding and oligomerization domains of p53 (see Figure 1). Human p63 and p73 also share functional homology with p53; both proteins are capable of binding to p53-responsive elements, can transactivate p53-responsive genes and can induce apoptosis when overexpressed (Jost *et al*., 1997; Kaghad *et al*., 1997; Yang *et al*., 1998; Zhu *et al*., 1998; Di Como *et al*., 1999). These structural and functional similarities to p53 suggest that p63 and p73 may activate similar growth-suppressive pathways as p53. However, p63 and p73 do not appear to be responsive to the same genotoxic stresses that trigger p53 up-regulation (Kaghad *et al*., 1997). Rather, they appear to be involved in more fundamental pathways associated with development and differentiation (Schmale and Bamberger, 1997; Almog and Rotter, 1998; Mills *et al*., 1999; Yang *et al*., 1999). A gene from squid which originally was thought to be squid p53 (Ishioka *et al*., 1995) appears to be more closely related to p73 and p63, suggesting that the specialized role of p53 may have evolved from a more basic and primitive p63/p73-like gene.

p63, p73 and squid p53 all contain a C-terminal extension not found in human p53 (Figure 1). The sequence alignment of the C-terminal regions of p63α, p73α and squid p53 (Figure 1) reveals a 100 residue region that is conserved among all three proteins. This C-terminal region of p63 and p73 is subject to alternative splicing, resulting in multiple isoforms of both proteins (Kaghad *et al*., 1997; De Laurenzi *et al*., 1998; Yang *et al*., 1998). There is evidence that these C-terminal splice variants may also have differential transcriptional and biological properties. For example, p63γ was able to transactivate a p53 reporter gene to a level ~80% of that of wild-type p53, whereas p63α was unable to activate the same reporter gene (Yang *et al*., 1998). Differences in the degree of transactivation by p73 isotypes have also been reported (De Laurenzi *et al*., 1998; Zhu *et al*., 1998; Di Como *et al*., 1999). These data suggest that the C-terminal region of p73α and p63α may have regulatory properties that modulate the transcriptional activity of these proteins and therefore may contribute to the distinctive biological properties of these proteins relative to p53.

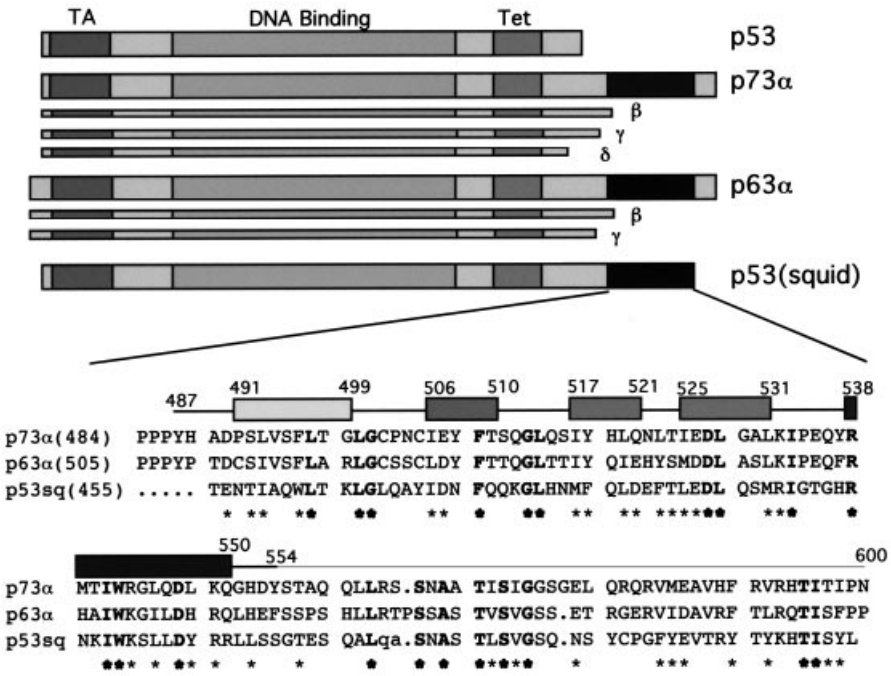

Fig. 1. A schematic of the primary structure of p53 family members. Conserved regions are indicated as: TA, transcriptional transactivation domain; DNA Binding, DNA-binding domain; Tet, tetramerization domain. A sequence alignment of the C-terminal conserved domain is shown for human p73, p63 and squid p53. Residues identical in all three proteins are shown in bold. Positions with similar residues are indicated with an asterisk. The secondary structure determined here by NMR is indicated along the top. Helices are shown as rectangles.

A distant relationship at the sequence level has been noted between a portion of the C-terminal conserved region of p53 homologs and members of the SAM (sterile alpha motif) superfamily (Schultz *et al*., 1997; Bork and Koonin, 1998). Despite sequence identities of $<18\%$, it was proposed that p73 contains a SAM domain protein–protein interaction module (Bork and Koonin, 1998). SAM domains are small (70 residues), protein–protein interaction modules found in a variety of proteins involved in developmental regulation (Schultz *et al*., 1997). The most notable type of protein–protein interaction identified for SAM domains to date is that of homo- and hetero-oligomerization among similar SAM domains. The SAM domains of the ephrin (Eph) receptors have been shown to homo-dimerize in a crystal environment and weakly dimerize in solution (Stapleton *et al*., 1999; Thanos *et al*., 1999). This SAM-mediated dimerization has been proposed as a mechanism for Eph receptor activation. The pointed domain (a SAM-like domain) of the ETS transcription factor, TEL, can also self-associate (Jousset *et al*., 1997), and has been found fused to other signaling and regulatory proteins in many leukemias, resulting in constitutive activation of these fusion proteins via dimerization (Golub *et al*., 1994; Golub, 1997). Finally, members of the polycomb group of homeotic transcriptional regulators form homo- and hetero-oligomers via their SAM domains (Peterson *et al*., 1997; Kyba and Brock, 1998).

We have used nuclear magnetic resonance (NMR) spectroscopy to determine the three-dimensional solution structure of the conserved C-terminal domain from human p73. Residues 487–554 of this region form a five-helix bundle with marked similarity to the structures of SAM domains from Eph receptor tyrosine kinases (Smalla *et al*., 1999; Stapleton *et al*., 1999; Thanos *et al*., 1999). This structural homology with a putative protein–protein interaction and signaling domain suggests a similar role for this region in p63/p73 function.

Results

p73(487–554) contains a folded, globular α-helical domain

Initial NMR analysis of p73(487–600), which contains the entire conserved domain, indicated that this protein fragment contains both a structured, globular region and a significant proportion of random coil-like residues (Figure 2, blue). This protein was very susceptible to degradation by trace proteases in the sample, also suggestive of the presence of a region that is both solvent accessible and flexible. N-terminal sequencing and mass spectroscopy of the proteolytic fragments indicated that there was a stable, protease-resistant domain at the N-terminus of p73(487–600) with a mol. wt of ~8 kDa. Based on this result, we subcloned an additional construct of p73 corresponding to residues 487–554 and expressed and purified the corresponding protein domain. NMR ^{15}N-HSQC analysis of this domain showed that the resonance frequencies of the dispersed cross-peaks were the same as those in p73(487–600), indicating that the globular domain is not significantly influenced by residues 555–600 (Figure 2, red). Furthermore, p73(487–554) lacked the intense, overlapping peaks in the central part of the spectrum, confirming that these peaks correspond to an unstructured C-terminal region. Comparison of the circular dichroism spectra of the two proteins indicates that p73(487–554) retains the same amount of α-helical structure as p73(487–600) (data not shown). These data taken together demonstrate that residues 487–554 of p73 form

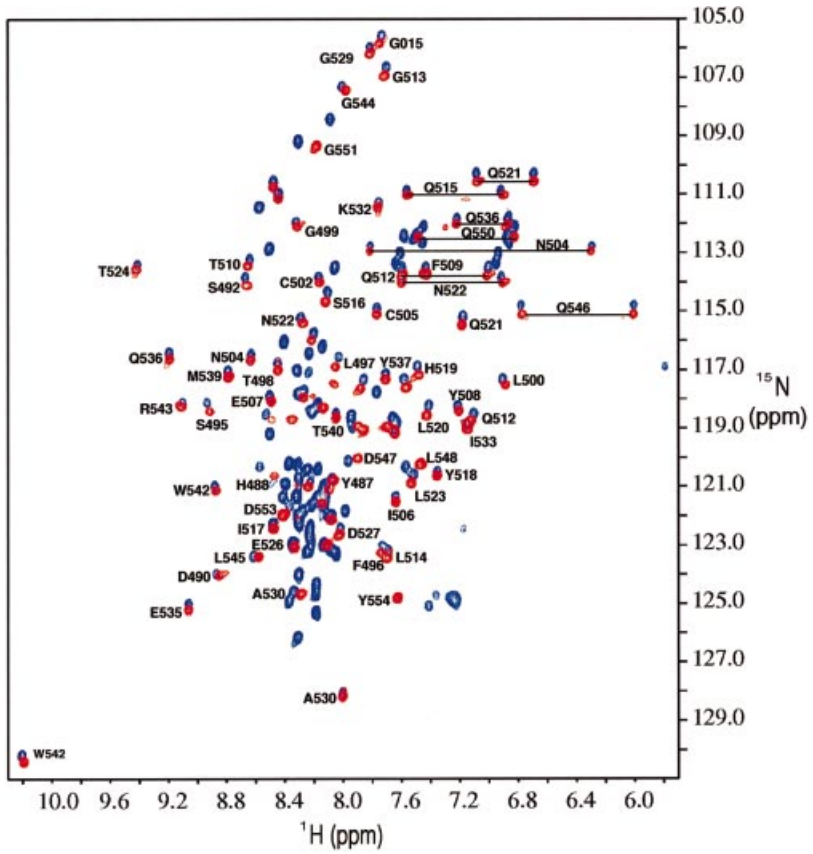

Fig. 2. A superposition of the two-dimensional ^{15}N-HSQC NMR spectra of p73(487–600) in blue and p73(487–554) in red. The cluster of intense blue peaks between 8.0 and 8.5 p.p.m. (^{1}H dimension) in p73(487–600) is indicative of flexible, unstructured regions of polypeptides. Most of these peaks are absent from the spectrum of p73(487–554). Peaks assigned to specific residues of p73(487–554) are labeled.

Table I. Structure statistics for the 18 lowest energy structures of p73(487–554)

R.m.s.d.s from experimental restraints[a]		
NOE distance restraints (1166) (Å)	0.017 ± 0.003	
Unambiguous (1053)	0.016 ± 0.003	
Ambiguous (93)	0.018 ± 0.005	
Hydrogen bond restraints (20) (Å)	0.005 ± 0.004	
Dihedral angle restraints (38) (°)	0.180 ± 0.045	
R.m.s.d. from the average structure (residue 491–550)		
Backbone atoms (Å)	0.35 ± 0.06	
All heavy atoms (Å)	1.16 ± 0.11	
Quality index (PROCHECK):		
	Overall G factor	% residues in most favored φ/ψ regions
All residues	0.04 ± 0.04	76 ± 3
Residues 491–499, 506–531, 538–550	0.12 ± 0.04	88 ± 3
Residues 491–499, 506–510, 517–519, 525–531, 538–550	0.20 ± 0.05	91 ± 3

[a]The number of each type of restraint is given in parentheses.

a folded globular domain with a significant amount of α-helical structure.

We determined the three-dimensional structure of p73(487–554) in solution using multidimensional NMR spectroscopy and simulated annealing. Table I shows the NMR-derived structural information and the results of structure calculations for p73(487–554). The 18 lowest energy structures that satisfy the NMR-derived structural restraints (Figure 3A) form a well-defined family of structures representative of the conformation of p73(487–554) in solution. Residues 491–550 form a well-defined five-helix bundle composed of four α-helices [residues 491–499(α1), 506–511 (α2), 525–531 (α4) and 538–550 (α5), respectively] and residues 517–520 form a small 3_{10} helix (H3). The α1 and α5 helices associate in a roughly antiparallel manner with α2, H3 and α4 associating with the hydrophobic surface formed by α1 and α5. Most of the hydrophobic residues that mediate association of all five helices are conserved among p73, p63 and squid p53 (Figure 3B).

p73(487–554) is structurally homologous to the SAM domain

To find clues about the function of the C-terminal domain of p63 and p73, we compared the structure of p73(487–554) with other known three-dimensional structures using the program DALI (Holm and Sander, 1993, 1994). This program identified the SAM domains from two Eph receptor tyrosine kinases, EphB2 (Smalla *et al.*, 1999; Thanos *et al.*, 1999) and EphA4 (Stapleton *et al.*, 1999), as having strong structural similarity to p73. The Cα carbons of residues 491–550 of p73 can be superimposed with those of a single subunit of the dimeric Eph receptor SAM domains with a root mean square deviation (r.m.s.d.) of ~2 Å despite sequence identities of only 15–17% (see Table II and Figure 4A). The pointed domain of the

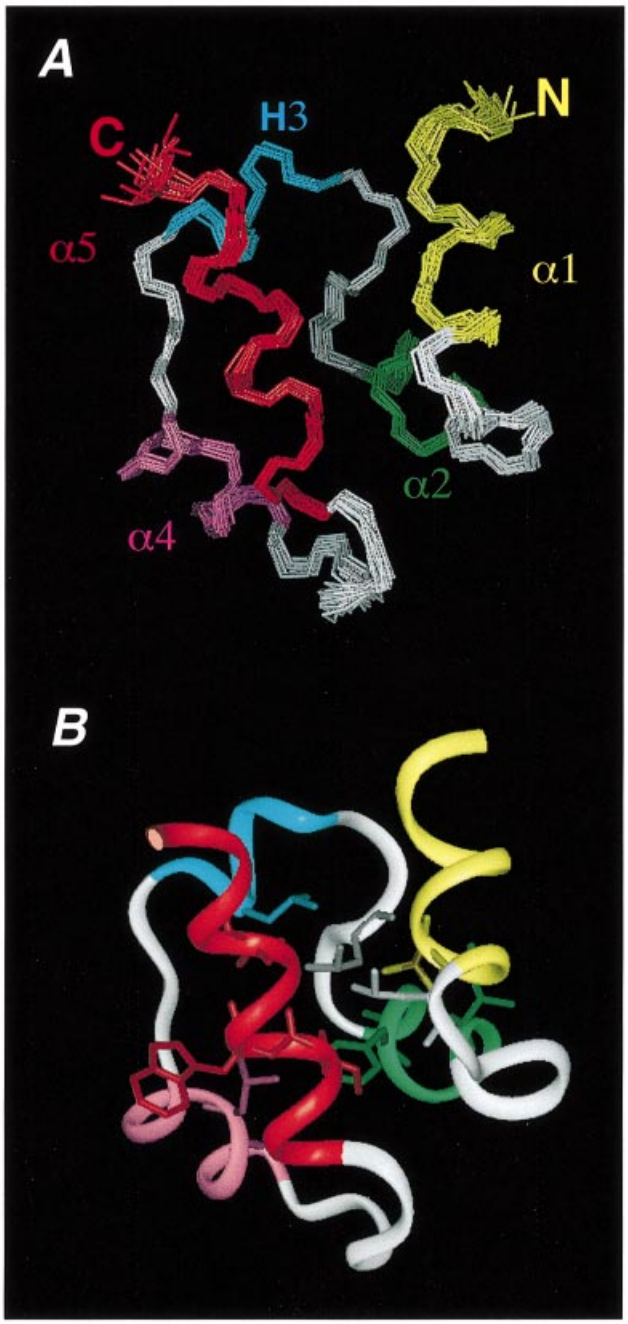

Fig. 3. A backbone representation of the solution structure residues 491–550 of p73. Residues 487–490 and 551–554 are unstructured and are not displayed. (**A**) The superposition of the 18 lowest energy structures. Helices 1–5 are colored yellow, green, cyan, pink and red, respectively. (**B**) A ribbon display of a representative structure. The side chains are displayed for conserved hydrophobic residues involved in the association of the five helices: L497 (yellow); L500 (white); I506, F509 (green); L514 (white); L520 (blue); L528 (pink); and I541, W542, L545 (red). Each of these residues corresponds to a hydrophonic or aliphatic residue in the SAM domain consensus sequence derived by Shultz *et al.* (1997). Graphics were generated with InstightII. The atomic coordinates have been deposited in the Protein Data Bank, accession number 1cok.

transcription factor Ets-1 (Slupsky *et al*., 1998) also has structural similarity to p73(487–554). The Ets-1 pointed domain is a more distantly related member of the SAM superfamily. Finally, a small region of endonuclease III also has a region with structural similarity to the p73 C-terminal domain, although the functional significance of this is not clear.

p73 and p63 SAM-like domains do not homo- or hetero-oligomerize

Many SAM domains can homo- and hetero-oligomerize with one another. Therefore, we investigated whether the p63 and p73 SAM-like domains were capable of homo- and/or hetero-oligomerization. Equilibrium sedimentation analysis of p73(487–554) (Figure 5A) and our original NMR data indicate that this domain is monomeric up to concentrations of 1.2 mM. However, based on analogy with the Eph receptors, it is formally possible that p73(487–554) does not contain all the sequence necessary for oligomerization. The EphB2 and EphA4 SAM domains homodimerize via an extended C-terminal α-helix and several N-terminal residues immediately preceding helix A. Therefore, we also performed equilibrium sedimentation on p73(487–600) which encompasses the entire conserved C-terminal domain shown in Figure 1. Again, both p73(487–600) and p63(469–679), in isolation or in combination, eluted from a gel filtration column as monomers, indicating that neither of these domains forms homo- or hetero-oligomers (data not shown). Their inability to interact was illustrated using protein–protein interaction studies of differentially tagged proteins. When His_6-tagged p73(487–600) was incubated with p63(469–679) and the mixture passed over Ni^{2+} resin, only p73(487–600) was retained by the resin, indicating that these two constructs of p63 and p73 do not interact. Similarly, His_6-tagged p63(469–679) was unable to retain p73 (487–600) on the Ni^{2+} resin (data not shown).

Given that SAM domains are known primarily as protein–protein interaction domains, we analyzed the p73(487–554) structure in terms of conserved surface residues which could form potential binding sites. A number of hydrophobic and hydrophilic residues conserved between p63 and p73 are evident on the surface (S492, S495, G501, Y508, Q512, Y518, K532, E535, Q536, D547 and Q550). These conserved residues appear to cluster at the 'top' and 'bottom' of the five-helix bundle (Figure 4B). Charged residues are clustered primarily along the surface formed by the two C-terminal helices.

Discussion

We have determined the three-dimensional solution structure of the C-terminal domain of p73. Residues 487–554 form a compact five-helix bundle with strong structural similarity to several members of the SAM domain superfamily. The helices and intervening loops of the p73 SAM-like domain align remarkably well with those of the Eph receptor SAM domains. However, the sequence identity between p73 and the Eph receptor SAM domains is quite low (15–17% identity). This distant relationship at the sequence level has been noted previously (Schultz *et al*., 1997; Bork and Koonin, 1998), and it was suggested that p73 and its homologs may be involved in protein–protein interactions involved in developmental regulation of transcription (Bork and Koonin, 1998). The structural similarity between the p73 SAM-like domain and the Eph receptor SAM domains presented here strengthens this argument. The key hydrophobic residues that form the hydrophobic

Table II. Proteins with structural homology to residues 491–550 of p73

Protein	PDB[a]	R.m.s.d.[b]	*Z*[c]	No. of residues aligned	% Identity
EphB2 SAM domain (NMR)	1sgg	1.8	8.2	60	17
EphB2 SAM domain (A)[d]	1b4f	2.0	7.4	60	15
EphB2 SAM domain (B)[d]	1b4f	2.9	4.1	59	14
EphA4 SAM domain	1b0x	1.9	5.1	60	17
Endonuclease III	2abk	2.6	3.7	56	9
Pointed domain from Ets-1	1bqv	2.8	2.1	49	18

[a]Filename from the Protein Data Bank (PDB).
[b]Positional root mean square deviation of superimposed C_α atoms in Angstroms, computed by Dali (Holm and Sander, 1993).
[c]Z score computed by Dali. A measure of structural similarity in standard deviations above that expected. A score of <2.0 indicates two proteins are structurally dissimilar (Holm and Sander, 1993).
[d]The EphB2 SAM domain dimer is asymmetric (Thanos *et al.*, 1999). Two subunits, A and B, from 1b4f were compared separately.

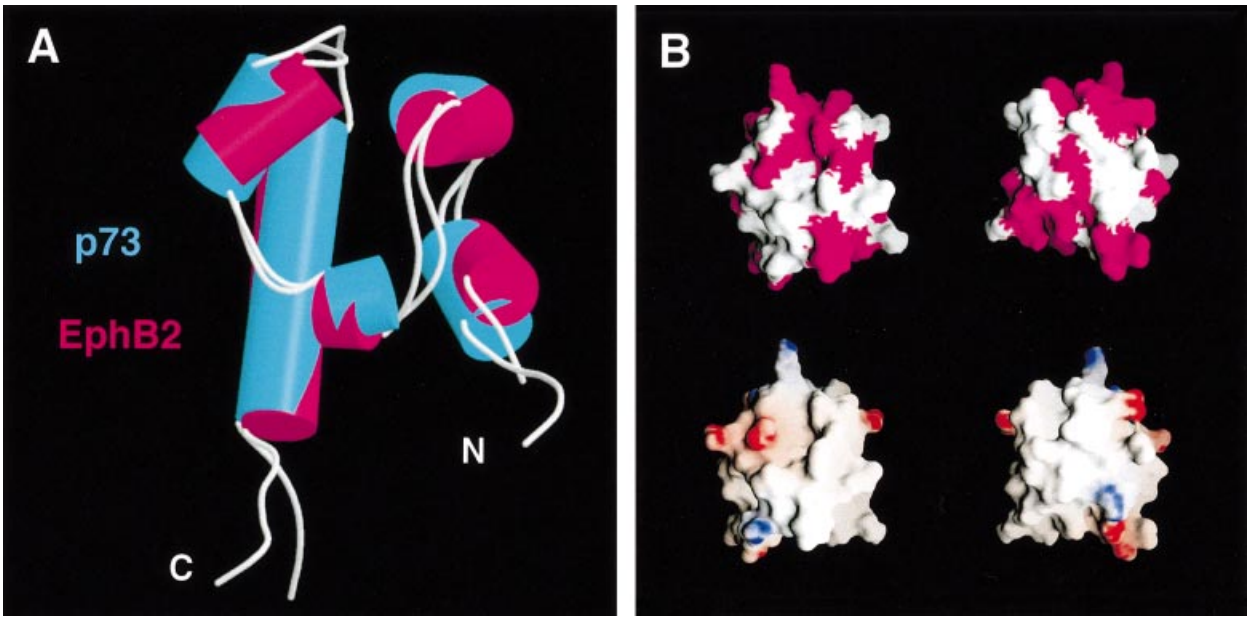

Fig. 4. (**A**) A superposition of SAM domains of p73 (cyan) and subunit A of the EphB2 receptor (magenta; Thanos *et al*., 1999). The helices are represented as cylinders. The orientation is rotated ~180° about a horizontal axis relative to that of Figure 3. The two structures were aligned with InsightII and the figure generated with Molscript (Kraulis, 1991) and RASTER3d (Merritt and Murphy, 1994). (**B**) Two views of the molecular surface of the p73 SAM-like domain (residues 491–550 displayed). The top panel shows residues that are conserved between p73 and p63 (magenta). The right hand view is the same as that of (A). The left hand view is rotated by 180° about a vertical axis. The lower two views show electrostatic surface potential from 13.9 kT (blue) to –8 kT (red). Surfaces were generated with the program GRASP (Nicholls *et al*., 1991).

core of p73(487–554) (Figure 3B) align with positions of hydrophobic residues in a consensus sequence that was derived from a multiple alignment of >40 members of the SAM superfamily (Schultz *et al*., 1997). However, the pattern of surface residues of p73(487–554) is most similar to that of p63 (Figure 4B). Thus, the p73/p63 C-terminal domains may constitute a unique subclass of human SAM-like domains.

We found no evidence of homo- or hetero-oligomerization of the SAM-like domains of p73 and p63. This result is not surprising, since both p63 and p73 have separate, very efficient tetramerization domains similar to that of p53 (Davison *et al*., 1999). If the p73/p63 SAM-like domain is involved in protein–protein interactions, the partner proteins must be either a more distantly related SAM domain, or quite possibly a non-SAM domain protein(s). Further protein–protein interaction studies are under way to identify possible interacting partners for the p73/63 C-terminal domains. We note that in this regard, it is quite possible that more than just the SAM-like region may be involved, since the region of C-terminal conservation between the p53 family members is ~100 residues (Figure 1). For example, residues upstream and downstream the SAM consensus region were found to mediate dimerization of the Eph receptors (Stapleton *et al*., 1999; Thanos *et al*., 1999).

There is mounting evidence that the various C-terminal isoforms of p63 and p73 may have differential transcriptional and biological activities (DeLaurenzi *et al*., 1998; Yang *et al*., 1998; Zhu *et al*., 1998; Di Como *et al*., 1999). The DNA-binding activity of p53 is negatively regulated by its C-terminal domain (Hupp *et al*., 1992) and the status of this regulation is subject to modifications or removal of this domain. p63 and p73 have a completely different C-terminal region (relative to p53) and do not appear to be regulated by the same genotoxic stimuli that activate p53, suggesting that the C-terminal domains of these proteins also constitute unique regulatory regions which can affect their transcriptional and/or other biological activities. The presence of a SAM-like protein–

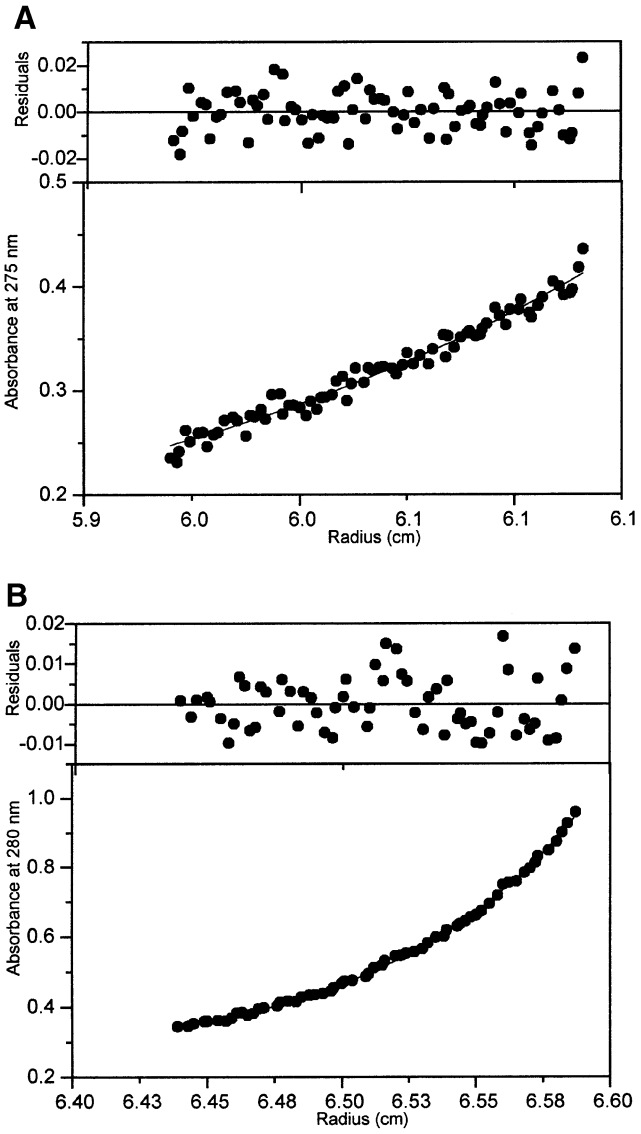

Fig. 5. Representative sedimentation equilibrium data, fitted curves and residuals for p73 C-terminal proteins. (**A**) p73(487–554) (0.2 mg/ml, 22 500 r.p.m.). The apparent molecular weight obtained from the fitting procedure was 9395 Da (expected molecular weight for the mononer is 10 096 Da). (**B**) p73(487–600) (0.5 mg/ml, 35 000 r.p.m.). The apparent molecular weight obtained from the fitting procedure was 13 922 Da (expected molecular weight for the monomer is 15 124 Da).

protein interaction domain in the C-terminus of p63α and p73α strongly suggests mechanism(s) of regulation in which protein–protein interactions are involved. It may also be reasonable to hypothesize that the SAM-like C-terminal domains of p53 homologs mediate developmental processes since most SAM domain-containing proteins studied thus far are involved in development or differentiation.

Materials and methods

Protein expression and purification

C-terminal segments of human p73 (residues 487–554 and 487–600) and p63 (residues 469–679) were subcloned into the pET-15b vector (Novagen) using PCR techniques (Sambrook *et al.*, 1989). The resulting plasmids express the protein of interest with an N-terminal hexahistidine tag followed by a thrombin cleavage site. Recombinant proteins were expressed in *Escherichia coli* and purified using Ni^{2+}-NTA chromatography. Typical yields were 10–20 mg of purified protein per 2 l of culture. The final protein NMR samples were typically 1.2 mM protein in a buffer of 25 mM sodium phosphate, pH 6, 150 mM NaCl, 3 mM dithiothreitol (DTT) and 1 mM benzamidine. Unless otherwise noted, the hexahistidine tag was not removed.

NMR spectroscopy and spectral assignments

NMR data were acquired at 25°C on a Varian Unity 600 spectrometer or a Varian Unity + 500 spectrometer equipped with pulse-field gradient units and actively shielded *z*-gradient triple resonance probes. Pulse-field gradient (PFG) techniques were used for water suppression (Key *et al*., 1994). NMR experiments involving correlations of amide protons were conducted with gradient-enhanced versions (Kay *et al.*, 1992) of the originally published pulse sequences. All data were processed using the NMRpipe software system (Delaglio *et al*., 1995) and analyzed with the program NMRview (Johnson and Blevins, 1994). Sequence-specific resonance assignments, nuclear Overhauser effect (NOE) and coupling constant data were obtained using standard multidimensional double and triple resonance NMR techniques as described in Morin *et al*. (1996).

Structure calculation

An initial set of 50 structures was calculated using the simulated annealing protocol from X-PLOR 3.851 (Brünger, 1996) using three types of restraints: (i) 502 manually assigned inter-residue unambiguous NOE-derived distance restraints; (ii) dihedral angle restraints derived from coupling constants ($\phi = -120 \pm 20°$ for $^3J_{NH-H\alpha} > 8.5$ Hz, or $\phi = -65 \pm 20°$ for $^3J_{NH-H\alpha} < 6.0$ Hz) (Redfield and Dobson, 1990; Smith *et al.*, 1991); and (iii) main chain hydrogen bond restraints (H–O, 2.2 Å; N–O, 3.3 Å). Floating chirality assignment (Weber *et al.*, 1988; Folmer *et al.*, 1997) was employed for all methylene and isopropyl groups. From this data set, a family of 30 preliminary structures was generated.

Using these initial structures as starting conformations, further refinement was performed using ARIA as described (Nilges, 1995; Nilges *et al.*, 1997) and a full set of NOE distance restraints generated by automated peak picking of the NOE spectra (including the original unambiguous NOEs). In the final iteration, the NMR-derived experimental restraints contained 1053 unambiguous NOE restraints [365 intraresidue, 307 sequential, 236 medium-range ($1 < i - j \leq 4$) and 145 long-range ($i - j \geq 5$) interproton restraints], 93 ambiguous NOE restraints, 20 restraints from main chain hydrogen bonds and 38 backbone ϕ angle restraints (Table I).

From a total of 30 calculated structures, the 18 lowest energy structures were selected as representative of the conformations of p73(487–554) in solution. These structures had no NOE violations greater than 0.5 Å and no dihedral violations greater than 5°. Structure statistics for this ensemble are listed in Table I. A quality analysis of the final superimposed ensemble was performed using the program PROCHECK-NMR (Laskowski *et al.*, 1996).

Protein oligomerization assays

Equilibrium sedimentation experiments were performed on His_6-tagged p73(487–554) and p73(487–600) at 20°C on a Beckman XLI Analytical Ultracentrifuge and AN50-Ti rotor. The sedimentation equilibrium runs using six-channel charcoal–Epon cells were performed for ~24 h before equilibrium absorbance measurements were taken. Molecular weight determinations involved global analysis of three different sample concentrations (0.2, 0.50 and 1.0 mg/ml) centrifuged at three different speeds [30 000, 35 000 and 40 000 r.p.m. for p73(487–600) and 18 000, 20 000 and 22 500 r.p.m. for p73(487–554)]. Molecular weights were calculated using Beckman XLI data analysis software in which absorbance versus radial position data were fitted to the sedimentation equilibrium equation using non-linear least-squares techniques (Johnson *et al*., 1981). The partial specific volume and density of the sample were calculated using the program SEDINTERP (Laue *et al*., 1992) from the amino acid sequence and buffer composition, respectively.

Size exclusion chromatography and protein affinity chromatography of differentially His_6-tagged p73 and p63 SAM domains was performed as described in Davison *et al*. (1999).

Acknowledgements

We thank Peter Yin and Sandy Go for excellent technical assistance, Dr D.Caput for fruitful discussions, and Drs F.Sicheri, M.Smalla and

H.Oschkinat for providing atomic coordinates of the EphA4 and EphB2 SAM domains prior to release by the PDB. We thank Lewis Kay for NMR pulse sequences. This research was supported by the National Cancer Institute of Canada with funds from the Terry Fox Run. C.H.A. is a Scholar of the Medical Research Council of Canada.

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Received April 19, 1999; revised and accepted June 23, 1999