CMLS, Cell. Mol. Life Sci. 55 (1999) 88–95
1420-682X/99/010088-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Regulation of p53 protein function through alterations in protein-folding pathways

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Abstract. The tumour suppressor protein p53 is a stress-activated transcription factor whose activity is required for regulating the cellular response to stress and damage. The biochemical activity of p53 as a transcription factor can be regulated by partner proteins affecting stability, nuclear transport, signalling pathways modulating phosphorylation and interactions with components of the transcriptional machinery. The key structural determinants of p53 protein that drive sequence-specific DNA binding include the core specific DNA-binding domain and the tetramerization domain. Flanking these domains are more evolutionarily divergent carboxy- and amino-terminal regulatory motifs that further modulate tetramerization and sequence-specific transactivation. This review will mainly focus on the mechanisms whereby the tetramerization domain modulates sequence-specific DNA binding and how missense point mutations in p53 protein and the activity of molecular chaperones may lead to unfolding of mutant p53 tetramers in human tumours.

Key words. p53; heat shock proteins; phosphorylation; protein folding; oligomerization.

Regulation of p53 function

P53 protein is a stress-activated transcription factor that drives the expression of gene products implicated in growth control. The assembly of p53 into an active transcription factor can be mediated by various factors such as intradomain communication within tetramers which may be regulated by phosphorylation; components of the transcriptional machinery including p300/CBP [1, 2], TAFII40/60 [3] and p33ING1 [4]; and components of the mdm2-dependent proteosome pathway [5, 6]. Naturally occurring point mutations within p53 protein found in human tumours inactivate sequence-specific DNA binding through alterations in the folding of the core domain which can further promote interactions with the protein-folding machinery. Highlighted below are new developments in the p53 field related to oligomerization, protein folding and interactions with molecular chaperones.

p53 protein-nucleic acid-binding properties

Sequence-specific DNA binding by the core domain of p53: the physiological DNA-binding sites of p53 protein

p53 protein is a tetrameric protein [7, 8] which can drive transcription by interaction with specific DNA elements in promoters of genes induced during growth arrest or apoptosis. The minimal p53 consensus DNA-binding site contains four pentanucleotide repeats in inverted orientation, with the basal sequence containing two copies of the consensus sequence PuPuPuC(A/T)(T/A)GPyPyPy separated by 0 to 13 base pairs [9]. Stereospecific alignment has suggested that spacing distance between two decamers is an important determinant of p53 affinity and that binding is optimal when the two decamers are contiguous or separated by insertions such that the two half sites are orientated on the same helical face [10]. Many genes containing p53-binding sites in the promoter or intron regions have been

identified and include p21WAF1 [11], gadd45 [12], mdm2 [13], IGF-BP3 [14] and BAX [15]. As these gene products can play a role in growth control, models have been developed linking these mediators of p53 protein to cell-cycle checkpoints and damage responses [16].

The likelihood that transcription factors like p53 regulate the expression of a host of genes has promoted efforts into identifying novel DNA elements under control of p53. The first strong evidence that many genes contain p53-binding sites was developed using a yeast-based method to identify human genomic DNA elements that could drive p53-dependent transcription [17]. Such cloning of over 50 distinct DNA elements suggested that up to 300 binding sites for p53 protein might exist in the human genome. Serial analysis of expression of over 7000 genes induced during the p53-dependent apoptotic (programmed cell death) process has also revealed a family of approximately 50 genes whose expression may be directly regulated by p53 protein [18]. The identity and significance of these gene products in modulating stress responses induced by reactive oxygen intermediates provides a link between p53 function and apoptosis dependent upon mitochondrial function [19].

Independent computer searches have also recently been developed for scanning genomic databases for DNA elements which hold homology to the known p53 consensus site in efforts to identify novel p53-responsive genes [20]. Such analyses identified novel DNA elements with putative p53-binding sites, some of which include N-myc; int-1; urokinase plasminogen activator; collagen; LMP2 proteosome subunit; TcR α and β chain; T3-δ; C4A complement protein; CD8 α chain; PCNA; thrombospondin; and kinases implicated in energy metabolism and adenosine triphosphate (ATP) generation. The identification of novel p53-binding sites within previously known p53-responsive genes has further led to the refinement of the architecture of a p53-responsive promoter. For example, although one original study has identified one pair of 10-bp consensus sites within the p21WAF1 promoter [11], the data of Bourdon and colleagues has defined four novel 10-bp consensus elements at a site more proximal to the transcription start site. In addition, although previous consensus sites for p53 within the IGF-BP3 gene were localized within intron 1 [14], up to eleven 10-bp binding sites were observed within the IGF-BP3 promoter [20]. The sequence divergence from a 'perfect' consensus fit within these newly identified p53-binding sites suggests that the additional copies may be needed to facilitate nucleation of p53 at a promoter. The data suggest that an important component of p53 transactivation is generally multiple copies of the 10-bp consensus site in the promoter, whose affinity for p53 may be further regulated by chromosomal looping [21] and the degree of superhelicity [22].

Single-stranded nucleic acid binding by the C-terminal domain

Although the core domain of p53 protein harbours the sequence-specific DNA binding activity, biochemical characterization has also revealed a distinct nucleic acid-binding activity residing within the C-terminal domain [23]. Subsequent work has demonstrated that p53 protein or fragmented derivatives that contain both the tetramerization domain and an extreme C-terminal basic domain (amino acids 315–393) exhibit a remarkably high affinity for single-stranded RNA and DNA, reflected in its ability to renature complementary strands of RNA or DNA into duplex hybrids [24, 25]. Interactions of p53 protein with DNA ends, irradiated DNA [26], mismatched DNA [27] and Holliday junctions [28] also require the C-terminal nonspecific DNA-binding domain. The higher affinity of p53 for nucleic acid in single-stranded form suggests that hydrophobic interactions with nucleotides may play a role in defining such specificity; thus the avidity for 'damaged' DNA may be related to the extent of single-stranded character in the DNA structures. Although the binding of p53 protein at such sites is relatively difficult to address in vivo, this function may be important to facilitate participation in RNA transcription, processing, translation or repair of damaged DNA. In addition, the observation that p53 protein can reduce tortional stress in a supercoiled DNA without changing the linking number suggests that a further link between p53 function and the rate of gene expression may relate to the control of tortional stress at a transcription fork [22].

Regulation of p53 protein-DNA interactions through modulation of the tetramerization domain

The tetramerization domain of p53 facilitates core sequence-specific DNA-binding activity

The tetramerization domain of human p53 from amino acid residues 320–356 flanks the core DNA-binding domain and consists of a monomeric core containing a β-strand-turn-α-helix, which is organized into a dimer via an antiparallel β sheet and an antiparallel helix-helix interface [29, 30]. The dimers associate across hydrophobic interfaces to form a four-helix bundle arranged orthogonally into tetramers. The tetramerization domain is required for effective p53 activity and its conservation in the

p53 homologues [31, 32] at key hydrophobic amino acids suggest that these p53 family members have also conserved this oligomerization domain. The need for a tetramerization domain in modulating p53 activity is at first glance unclear, since replacement of this domain with a coiled-coil domain of GCN4 is sufficient to confer sequence-specific transcriptional activity in cells [33]. However, reviewed below are new approaches that have shed light on the role of the tetramerization domain in the conformational regulation of sequence-specific DNA binding by the core domain of p53.

Point mutations in the tetramerization domain alter its folding and oligomeric state

Based on the dihedral symmetry adopted by the tetramerization and the twofold cyclic symmetry of the four pentanucleotide inverted repeats in the consensus DNA element of p53, a symmetry discrepancy exists in the p53-DNA complex that has been resolved by predicting that asymmetric folding of the linker polypeptide between the core domain and the tetramerization domain is required to permit DNA binding [34]. Such predictions have been supported by data showing that altering the polypeptide linker distance between the tetramerization domain and core domain can prevent conformational alterations in p53 and reduce DNA binding of p53 protein. In addition, changes in the hydrophobic amino acid side chains in the tetramerization domain, at positions mutated in human tumours, promote a conversion from a tetrameric to dimeric structure with changes in α-helical packing from antiparallel to parallel and altered positioning of the α helices compared with the β strands [35]. These results predict that altering tetramerization of p53 protein by missense mutation can impede its tumour suppressor function and further supports a role for this domain in modulating p53 activity in cells. In addition, recent work has shown that naturally occurring point mutations in the tetramerization domain that occur in patients with Li-Fraumeni-like syndrome can change the thermal stability of the tetramerization domain (C. Arrowsmith, personal communication) and impede mdm2 binding (R. Camplejohn, personal communication), suggesting that intradomain communication within the p53 tetramer is important for both DNA binding and mdm2 binding.

A link between CK2 site phosphorylation, tetramerization domain stability and sequence-specific DNA binding

The regulation of DNA binding of p53 by phosphorylation in its extreme C-terminus (fig. 1) has been proposed to result from allosteric mechanisms, largely based on kinetic data showing (i) an uncoupling of serine-392 phosphorylation [casein kinase 2 site (CK2)] from latent p53 activation [36]; (ii) the inability of some mutants, with the machinery to engage in specific DNA binding (LYS285 and HIS273) to be activated by phosphorylation [37]; and based on the stoichiometry of an antibody-activated p53-DNA complex ($[PAb421]_2[p53]_4$; ref. 38). This conformational model for p53 activation is supported by independent approaches that include changes in the proteolytic cleavage products generated in an ATP-dependent manner by some cyclin-dependent

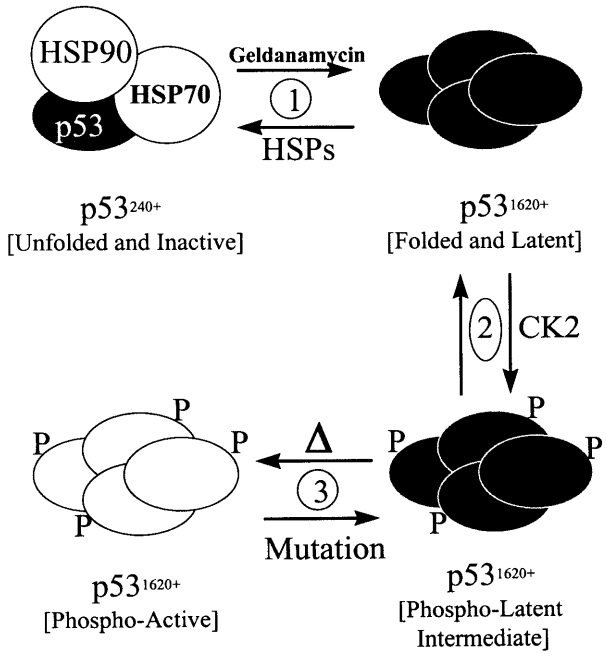

Figure 1. Key biochemical stages in the assembly of an activated p53 tetramer. Three distinct stages in the regulation of p53 activity and conformation are based on the existence of four distinct biochemical forms of p53 protein. (1) p53 protein unfolding into the $PAb240^+$ reactive conformation can be promoted by point mutation [55, 58] or heat [44] and can be bound stably by HSP90 and HSP70 family members through interaction with internal hydrophobic regions [49, 56]. Drugs like geldanamycin that inactive HSP90 can displace the molecular chaperone complex from p53 protein and permit the refolding of p53 into the $PAb1620^+$ reactive conformation [66]. (2) Phosphorylation of latent p53 protein tetramers in the $PAb1620^+$ reactive conformation [76] by CK2 does not spontaneously activate the tetramer for sequence-specific DNA binding, and a phosphorylated, latent tetrameric intermediate can be isolated [36]. (3) Conformational changes within the C-terminal domain catalysed by phosphorylation at serine-392 leads to tetramer stabilization [40], possibly through interaction with the CDC2 phosphorylation domain [40, 42, 77]. However, certain point mutations in p53 (LYS285 and HIS273) prevent CK2 phosphorylation from activating the mutant protein [37], presumably by increasing the energy barrier to tetramer stabilization.

protein kinases [39] and stabilization of p53 protein (with deletion of the N-terminal negative regulatory motif) from heat denaturation by PAb421 binding to the C-terminus (S. Hansen, personal communication). Presumably, these conformational changes are mediated in part by the tetramerization domain, and efforts to link the function of the C-terminus to changes in the conformation of the tetramerization motif have been recently reported.

Specifically, the effects of CK2-site phosphorylation at serine-392 on p53 activity may be related to the ability of this modification to induce conformational changes in the tetramerization domain [40], which presumably will be directed to changes in the conformation and activity of the core DNA-binding domain. Using analytical ultracentrifugation, phosphorylation at serine-392 can enhance tetramer formation by 4 kcal/mol (30 °C) with an eightfold decrease in the K_d of tetramer formation. Consistent with ultracentrifugal data, isothermal titration calorimetry also demonstrated that serine-392 phosphorylation can increase the stability of the tetramerization domain. The thermodynamic parameters suggest that the stability is due to the formation of new intramolecular or intermolecular polar side-chain interactions. The striking feature of these studies is (i) the inability of serine-315 (CDC2 site) or serine-378 phosphorylation (protein kinase C, PKC site) to effect tetramer stability and (ii) the destabilizing effects of serine-315 phosphorylation on serine 392 phosphorylation. These latter results would not be expected if serine-315 phosphorylation [39], like CK2 phosphorylation [41], activated specific DNA binding. However, Hansen and colleagues have shown that there is intradomain communication between the C-terminal CDC2 and CK2 sites [42], consistent with the biophysical data summarized above [40]. Resolution of this discrepancy will require further structure-function relationships to examine how unique modifications on p53 effect its function. Regardless, the biophysical data permit the development of more refined conformational models that predict how serine-392 phosphorylation can modulate the equilibrium states of the p53 tetramer (fig. 1).

A conformational link between N-terminal and C-terminal domains

The amino terminus of p53 harbours the transactivation domain that drives p53- dependent transcription. Implicit in such a structure are functional motifs that regulate the assembly of p53 into transcriptional complexes. Factors that bind these functional motifs include negative regulators like mdm2 or positive regulators including TAFII40/60 and p300/CBP. Despite the models that the N-terminal domain functions as a scaffold to regulate protein-protein interactions with trans-acting factors, modification of the N-terminus can also allosterically regulate core DNA-binding activity. The first clear evidence for this came from the observation that mdm2-p53 complexes synthesized in vivo are inherently inactive for DNA binding, suggesting mdm2 protein can function like T-antigen and possibly distort the core DNA-binding domain through conformational alterations [43]. The ability of the N-terminal antibody DO-1 to stimulate CK2-modified or HSP70-modified mutant p53 proteins [37] also suggest that the N-terminus contains regulatory domains that can effect core DNA-binding activity. Further, modification of the N-terminus of p53 protein by monoclonal antibodies can protect the wild-type and mutant forms of p53 protein from thermal denaturation [44, 45], and deletion of the N-terminal 22 amino acids can rescue a tetramerization-deficient mutant for DNA binding, providing solid evidence for intradomain communication within the tetramer [46].

Defects in protein folding and the role of molecular chaperones in controlling p53 function

Biochemical interactions of p53 and HSPs

The first cellular protein shown to bind to p53 included a member of the HSP70 family of proteins [47], whose associations with p53 have since been extended to include the molecular chaperones HSP40 [48] and HSP90 (fig. 1; ref. 49). These three heat shock proteins (HSPs) form a holoenzyme complex that can coordinately refold denatured proteins, protect proteins from unfolding or target irreversibly damaged proteins for degradation [50]. HSPs have also been shown to prevent drug- or radiation-dependent apoptosis in cells [51–54], highlighting the role these proteins may play in tumour cell survival. The relevance of the interaction of p53 within tumour cells has been unclear, but recent evidence described below suggests that one component of the antiapoptotic function of HSPs may be related to the control of the conformation and inactivation of p53. As a result, drugs which disrupt HSP-p53 interactions in tumours may hold promising therapeutic potential.

The altered folding of mutant p53 protein in tumour cells was first detected with a monoclonal antibody specific for denatured forms of p53 [55] and identified a biochemical pathway whose dysregulation in tumours may contribute to the inactivation of p53. The elevated stability of the HSP-mutant p53 complex in some tumours also provided a compelling link between dysregulation of the protein-folding machinery and inactivation of p53 protein function during carcinogenesis. Thus, studies aimed at dissecting the nature of the HSP-p53 interaction should shed light on molecular processes that contribute to p53 inactivation and assist

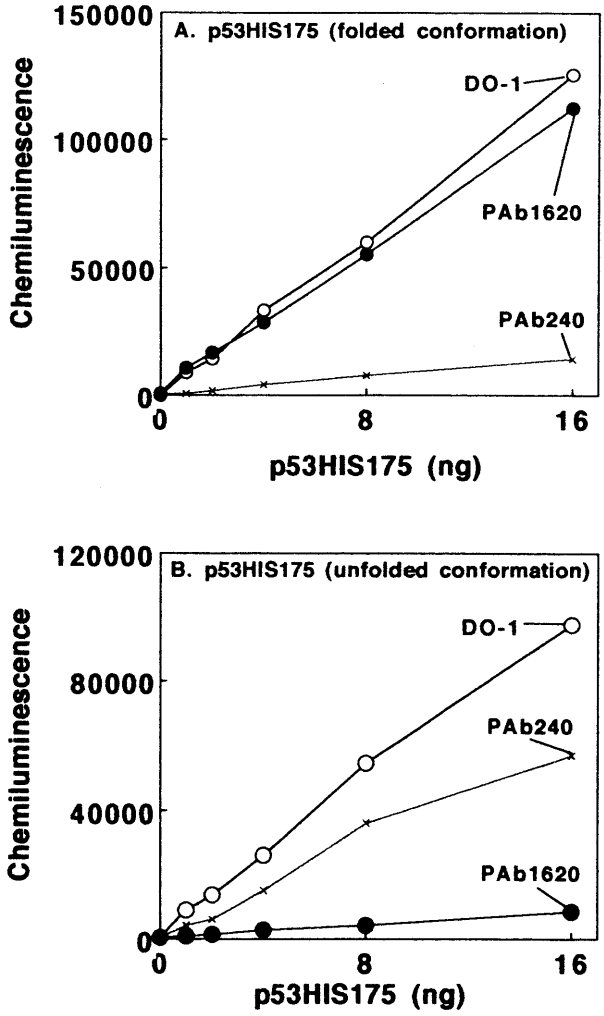

Figure 2. Assembly of the HIS175 mutant form of p53 into the PAb1620$^+$ reactive conformation. Mutant p53 protein encoded by the HIS175 allele was purified from recombinant insect cells as described [76]. A two-site native ELISA (as in ref. 76) was used to quantitate the relative amount of the mutant p53 protein in the folded (PAb1620$^+$ reactive) or unfolded (PAb240$^+$ reactive) conformation (*A*) without or (*B*) after heating at 37 °C for 30 min. The HIS175 protein is inactive as a sequence-specific DNA-binding protein (data not shown), although it has assembled into the PAb1620$^+$ conformation. These results indicate that the HIS175 mutation does not unfold the core domain, as defined by PAb1620$^+$ reactivity, but indicates that a distinct structural defect is responsible for inactivation of the DNA-binding function of this mutant form of p53 protein.

in designing strategies to reactivate the normal p53 protein folding and assembly pathways in cells. Despite the observations that relatively stable interactions can be observed between HSP70 homologues and mutant p53 protein in tumour cells, native wild p53 or mutant p53 tetramers in the PAb1620$^+$ conformation do not bind stably to HSPs ([42, 44]; fig. 2). These results suggest that HSPs may actually bind directly to mutant p53 in tumours via recognition of hydrophobic motifs in the core domain which are exposed or unfolded in tumour cells.

To locate such high-affinity binding sites for HSPs within the core domain of p53, a quantitative HSP-p53 peptide-binding assay was developed [56]. Using this assay, the predominant peptides from p53 protein which bind to HSC70 derive predominantly from secondary structural elements, like β strands, which reside in the hydrophobic core domain. These results are consistent with the model that unfolding of p53 protein in tumour cells contributes to a stable HSP-p53 complex. However, the failure of native p53 tetramers to bind stably to HSPs despite having binding sites in the C-terminal negative regulatory domain exposed [42, 44] suggest that structural constraints imposed within the native tetramer prevent HSP70 isoforms from binding stably within this region.

Despite a growing understanding of the HSP-binding sites within the core domain of p53, we still lack an understanding of whether the binding of HSPs to p53 plays a role in p53 inactivation or whether the mutant proteins inherently unfold in tumour cells and the binding to HSPs is just a consequence of unfolding. Two pieces of growing evidence point to a combination of both. First, the same point mutant p53 proteins can exist in either PAb1620 or PAb240 conformation, depending upon the environment of the tumour cell [57]. In addition, pure preparations of the most 'oncogenic' allele of p53 (encoded by the HIS175 allele) assembles readily into the PAb1620$^+$ wild-type conformation (fig. 2), indicating that the point mutation in the core does not inherently predispose the protein to spontaneous unfolding. These data are consistent with trans-acting factors (like HSPs) playing a role in p53 folding and oligomerization in vivo and that mutations do not spontaneously unfold but change the protein-folding equilibrium which can predispose the mutant proteins to unfolding.

In support of this concept, biophysical studies have shown that common mutations in p53 protein do predispose the protein to urea-dependent denaturation or aggregation [58]. Cooperative two-state unfolding transitions demonstrate that the wild-type p53 core domain is moderately unstable (thermodynamic stability of 6.0 kcal mol^{-1}; 25 °C) with the mutants destabilized from 0.4, 1.9, 1.9, 2.9 and 3.0 kcal/mol, respectively, for the HIS273, SER249, TRP248, SER242 and HIS175. The pronounced instability of the HIS175 or SER242 mutants is not unexpected due to their proximal location to Zn^{2+} in the core domain. The surprising feature of these studies, however, is that the active site mutation TRP248 (presumed to have no role in stability as it makes direct contacts to the DNA) also can predispose

the core domain to unfold, indicating that the active site mutants also can effect the conformation of the core domain. The degree of destabilization of the mutant p53 proteins is sufficiently small to suggest that protein engineering will permit the development of small agents that can stabilize the conformation of the temperature-sensitive mutant proteins [59].

Molecular chaperones as effective anticancer drug targets

A striking breakthrough in dissecting molecular pathways that regulate mutant p53 protein conformation and stability in tumour cells came from independent studies examining the mechanism of function of the benzoquinone ansamycin class of antitumour compounds, which include geldanamycin [60]. The antiproliferative activity of geldanamycin is attributed to depletion of oncogenic proteins including erbB2 receptor kinases and RAF-1 [61, 62]. The mechanism whereby these kinases are inactivated by geldanamycin appear to depend in part upon the binding to and inhibition of the molecular chaperone HSP90 [63], which is involved in assembly of the activated kinases.

Given that one of the major HSPs bound to mutant p53 in tumours appears to be a member of the HSP90 family of chaperones [49] and that HSP90 is the major target of geldanamycin, studies were initiated to examine whether mutant p53 folding in vitro and stability in vivo is regulated by molecular chaperones. A pronounced reduction in mutant p53 protein levels upon treatment of tumour cells with geldanamycin [64] mediated by the ubiquitin-dependent proteasome [65], and the reassembly of the mutant and unfolded p53 into the wild-type conformation in vitro [66] demonstrates that HSP90 may play a significant role in modulating mutant p53 conformation and stability. These data further support the model that mutant p53s are not inherently unfolded, but the HSPs can affect p53 folding pathways in tumours by binding to hydrophobic amino acids within the core domain whose rate of unfolding may be promoted by point mutation (fig. 1).

T-antigen transformation and dysregulation of protein-folding pathways

The attraction for studying the function of transforming viral oncogenes such as T-antigen has been and remains to identify cellular factors that modulate the molecular switch that controls carcinogenesis. The identification of p53 protein as one of the major targets of T-antigen [67, 68] and its subsequent identification as a tumour suppressor justified such a rationale. Other known targets of T-antigen are also now known to comprise other components of the cell-cycle machinery, including the retinoblastoma a family of proteins [69] and the p300/CBP family of transcriptional adapter binding proteins [70]. Recent data summarized below suggests that components of the protein-folding machinery are also directly targeted by T-antigen, highlighting the possibility that dysregulation of the molecular chaperones play a role in cellular transformation.

Such studies stemmed from the observation that an N-terminal motif of T-antigen termed the J BOX domain has a high homology to the bacterial DNAJ or human HSP40 family of molecular chaperones. It is the holoenzyme complex of HSP40, HSP70 and HSP90 that is implicated in protein folding and in binding to and stabilizing mutant p53 in some tumours. The J BOX domain of T-antigen can replace the human HSP40 in vitro by stimulating HSC70 ATPase activity [71, 72]. Biochemical studies have shown that modification of the J BOX region of T-antigen is required to promote a stable complex between p53 and T-antigen in vitro [73], pinpointing a novel regulatory domain within T-antigen that affects its conformation and activity. If the N-terminal J BOX domain of T-antigen plays a significant role in cellular transformation, then one would expect it to compete with endogenous HSP40 for chaperone functions. Consistent with this, transfection of the J BOX domain into cells can block T-antigen-dependent processes [74], further linking the dysregulation of molecular chaperone function and protein-folding pathways to key events in tumorigenesis.

Summary: prospects for correcting temperature-sensitive folding defects in mutant p53 protein

This review has aimed to focus on some of the newly identified factors that regulate p53 oligomerization, in vitro protein-folding pathways and stability in tumour cells. p53 protein is a tetrameric protein whose assembly can be controlled intramolecularly by a tetramerization domain and in trans by the HSP family of molecular chaperones. Subtle changes in the equilibria of mutant p53 protein-folding pathways induced by missense mutations [58], by phosphorylation [37] or by monoclonal antibody binding [42, 44, 45] suggest that the design of agents, like peptidomimetics, which affect such equilibria may promote the activation of latent or inactive forms of p53 protein in tumour cells [36]. Early successes at the development and exploitation of agents that affect p53 conformation include the benzoquinone ansamycin antitumour antibiotics that can be used to control p53 folding pathways in vitro [66] and synthetic peptides derived from the C-terminus of p53 that can be used to induce p53-dependent apoptosis [75].

The realization that many point mutations in p53 protein predispose the protein to thermal instability [45,

58] raises the hope that strategies can be developed to manipulate protein-folding pathways in cells to reactivate temperature-sensitive p53 proteins. Recent precedents for the success of such approaches come from examining the ability of chemical solvents to activate temperature-sensitive mutant proteins, like p53, in vivo [59]. The well-documented ability of such chemicals to stabilize polypeptide conformation in vitro provides the precedent for rational approaches aimed at manipulating polypeptide conformation and underscores the possibilities for intervening in protein-folding pathways in human disease with therapeutic benefit.

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