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The p53 Tumour Suppressor Protein

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Introduction

Cancer is a genetic disease dependent upon the accumulation of mutations within the genes that control cellular proliferation. Mutations may arise, either as a consequence of errors made during DNA replication, or after exposure to physical or chemical mutagens. DNA damaging agents, such as the oxygen free radicals produced by the mitochondria during respiration, are continually generated as a result of normal cellular activity (Kaufmann and Paules, 1996). Disruption of the genes responsible for cell growth regulation, programmed cell death (apoptosis), differentiation and motility may contribute to tumour formation, either promoting unrestrained cell division or allowing inappropriate cell survival. The earliest studies revealed that tumours contain gain-of-function mutations within genes that normally signal cell division under specific growth conditions. The products of these genes are often components of the signal transduction pathways that are either overexpressed or expressed as overactive mutant proteins (Cantley *et al.*, 1991). The constitutive activation of these pathways favours proliferation under conditions that would otherwise be growth prohibitive. However, it is clear that a single oncogenic mutation is insufficient to induce the formation of a tumour. The creation of artificial tumour cell lines by exogenous expression of oncogenes has demonstrated that at least four growth regulatory pathways must be disrupted to enable tumourigenic conversion of primary cells (Hahn *et al.*, 1999). The requirement for additional mutations is explained, at least in part, by the existence of cell cycle checkpoints that have evolved to protect multicellular organisms against tumourigenesis. It is now clear that these checkpoints are governed by a second class of genes, the tumour suppressor genes, which repress cellular proliferation and which are frequently mutated in tumours. In normal tissues, the growth restraint exerted by the tumour suppressor gene products

may be overcome by signalling from the extracellular environment. Although a single oncogenic mutation might bypass a tumour suppressor checkpoint, it is insufficient to give rise to a fully transformed tumour cell. Thus, our current view of the tumour cell is one in which both dominant oncogenic mutations and recessive tumour suppressor gene mutations co-operate to bypass the control mechanisms that govern DNA replication and cell division.

TUMOUR SUPPRESSOR GENES

The earliest evidence for the existence of tumour suppressor genes came from the observation that fusion of tumour cells with primary fibroblasts results in the formation of non-tumourigenic hybrid cells. This implies that dominant growth suppressive factors within normal cells inhibit the proliferation of transformed cells (Sager, 1985). Since tumour suppressor gene mutations are generally recessive (with some exceptions, explained later in this chapter) they are often associated with genetic disorders in which patients are predisposed to the development of specific tumours. In these cases, a tumour suppressor gene mutation is present in the germ-line but, due to its recessive nature, does not interfere with development, only giving rise to tumours when the remaining allele is mutated or deleted. A germ-line mutation in the *RB-1* tumour suppressor gene, for example, was the first tumour suppressor to be linked to such a syndrome, being responsible for inheritable predisposition to the rare tumour, retinoblastoma (Knudson, 1971; Benedict *et al.*, 1983; Cavenee *et al.*, 1983; Dryja *et al.*, 1986). The association of both the *APC* gene mutation with the development of colon carcinoma (Tanaka *et al.*, 1991; Powell *et al.*, 1992) and *BRCA-1* gene mutations with predisposition to breast and ovarian cancers (Vogelstein and Kinzler, 1994; Merajver *et al.*, 1995) have provoked intense study in recent years, adding to an ever growing list of tumour suppressor genes and their associated genetic disorders. In addition to these congenital disorders, which are relatively rare, tumour suppressor gene mutations are also vital to the development of sporadic tumours. The recessive nature of the tumour suppressor gene function means that both alleles must be inactivated. This event should be relatively infrequent if these mutations were to occur independently. However, analysis of tumour samples has revealed that mutated tumour suppressor alleles often contain identical missense mutations, accompanied by loss of heterozygosity (LOH) at these loci. This suggests that inactivation of the second allele is frequently achieved through recombination during mitosis (Weinberg, 1991).

THE *TP53* GENE AND TUMOURIGENESIS

The *TP53* tumour suppressor gene, the main focus of this review, initially escaped classification as a tumour suppressor due to the fact that the first p53 sequence identified encoded a dominant negative mutant protein, thereby breaking the rule that tumour suppressor mutations are recessive. This mutant form of p53, which was identified as a protein bound to the SV40 large T antigen (DeLeo *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; McCormick and Harlow, 1980), was originally classified as an oncogene as it was able to cooperate with known oncogenes to promote cellular transformation (Eliyahu *et al.*, 1984). However, the wild-type p53

sequence was subsequently identified and shown to encode a protein which strongly suppressed cell growth (Finlay *et al.*, 1989; Baker *et al.*, 1990; Lane and Benchimol, 1990). In these experiments, the wild-type p53 protein was able to suppress the transforming activity of various oncogenes and, most importantly, inhibit the growth and tumourigenicity of p53-negative tumour cell lines. The dominant-negative effect exhibited by some mutant p53 proteins is a result of their ability to associate with wild-type p53 and abrogate its function (Herskowitz, 1987; Kraiss *et al.*, 1988; Schmieg and Simmons, 1988; Milner *et al.*, 1991).

In the years following these initial studies, an intense research effort revealed that disruption of *TP53* is a prevalent factor in carcinogenesis. A wide variety of human malignancies were shown to contain deletions and rearrangements at the p53 locus (17p13) and approximately 50% of all human tumours demonstrate perturbation of wild-type p53 function (Hollstein *et al.*, 1991; Levine *et al.*, 1991). In accordance with the genetics of other tumour suppressor genes, the disruption of a single p53 allele in the germ-line is associated with Li-Fraumeni syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990), a condition characterized by high susceptibility to the development of a wide range of tumours at an early age. Importantly, the role of p53 in tumourigenesis has been substantiated by studies in transgenic and 'knockout' mouse models. As a parallel of Li-Fraumeni patients, p53-heterozygous mice are highly susceptible to diverse tumours as a result of mutation within the remaining p53 allele. Both transgenic mice expressing a tumour-derived dominant negative p53-mutant and p53-'knockout' mice develop a large number of tumours at an early age but embryonic development generally proceeds as normal (Donehower *et al.*, 1992; Hann and Lane, 1995). One group has reported an elevated incidence of exencephaly in p53-null embryos but this effect is strain dependent and restricted to a small subset of female embryos (Sah *et al.*, 1995). Thus, it appears that, in most circumstances, p53 is neither required during embryonic development nor involved in the control of the cell cycle under normal conditions. Rather, it is hypothesized that p53 acts to suppress aberrant cell proliferation. Much of the work investigating the function of p53 has indicated that its major role lies in the mediation of the cellular response to DNA damage (see below). In this model, p53 would be expected to protect against the accumulation of genetic aberrations that could potentially lead to cellular transformation and tumourigenesis.

The structure and function of the p53 protein

The human p53 protein is a transcription factor consisting of 393 amino acids divided into four structural domains (*Figure 7.1*). The protein contains an N-terminal trans-activation domain (Fields and Jang, 1990; O'Rourke *et al.*, 1990) and a DNA binding domain that recognizes the loose consensus site, 5'- PuPuPuC(A/T)(A/T)GPyPyPy-3' (Kern *et al.*, 1991; El-Deiry *et al.*, 1992; Funk *et al.*, 1992). The 42 amino acid trans-activation domain is essential for association with the basal transcriptional machinery, and substitution of amino acids Phenylalanine 19, Leucine 22 or Tryptophan 23 results in trans-activation deficient mutant proteins (Chen *et al.*, 1993; Lu and Levine, 1995; Thut *et al.*, 1995). The sequence-specific DNA binding domain of p53, between amino acids 100 and 300, forms a protease resistant core (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). The domain is folded into a
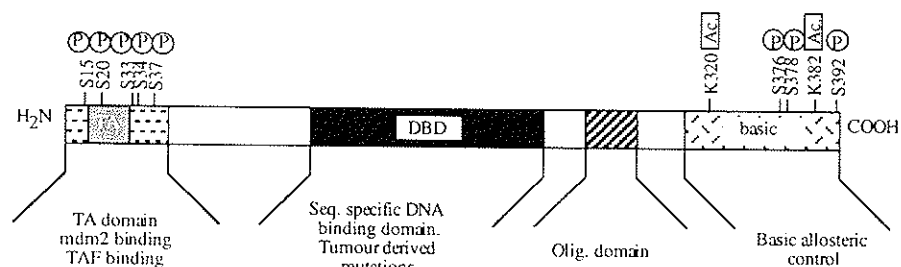
Figure 7.1. The structure of the p53 protein. The positions of the four functional domains are indicated. From the N terminus (H₂N), these domains include the trans-activation domain (TA); the DNA binding domain (DBD); the oligomerization domain (Olig.) and the basic allosteric control region (basic). The sites of *mdm-2* and TAF binding are indicated. The phosphorylation (P) and acetylation (Ac.) sites that are relevant to this discussion are also shown, 'S' indicating a serine residue and 'K' a lysine residue.

four- and five-stranded anti-parallel β sheet scaffold and two α helices that contact DNA (Cho *et al.*, 1994). Notably, almost all of the tumour-derived p53-mutant proteins contain point mutations within this region, indicating that the role of p53 as a transcription factor is essential to its tumour suppressor function. These mutations may be one of two types. The mutations either involve amino acids which contact DNA directly, thereby cancelling specific interactions between p53 and its DNA binding site, or may lie outside of the DNA:protein interface causing conformational changes in the domain that mask the critical residues (Cho *et al.*, 1994).

The third domain from the N-terminus, the oligomerization domain, consists of amino acids 324 to 355 and is linked to the DNA binding domain by a flexible region. The substitution of hydrophobic amino acids in this region interferes with the ability of p53 to form tetramers (McCoy *et al.*, 1997). The DNA binding consensus described above is an inverted repeat, which is paired with a second consensus sequence in p53-responsive promoters, providing four binding sites for p53 monomers. This suggests that p53 associates with DNA as a tetramer, each monomer contacting a single half site. The p53 tetramer is more correctly envisaged as a dimer of dimers on the basis of crystallography data (Jeffrey *et al.*, 1995). Although p53 tetramers bind DNA more tightly than p53 monomers, the oligomerization domain is apparently dispensable for the suppression of cell growth (Pellegata *et al.*, 1995). Recent data, however, show that a nuclear export signal, NES, is located within this region (Stommel *et al.*, 1999). These researches demonstrate that tetramerization of p53 masks the NES and traps p53 in the nucleus, whilst monomers and dimers are exported to the cytoplasm, suggesting that p53 may be compartmentally regulated via tetramerization. The fourth functional domain of p53 is situated at the extreme C-terminus and consists of a 26 amino acid highly basic region. This domain was shown to associate with both DNA and RNA in a structure dependent manner but without requirement for a specific sequence. *In vitro* DNA binding studies demonstrated that a number of modifications at this site could promote association between p53 and its consensus DNA binding site (Hupp and Lane, 1994). These modifications include the phosphorylation of serine 378 by Protein Kinase C (PKC), phosphorylation of serine 392 by Casein Kinase II (CKII), and incubation with the anti-p53 antibody PAb421, whose epitope lies between amino acids 370 and 378. Furthermore, short nucleotide sequences that bind to the C-terminal domain were shown to activate DNA binding

(Jayaraman and Prives, 1995). Since a wealth of data supports the notion that p53 exists in an equilibrium state between latent and DNA binding conformations, it has been suggested that this C-terminal domain may mediate control over the transcriptional activity of p53. The basic domain may therefore either influence the conformation of the DNA binding domain via allosteric mechanisms, or may otherwise block the DNA binding site directly in the absence of activating modifications. A final note should be made to the effect that, despite its transcriptional trans-activating activities, p53 also has the potential to repress expression from a wide variety of promoters. The mechanism of repression is unclear but may be mediated by association between p53 and the basal transcription factors $TAF_{II}40$, $TAF_{II}60$ and TBP (Chen *et al.*, 1993; Thut *et al.*, 1995).

Mechanisms of p53-induced tumour suppression

P53 ACTIVATES CELL CYCLE CHECKPOINTS

Following DNA damage, non-transformed cells retain the ability to undergo cell cycle arrest, thereby avoiding the replication and segregation of damaged chromosomes. The transfection of wild-type p53 into p53-deficient cells was shown to induce a cell cycle arrest just prior to the G1/S phase transition (Baker *et al.*, 1990). Conversely, cells which express a dominant negative mutant p53 protein are compromised with respect to the G1/S phase cell cycle arrest in response to treatment with DNA damaging agents (McIlwrath *et al.*, 1994). Similarly, following X-ray treatment, mouse ML-1 cells carrying wild-type *TP53* are able to elicit a G1/S arrest, whilst similar myeloid cell lines lacking p53 function are not subject to this checkpoint (Kastan *et al.*, 1991). The first step in understanding the mechanism by which p53 suppresses cell proliferation has been the identification of the p53-transcriptional target genes. Most notable amongst these genes, the p21[WAF1/CIP1] G1 cyclin dependent kinase (CDK) inhibitor was identified in a subtractive hybridization screen for genes which were activated by wild-type p53, but not by a transcriptionally inactive mutant protein (El-Deiry *et al.*, 1993). Progression of the cell cycle from G1 phase to DNA synthesis requires the presence of the active G1 cyclin/CDK complexes. Expression of p21[WAF1/CIP1] has been shown to inhibit cyclin D/CDK4 and cyclin E/CDK2, thus blocking the phosphorylation of protein substrates required for the onset of S phase (Xiong *et al.*, 1993; Harper *et al.*, 1993). One of the targets of these kinase complexes is the retinoblastoma tumour suppressor protein, pRB (reviewed in Weinberg, 1995) which, when phosphorylated, dissociates from the E2F family transcription factors and allows DNA synthesis to proceed (*Figure7.2*). The consequence of p21[WAF1/CIP1] induction by p53, therefore, is the prevention of pRB phosphorylation and subsequent cell cycle block, linking these two tumour suppressor genes into the same cell proliferation checkpoint.

Several lines of evidence corroborate the model described above. Gamma irradiation has been shown to induce accumulation of p53 and expression of p21[WAF1/CIP1], causing inhibition of cellular CDK activity (Dulic *et al.*, 1994). Mouse embryo fibroblasts (MEF) derived from p21[WAF1/CIP1]-deficient mice are also defective in the DNA damage induced G1/S checkpoint (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). However, this defect is incomplete, suggesting that other genes may also be involved. The cell cycle
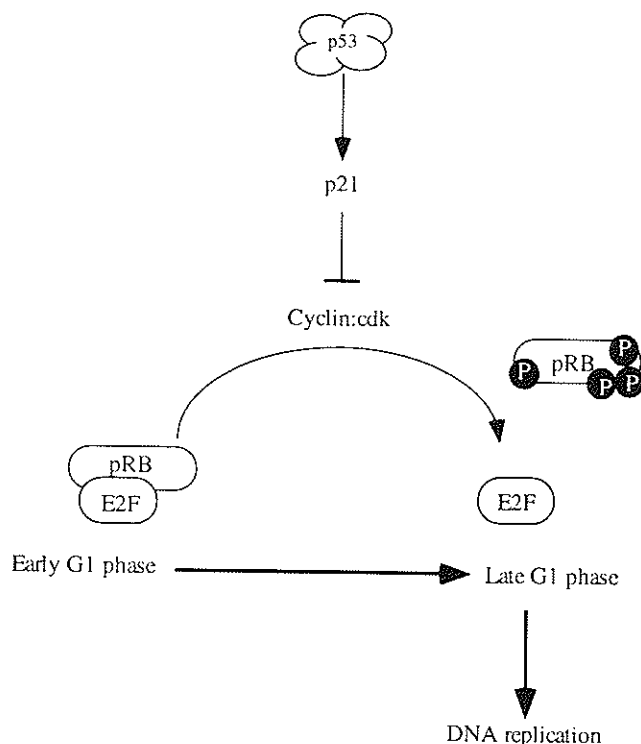
Figure 7.2. p53 mediates a G1/S phase cell cycle checkpoint. Transcriptional activation of p21^WAF1/CIP1 blocks the activity of G1 cyclin /CDK complexes. This, in turn, prevents the phosphorylation of the pRB family members (shown here as pRB only, for simplicity) and subsequent release of the E2F family transcription factors from pRB family control. Since the activity of the E2F proteins is required for entry into S phase, p53 activation blocks cell cycle progression just prior to the G1/S transition.

inhibitory effect of the p21^WAF1/CIP1 protein is well documented, but p53-induced expression of a number of cellular genes has been reported (see *Table 7.1*), many of which could potentially be involved in cell cycle arrest. Such p53-inducible genes, including the growth-suppressive protein phosphatase WIP1 (Fiscella *et al.*, 1997) and cyclin G1 (Okamoto and Beach, 1994; Bates *et al.*, 1996) may also contribute to the G1/S phase block. The use of a panel of p53 mutants has demonstrated a correlation between the p53 trans-activation function and the ability of these proteins to initiate a G1 arrest (Crook *et al.*, 1994; Pietenpol *et al.*, 1994), but transcriptional trans-repression may also be involved. Overexpression of p53 represses transcription from a variety of cellular promoters. In accordance with p53 function, some of the gene targets of p53 trans-repression are involved in stimulation of cell proliferation (Ginsberg *et al.*, 1991).

Some of the p53-induced genes in *Table 7.1* may, theoretically, participate in a G2/M phase delay, although data upon the requirement of p53 for the DNA damage induced G2/M checkpoint is conflicting. Two laboratories independently demonstrated that inducible overexpression of p53 in p53-deficient cells led to both G1/S and G2/M phase cell cycle arrest (Agarwal, *et al.*, 1995; Stewart, *et al.*, 1995). Following treatment with ionizing radiation, however, both a p53-dependent G2

Table 7.1. p53-regulated genes

Gene	+/-	Gene function (p53 response)	Reference
P21[WAFI/CIPI]	+	CDK inhibition (G1/S arrest)	El-Deiry *et al.*, 1993 Harper *et al.*, 1993
mdm2	+	p53 degradation (Negative feedback)	Momand *et al.*, 1992 Oliner *et al.*, 1992 Barak *et al.*, 1993
Bax	+	Membrane channel (Apoptosis)	Miyashita *et al.*, 1994 Selvakumaran *et al.*, 1994 Miyashita and Reed, 1995
gadd 45	+	Binds Cdc2 (G2 /M arrest) DNA repair (DNA repair)	Kastan *et al.*, 1992 Smith *et al.*, 1994
IGF-BP3	+	IGF inhibition (Growth arrest and Apoptosis)	Buckbinder *et al.*, 1995
Fas/Apo1	+	Membrane receptor (Apoptosis)	Owen-Schaub *et al.*, 1995
Killer/DR5	+	Membrane receptor (Apoptosis)	Wu *et al.*, 1997
Wip1	+	Phosphatase (G1/S arrest)	Fiscella *et al.*, 1997
PCNA	+	DNA replication/repair (Cell cycle arrest)	Shivakumar *et al.*, 1995
14-3-3σ	+	Binds Cdc25C (G2/M arrest)	Hermeking *et al.*, 1997 Peng *et al.*, 1997
Bcl-2	-	Cell survival (Apoptosis)	Miyashita *et al.*, 1994
c-myc	-	Growth signal (Growth arrest)	Ragimov *et al.*, 1993
c-fos	-	Growth signal (Growth arrest)	Ginsberg *et al.*, 1991

A plus sign (+) indicates that a gene is transcriptionally induced by p53 whereas a minus sign (-) denotes p53-mediated transcriptional repression. In each case, the main function of the gene is indicated with the influence of each gene upon the p53 response given in parentheses.

checkpoint (Bunz *et al.*, 1998) and a p53-independent G2/M arrest pathway (Kastan *et al.*, 1991; O'Connor *et al.*, 1993) have been observed. The progression from late G2 phase to mitosis is under the control of the cyclin B1/cdc2 kinase complex and, consequently, the mitotic checkpoint may be activated by down-regulation of cyclin B1 or phosphorylation of cdc2 (Krek and Nigg, 1991; Maity *et al.*, 1995). The p53-induced GADD45 protein, which disrupts the cyclin B1/cdc2 complex, may possibly play a role in G2/M phase arrest (Zhan *et al.*, 1998). Alternatively, p53-mediated transcriptional up-regulation of 14-3-3σ expression may be an important determinant in the establishment of a pre-mitotic arrest. The 14-3-3 proteins are known inhibitors of cdc25c, which is in turn required for cdc2 activation, and have been shown to activate a G2/M arrest when exogenously expressed in cells (Hermeking *et al.*, 1997; Peng *et al.*, 1997). There is some evidence that p53 also participates in a mitotic spindle checkpoint (Cross *et al.*, 1995). In contrast to normal cells, p53-null cells that are artificially blocked in mitosis undergo repeated rounds of DNA duplication without intervening chromosome segregation. Furthermore, p53-null MEF, appear to accumulate a surplus of functional centrosomes (Fukasawa *et al.*, 1996). Endoreduplication in p53-null MEF in the presence of spindle inhibitors was confirmed independently. However, in these studies, pRB-null MEF also display this phenotype (DiLeonardo *et al.*, 1997; Khan and Wahl, 1998). These observations may be resolved by the findings of two laboratories that show that this p53-dependent checkpoint may, in fact, be an early G1 phase checkpoint dependent upon p21[WAFI/CIPI] (Lanni and Jacks, 1998; Stewart *et al.*, 1999). Perhaps p53 may mediate a post-mitotic checkpoint that is activated following defective mitosis. Loss of this checkpoint would almost certainly contribute to genetic instability in p53-null cells but the signalling pathways involved in p53 activation remain elusive.

P53 INDUCES APOPTOTIC CELL DEATH

Programmed cell death, or apoptosis, is an intrinsic property of most cell types, distinguishable from necrotic cell death by a defined set of morphological changes such as chromosomal condensation and nuclear fragmentation (Wyllie *et al.*, 1981). Apoptotic processes have been shown to be essential for normal embryonic development and for negative selection of autoreactive T cells in the embryonic thymus (Sentman *et al.*, 1991; Surh and Sprent, 1994). Ectopic overexpression of p53 had been shown to induce apoptosis in transformed myeloid cells in culture (Yonish-Rouach *et al.*, 1991). Apoptosis is also initiated in response to a wide range of physical and chemical DNA damaging agents in a p53-dependent manner (Lowe *et al.*, 1993a; Clarke *et al.*, 1993). Furthermore, the apoptotic response of haematopoietic cells to serum deprivation also requires wild-type p53 expression (Gottlieb *et al.*, 1994).

Apoptosis is characterized by a proteolytic cascade of events involving a series of apoptosis-specific cysteine proteases known as the caspases (for review see Nunez *et al.*, 1998). Activation of an initial caspase, via cleavage of its pro-domain, leads to the activation of other caspases. This cascade culminates in the cleavage of specific substrates, each of which is linked to the characteristic morphological changes that occur during apoptosis. These substrates include structural proteins, such as nuclear lamins and proteins of the cytoskeleton, whose degradation contributes to nuclear breakdown and other events associated with the late stages of apoptosis. Many of the p53-transcriptional target genes listed in *Table 7.1* may be potential mediators of the apoptotic response, feeding in to the caspase cascade at several points. Indeed, transcriptional trans-activation appears to be essential for p53-induced apoptosis in several systems (Sabbatini *et al.*, 1995; Attardi *et al.*, 1996). In other studies, however, p53-induced apoptosis was observed in the absence of new RNA or protein synthesis (Caelles *et al.*, 1994; Wagner *et al.*, 1994). Thus, the mechanism of p53-induced apoptosis remains unclear and is apparently diverse in different cell types. Induction of apoptosis may be partially mediated via transcriptional regulation of the bcl-2 family member proteins. These proteins are divided into pro-apoptotic and anti-apoptotic groups, which seem to have independent functions but which homodimerize and heterodimerize with each other (for review see Reed, 1998). The bax protein, a pro-apoptotic bcl-2 family member, is a transcriptional target of p53 (Miyashita *et al.*, 1994) that either exists as a heterodimer with the anti-apoptotic factor, bcl-2 or, in the apoptotic cell, exists as a homodimer when bax levels greatly exceed those of bcl-2 (Oltvai *et al.*, 1993). It is therefore satisfying that p53 has also been reported to mediate transcriptional repression of bcl-2. The mechanism of apoptosis as mediated by the bcl-2 family members in response to p53 is outlined simplistically in *Figure 7.3*. The anti-apoptotic proteins, bcl-X_L and bcl-2, and the pro-apoptotic bax each form membrane channels and are located to the outer mitochondrial membrane (for review see Schendel *et al.*, 1998). It is now becoming clear that these proteins control the release of cytochrome c from the mitochondria. Cyctochrome c release occurs in response to a variety of apoptotic stimuli and has been shown to be required for the activation of effector caspases (Liu *et al.*, 1996; Kharbanda *et al.*, 1997; Kluck *et al.*, 1997). Cytosolic cytochrome c associates with Apaf-1 allowing the latter to associate and activate procaspase 9, forming a structure that is referred to as the apoptosome (Zou *et al.*, 1997). Apaf-1 and procaspase 9 have both been shown to be necessary for
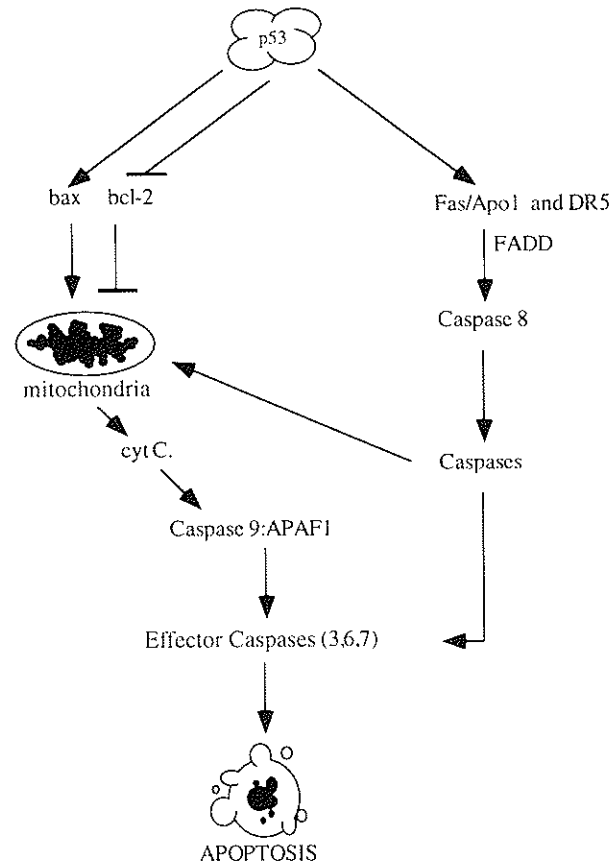
Figure 7.3. Mechanisms of p53-induced apoptosis. Two pathways of p53-mediated apoptosis are shown. p53-mediated transcriptional activation of bax, together with transcriptional repression of Bcl-2 results in the release of cytochrome *c* from mitochondria. Subsequent assembly of the apoptosome (Caspase 9, APAF1 and cytochrome *c* complex) precedes the activation of effector caspases and the cleavage of death substrates. A parallel pathway involving the Fas/Apo1-initiated caspase cascade, which may also feed back to the mitochondria to cause cytochrome *c* release. Other pathways by which p53-induced apoptosis is executed have been omitted for clarity.

p53-induced apoptosis in response to *c*-myc overexpression (Soengas *et al.*, 1999). The exact mechanism by which the bcl-2 family members interact to determine whether cytochrome *c* is released or whether it is retained is still a subject for intense speculation and research. Most recently, bax and another pro-apoptotic family member, bak, have been shown to mediate the opening of the mitochondrial voltage-dependent anion channel (VDAC), which allows cytochrome *c* to permeate (Shimizu *et al.*, 1999).

Although bax induction is clearly associated with apoptosis, cells from bax deficient mice are nonetheless able to undergo apoptosis in response to p53 (Brady *et al.*, 1996). These data would imply that regulation of the relative ratios of bcl-2 family members might be a vehicle for p53-mediated apoptosis, but that other pathways are evidently involved. p53-mediated transcriptional up-regulation of cell surface receptor

proteins may also contribute to the initiation of apoptosis. Active p53 induces expression of two tumour necrosis factor receptor (TNFR) family members, the Fas/Apo1 receptor (Owen-Schaub *et al.*, 1995) and Killer/DR5 (Wu *et al.*, 1997). Activated Fas/Apo1 recruits the FADD protein via a protein:protein interaction motif known as the death domain, which in turn recruits caspase 8 at the head of a caspase proteolytic cascade (Enari *et al.*, 1995), thereby initiating apoptosis (*Figure 7.3*). A further study, however, has demonstrated that p53 can activate this pathway in the absence of protein synthesis on account of its ability to mediate cell surface trafficking of Fas/Apo1 (Bennett *et al.*, 1998), and that p53-induced apoptosis is at least partially dependent upon this receptor pathway. Therefore, at least two mechanisms of p53-induced apoptosis exist, but there are undoubtedly other p53-induced factors that contribute to cell death in response to stress factors. Some of the other p53-induced genes are also potentially involved in apoptosis. Genes such as IGF-BP3 (Buckbinder *et al.*, 1995), for example, which may compromise both the cell growth and the cell survival signals generated by the insulin-like growth factor receptor. There is also evidence that, under some circumstances, p53 might contribute to apoptosis via transcription-independent pathways (Caelles *et al.*, 1994). However, little progress has been made towards identifying the mechanisms by which p53-mediated apoptosis might occur in the absence of protein synthesis.

The regulation of p53 activity *in vivo*

P53 IS STABILIZED IN RESPONSE TO VARIOUS STRESS STIMULI

In most cell types, and under normal growth conditions, the p53 protein is relatively unstable, possessing a half-life of approximately 20 minutes. Upon exposure of cells to DNA damaging agents, p53 rapidly accumulates in the absence of any increase in transcription of *TP53*. This suggested that the increase in p53 protein levels observed following DNA damage is due to protein stabilization rather than increased protein production (Malzman and Czyzyk, 1984; Kastan *et al.*, 1991; Fritsche *et al.*, 1993; Hall *et al.*, 1993). Both the transcriptional activity of p53 and p53 stability are reported to increase in response to other stress stimuli, including overexpression and activation of oncogenes (Debbas and White, 1993; Hermeking and Eick, 1994; Lowe and Ruley, 1994; Wagner *et al.*, 1994); hypoxia (Graeber *et al.*, 1994); alterations in the ribonucleotide pools (Linke *et al.*, 1996) and cell adhesion (Nigro *et al.*, 1997). Thus, p53-mediated tumour suppression appears to rely on transduction of both extracellular and intracellular signals. Signalling to p53 upon DNA damage or in the absence of survival signals from the extracellular matrix serves to prevent the proliferation of cells under conditions that might otherwise give rise to potentially oncogenic mutations. DNA damaged cells may, therefore, undergo cell cycle arrest until repair of the DNA has been completed or, if the damage is irreparable, may be eliminated via apoptosis. Alternatively, disruption of the internal environment by, for example, oncogene expression may sensitize the cells to apoptosis. The observation that oncogene overexpression induces p53-dependent apoptosis provides strong evidence that p53 acts as a sensor of aberrant cell growth.

Recent data have implicated the *mdm-2* protein in the mediation of p53 degradation via the ubiquitin pathway (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Since *mdm-2* is

a well recognized transcriptional target of p53 (Juven *et al.*, 1993), these observations lead to the hypothesis that a negative feedback loop might function to maintain low levels of p53 in normal cycling cells. The link between p53 and *mdm-2* is supported by studies in 'knockout' mice, which have shown that the *mdm-2* negative phenotype results in embryonic lethality due to p53 deregulation (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Moreover, this phenotype may be rescued if p53 is also deleted. The association between *mdm-2* and the N-terminus of p53 is required for both transcriptional suppression and degradation. Additionally, *mdm-2* has been implicated in a shuttling pathway that actively exports p53 from the nucleus for degradation in the cytoplasm (Roth *et al.*, 1998). The search for protein factors and covalent modifications which influence *mdm-2* mediated degradation of p53, therefore, has recently been the focus of intense research. Physiologically, the modulation of *mdm-2* function appears to be important for the activation of p53 under at least two conditions, namely following DNA damage and the acquisition of oncogenic mutation. The mechanisms by which these two stimuli signal to *mdm-2*, however, appear to be diverse. Ionizing radiation-induced phosphorylation of p53 has been shown to destabilize the interaction between p53 and *mdm-2* (Shieh *et al.*, 1997). In contrast, oncogene expression appears to evoke a separate mechanism, inducing ARF, a novel *mdm-2* binding protein that abrogates p53 degradation (De Stanchina *et al.*, 1998; Zindy *et al.*, 1998). The conclusion that is currently emerging from the literature is suggesting that there are many levels of p53 regulation, each being specific for both the cell-type and the activating stress applied.

P53 STABILIZATION BY THE ARF TUMOUR SUPPRESSOR

ARF regulates mdm-2 mediated degradation of p53

The murine p19^{ARF} protein (corresponding to human p14^{ARF}) is derived from an alternatively spliced transcript of the INK4a locus. This locus was initially studied on account of the fact that the protein encoded by the INK4a gene, p16^{INK4a}, is a specific inhibitor of cyclin D/CDK4 and thus a potential tumour suppressor gene targeting the pRB checkpoint pathway. However, the ARF protein, which arises from an alternative reading frame of exon 2, is a basic nuclear protein which also provokes both a G1 phase and a G2 phase cell cycle arrest (Quelle *et al.*, 1995). The generation of INK4a/ARF 'knockout' mice gives rise to animals that are highly susceptible to tumour development (Serrano *et al.*, 1996) and yield fibroblasts which, unlike wild-type MEF, may be transformed by the overexpression of a single oncogene. Interestingly, these properties are reminiscent of the p53-null phenotype and of p53-null MEF. These phenomena were initially attributed to loss of the p16^{INK4a} mediated cell senescence pathway, but ARF-null mice which contain functional p16^{INK4a} were subsequently generated and shown to exhibit an identical phenotype (Kamijo *et al.*, 1997). Moreover, the tumours that arise in these mice also contain functional p16^{INK4a}. Taken together, these data suggest that there are two potential tumour suppressor genes encoded at the same locus. The relative importance of each gene with regards to tumour development will emerge with the generation of a p16^{INK4a}-null mouse, which retains wild-type ARF function.

Spurred by the similarity of the ARF-null phenotype to the p53-null phenotype,

researchers endeavoured to find a biochemical link between these two proteins. Ectopic overexpression of ARF stabilizes p53 and promotes the transcription of p53 target genes. The N-terminal portion of ARF associates with the C-terminus of *mdm-2*, thereby inhibiting both *mdm-2* mediated degradation of p53 and down-regulation of its transcriptional activity (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998). Human ARF, at least, is unable to associate with p53 in a *mdm-2* independent manner; rather, the three proteins exist as a ternary complex, p53 and ARF associating with opposing termini of *mdm-2*. One of these studies has reported that ARF overexpression leads to destabilization of *mdm-2*, but these observations are in conflict with other data, which report ARF-induced accumulation of *mdm-2*. As a transcriptional target of p53, an increase in *mdm-2* levels may simply reflect an increase of p53 activity. In most cell types, it appears that ARF interferes with the ability of *mdm-2* to target p53 to proteasome mediated degradation but that this mechanism is independent of *mdm-2* stability. Pomerantz *et al.* have suggested that cellular localization of the ternary complex may be a possible mechanism for p53 stabilization. In their studies, ARF and *mdm-2* are seen to co-localize in the nucleoli. Two recent papers have confirmed this observation, however p53 is not observed in the nucleoli (Weber *et al.*, 1999; Zhang and Xiong, 1999). It seems probable that ARF sequesters *mdm-2* in the nucleoli, thus blocking *mdm-2*-mediated nuclear export of p53. Importantly, some tumour-derived ARF mutants are unable to block p53 and *mdm-2* nuclear export (Zhang and Xiong, 1999).

ARF mediates p53 stabilization in response to oncogene expression

In order that primary cells may undergo oncogenic transformation, it appears that both pRB and p53 tumour suppressor pathways must be targeted (Greenblatt *et al.*, 1994; Weinberg, 1995). As mentioned above, the ectopic expression of oncogenes leads to the activation and accumulation of p53, and this in turn has been associated with both apoptosis and premature senescence. For this reason, DNA tumour viruses encode proteins which inactivate both pRB and p53 checkpoints, thereby promoting cell growth (and consequently, viral replication) whilst avoiding the activation of p53-induced growth suppressive pathways. The adenoviral oncoprotein E1A, *c*-myc and the human E2F-1 protein, each of which circumvents pRB mediated growth control, can elicit p53-dependent apoptosis (Debbas and White, 1994; Hermeking and Eick, 1994; Lowe and Ruley, 1994; Wagner *et al.*, 1994; Wu and Levine, 1994; Hiebert *et al.*, 1995). Conversely, E1A and *c*-myc can transform p53-null fibroblasts, whilst the transformation of p53-positive fibroblasts requires the expression of a cooperating oncogene. Until recently, the mechanism of oncogene-induced p53 activation remained unclear, but several new studies have implicated the ARF tumour suppressor in this pathway. Overexpression of E1A and *c*-myc in primary MEF induces ARF expression, whilst ARF-null MEF were shown to be partially resistant to oncogene induced apoptosis (De Stanchina *et al.*, 1998; Zindy *et al.*, 1998). Finally, the reintroduction of ARF into *c*-myc expressing ARF-null fibroblasts renders them susceptible to apoptosis. Taken together, these data provide strong evidence of a role for ARF in the p53 response to oncogene expression.

The induction of ARF expression by E1A is likely to proceed via pRB and the E2F transcription factors (*Figure 7.2*) since E1A mutants that fail to inactivate pRB and

deregulate the E2Fs are also defective in ARF signalling. Expression of E2F-1 can activate expression from the ARF promoter *in vivo* (Robertson and Jones, 1998), whilst activation of a conditional E2F-1 allele induces the accumulation of ARF mRNA (Bates *et al.*, 1998). The p53 protein itself appears to exert negative regulation on the ARF promoter, suppressing ARF protein levels in turn (Kamijo *et al.*, 1998; Robertson and Jones, 1998; Stott *et al.*, 1998). Together, these data suggest that a second, ARF-mediated, negative feedback loop compliment *mdm-2* mediated feedback inhibition to keep p53 levels in check under normal growth conditions. Presently, there are no further insights into the control of ARF and p53 in response to oncogenes, yet several discrepancies remain. Transfection of ARF alone leads to a cell-cycle arrest, and yet oncogene mediated induction of ARF is followed by apoptosis (Quelle *et al.*, 1995). Furthermore, E2F-1 deregulation can induce p53-independent apoptosis under certain conditions (Macleod *et al.*, 1996; Phillips *et al.*, 1997; Holmberg *et al.*, 1998). Further investigation is therefore required to clarify what might currently be described as cell specific differences.

P53 REGULATION BY COVALENT MODIFICATION

p53 regulation via phosphorylation

The activity of wild-type p53 must be subject to further levels of control in addition to the regulation of its stability. This is evident from the observations of Hupp *et al.* (1993), who showed that bacterially produced recombinant p53 did not bind efficiently to DNA in the absence of C-terminal modification. Moreover, UV-C treatment of non-proliferating mouse fibroblasts was shown to enhance p53 activity in the absence of any accumulation of the protein (Haapajarvi *et al.* 1997). The existence of two conformational forms of wild-type p53, the DNA binding and the non-DNA binding conformations, has long been recognized as a possible p53 regulatory mechanism. Wild-type p53 has been shown to exist in an equilibrium state between these two conformations, and factors that modulate this equilibrium may potentially modify the ability of p53 to activate transcription (McLure and Lee, 1999). Some of the mutations found in human tumours, although not directly associated with the DNA, prevent p53 activity by shifting the balance of the equilibrium to favour the non-DNA binding form. The conformational change induced by these mutations is apparent from the observation that these mutant proteins do not associate with *mdm-2*, although the mutation sites are not located within the *mdm-2* binding site, and are consequently more stable (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). It is only in very recent years that researchers have been able to identify pathways that link p53 conformational activation to the physical stresses that initiate p53-mediated responses. Both stabilization and activation of p53 may be achieved by phosphorylation in response to DNA damage, but these two events appear to be separable, being dependent upon phosphorylation at different sites (Chernov *et al.*, 1998).

Until recently, it has been difficult to evaluate the contribution of a given kinase or a given phosphorylation site to the activation of p53, probably owing to redundancy in the system. The p53 protein is regulated so tightly that several pathways apparently converge to the same endpoint. For example, ionizing radiation (IR) and ultraviolet irradiation (UV) induce the phosphorylation of serine 15 via different kinases. IR,

which causes double-stranded DNA breakage, does not optimally activate p53 in cells from ataxia-telangiectasisa (AT) patients that lack expression of the ATM kinase (Kastan *et al.*, 1992; Khanna and Lavin, 1993). This was linked to an inability of IR to induce p53 phosphorylation at serine 15 in these cells (Siliciano *et al.*, 1997). The observation that ATM can catalyze the phosphorylation of serine 15 *in vitro*, whilst IR induces ATM activity (Banin *et al.*, 1998; Canman *et al.*, 1998), suggests that the failure of ATM minus cells to induce serine 15 phosphorylation of p53 upon IR treatment is central to the inability of these cells to activate p53 under these conditions. In contrast, serine 15 phosphorylation in response to UV-C is not dependent upon ATM (Khanna and Lavin, 1993; Canman *et al.*, 1994; Siliciano *et al.*, 1997) and p53 accumulation and activation in response to UV are unaffected in AT cells. Other differences exist between IR- and UV-induced phosphorylation of p53. Phosphorylation of the casein kinase II site serine 392, for example, is observed specifically in response to UV irradiation (Kapoor and Lozano, 1998; Lu *et al.*, 1998). Evidence suggests that phosphorylation at this site may influence tetramerization of the protein (Sakaguchi *et al.*, 1997). Conversely, IR treatment leads to dephosphorylation of p53 at serine 376, a putative site of PKC phosphorylation, in an ATM-dependent manner. This latter modification facilitates the association between p53 and the 14-3-3 protein, thus enhancing DNA binding (Waterman *et al.*, 1998). Differences in the p53 modifications that are induced by individual stresses may indicate that each agent induces a distinct p53 response, dependent upon the effect that these combinations of modifications have on the overall activity of the p53 protein. As yet, the apparent complexity of p53 phosphorylation has foiled attempts to address this possibility.

Haupt and colleagues mutated six target serine residues in the N-terminus of p53 to alanine residues, creating six individual phosphorylation mutants at residues 6, 9, 15, 20, 33 or 37 and a multiple mutant in which all six sites were mutated (Unger *et al.*, 1999). The group observed that the multiple mutant protein and both site 15 and site 20 single substitution mutants were impaired in their ability to induce apoptosis in p53-negative tumour lines. Despite this, none of the mutant proteins were altered in their ability to suppress cell growth or trans-activate target promoters. These data imply that serines 15 and 20 are required for full activity of p53, but also indicate that single phosphorylation mutants may have subtle effects that have so far escaped detection.

There are many other consensus sites of known kinases within the p53 sequence. The stress activated *c*-Jun kinase, JNK, phosphorylates serine 34 and inhibits the association between p53 and *mdm-2* (Milne *et al.*, 1995; Fuchs *et al.*, 1998). In response to IR, the N-terminus of p53 is phosphorylated at serine 33 (Sakaguchi *et al.*, 1998), an *in vitro* target site of the CAK kinase (Ko *et al.*, 1997). Interestingly, if IR irradiation does indeed induce CAK activity, there are additional CAK sites located in the C-terminal domain that activate DNA binding activity when phosphorylated by CAK *in vitro* (Lu *et al.*, 1997). Furthermore, Casein kinase I has been implicated in drug-induced phosphorylation of the N-terminal domain (Knippschild *et al.*, 1997). Likewise, DNA-PK, a kinase that is activated by double-stranded DNA breakage, phosphorylates p53 at serines 15 and 37 (Lees-Miller *et al.*, 1992). However, whilst DNA-PK clearly phosphorylates p53 in response to DNA damage (Woo *et al.*, 1998), phosphorylation by this kinase does not appear to be necessary for p53 activation

(Jimenez *et al.*, 1999). The evaluation of the importance of each of these individual modifications is hampered by redundancy that prevents the use of many single phosphorylation site mutants in such studies. The data presented by Haupt and co-workers may indicate that some phenotypes may have been overlooked. Furthermore, single and double mutant p53 proteins that lack some of the consensus phosphorylation sites, including the ATM and JNK sites, are, nonetheless, fully functional and may still be stabilized by DNA damage (Blattner *et al.*, 1999). That many different kinases may phosphorylate an identical site in p53, and that p53 is subject to such diverse modification, imply that cell specific responses to p53 activation will always be apparent in the literature.

p53 regulation by acetylation

The p53 molecule is heavily modified *in vivo* and, as we will discuss briefly in this section, these modifications are not limited to phosphorylation. Notably, C-terminal modification of p53 and its subsequent conversion to the DNA binding conformation may also be achieved via acetylation. The association between the histone acetylase protein p300/CBP and the N-terminus of p53 enhances p53-mediated transcriptional activation (Avantaggiati *et al.*, 1997; Gu and Roeder, 1997; Gu *et al.*, 1997). Both p300/CBP and PCAF acetyl-transferases were subsequently shown to acetylate p53 directly at lysine 382 and lysine 320, respectively (Sakaguchi *et al.*, 1998). Although no direct correlation between stress signalling and acetylation of p53 had previously been described, Sakaguchi *et al.* were able to demonstrate that p53 phosphorylation at the N-terminus is required for these acetylation events. Acetylation form specific anti-p53 antibodies were used to show that lysine 320 is acetylated in response to UV irradiation, whilst lysine 382 is modified upon UV and IR treatments. These acetylation events appear to depend upon DNA damage induced phosphorylation of the N-terminal serines 33 and 37, suggesting that C-terminal acetylation of p53 is dependent upon a signalling cascade involving DNA damage induced phosphorylation. The mechanism by which acetylation of the C-terminus influences p53 activity is, as yet, subject to debate. A change in the conformation of the DNA binding site is one possibility, but acetylation may also inhibit the non-specific DNA binding associated with the C-terminal domain. PCAF and p300/CBP may conceivably enhance the function of the trans-activation domain by catalyzing histone acetylation around the transcription start site. Further work is required to distinguish between these possibilities.

OTHER MECHANISMS GOVERNING P53 ACTIVITY

Finally, we should cast our attention briefly to some of the many proteins that reportedly associate with p53 and modify its function. For example, p33[INGI], a potential tumour suppressor gene that demonstrates LOH in head and neck carcinomas, was recently shown to co-immunoprecipitate with p53 (Garkavtsev *et al.*, 1998) and to suppress cell growth in a p53-dependent manner. Ectopic expression of p33[INGI] enhances p53-mediated trans-activation, although the mechanism by which the two proteins cooperate is unknown. The p33[INGI]/p53 pathway may contribute to cellular senescence, as suggested by the observation that p33[INGI] expression is enhanced in

senescent fibroblasts, whilst antisense oligonucleotides increase the lifespan of primary fibroblasts in culture. Furthermore, two redox-responsive p53-binding proteins, Ref-1 (Jayaraman *et al.*, 1997) and HIF-1 (An *et al.*, 1998), may help to shed some light on the as yet elusive mechanism by which p53 is activated in response to hypoxia. Binding of the DNA repair proteins XPB, XPD and rad51 suggest that p53 may be somehow involved in the repair of damaged DNA (Wang *et al.*, 1995; Buchhop *et al.*, 1997). Finally, the observation that a proline rich region between the trans-activation domain and the DNA binding domain can mediate associations between p53 and SH3 domain containing proteins such as BP2 and *c*-abl could link p53 to a host of signal transduction cascades (Gorina and Pavletich, 1996; Yuan *et al.*, 1996). The possible consequences of these associations have barely been addressed and will surely provide fertile ground for future research.

The complexity of p53 regulation doubtless reflects the importance of this protein cell growth control. Indeed, either deregulated p53 expression or the loss of p53 inducibility would be expected to have catastrophic consequences for the multicellular organism. Targeting these regulatory mechanisms directly to the protein, as opposed to transcriptional targeting, theoretically permits p53 activity to be up-regulated and down-regulated rapidly. Clearly, many questions remain concerning the regulation of p53 activity, not least in the p53 response to redox stress, disruption of cell adhesion and ribonucleotide depletion.

p73 and p51 (p63), members of a p53 gene family

A characteristic of many of the well-known tumour suppressor genes is that they tend to belong to gene families that possess functional and structural similarities. The members of these families are not necessarily all classified as tumour suppressor genes. For example, the *RB-1* gene, which encodes the pRB tumour suppressor protein, is part of a multigene family that includes the genes encoding the cell cycle regulatory proteins, p107 and p130. The latter two genes do not appear to be involved in tumourigenesis. Likewise, the four INK4 genes also encode a family of CDK4 inhibitors, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, in addition to the functionally unrelated $p14^{ARF}$ alternative splice product of the INK4a locus. p53 was previously thought to be unique in this regard until the recent cloning of the p73 gene, which shares strong homology with p53 in the trans-activation, DNA binding and oligomerization domains (Kaghad *et al.*, 1997). Subsequently, another human p53 homologue was cloned, known as p51, and its murine and rat counterparts, p63 and KET respectively, were also identified (Osada *et al.*, 1998; Yang *et al.*, 1998). As is the case for p107 and p130, p73 and p51 share more homology with each other than they do with p53 itself. However, both p73 and p51 encoded proteins associate with p53 consensus binding sites *in vitro* and thus initiate both growth suppressive and apoptotic in tissue culture systems (Jost *et al.*, 1997; Kaghad *et al.*, 1997; Yang *et al.*, 1998). Unlike p53, both of these proteins exist in several alternatively spliced isoforms. p73 is expressed as α, β , γ and δ isoforms of which α, β and δ isoforms activate p53 promoters to varying degrees in cells, whereas the γ isoform has almost no transcriptional activity and cannot repress cell growth (DeLaurenzini *et al.*, 1998). Similarly, p51 is expressed as active A and B isoforms (which correspond to p73 β and α, respectively), in addition to dominant-negative forms which lack the N

terminal trans-activation domain. Another difference between p53 and the other p53 family members is their respective mechanisms of activation. Neither p73 nor p51 appear to be induced by the same pathways as those that activate p53, although there may be some overlap. The *mdm-2* protein, for example, can suppress the transcriptional activation function of p73 although it does not target the protein to ubiquitin mediated degradation (Zeng *et al.*, 1999). Very recently, the p73 protein has been shown to be either activated or stabilized in response to some forms of DNA damage, involving a phosphorylation cascade that acts via the non-receptor tyrosine kinase, *c*-abl (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). *C*-abl associates with p73, via a PXXP motif in the c terminus of p73, and this association is shown to enhance both p73-mediated apoptosis and the apoptotic cell death observed in response to cisplatin. p73 appears to be stabilized in response to cisplatin and tyrosine-phosphorylated in response to IR. Although it is not clear what the specific differences in p73 modification represent, both events seem to proceed p73-dependent apoptosis.

The respective 'knockout' mice suggest that the p53 family proteins participate in completely separate developmental pathways. In particular, deletion of the mouse gene that encodes p63, the homologue of p51, has not been linked to the development of tumours since the new-born p63-null mice die within days of birth as a result of severe developmental defects (Mills *et al.*, 1999; Yang *et al.*, 1999). In contrast to the p53-null mice that undergo essentially normal development, these mice fail to develop limbs and stratified epithelium, suggesting that p51 and its homologues are essential for ectodermal differentiation. Since p63 is highly expressed in the dividing basal layer of the epidermis and not in the non-proliferative differentiating layers, it is suggested that p63 is required to maintain the basal cell population. How this fits with the observation that p63 induces cell cycle prohibitive and death-inducing genes is not yet clear. Additionally, it is not understood whether it is the p51 A and B isoforms or their dominant-negative counterparts that are responsible for this phenotype.

Whether either of the p73 or p51 genes has tumour suppressor function has been a topic of much recent debate. Mutations in either gene appear to be rare in tumour samples. p73 disruption appears to be associated with some cancers, notably neuroblastoma, but biallelic disruption of the corresponding 1p36 locus has not been observed (Kovalev *et al.*, 1998; Mai *et al.*, 1998a; Nomoto *et al.*, 1998; Takahashi *et al.*, 1998). This may be due to its predicted monoallelic expression (Kaghad *et al.*, 1997). Thus, if the p73 gene is imprinted, one would expect that loss of the transcribed allele should be sufficient to disrupt expression of the proteins. At present, however, there is some confusion as to whether p73 is monoallelically expressed in all tissues or if there is some tissue type dependence. Moreover, several groups have reported biallelic expression specifically in tumour tissue as opposed to monoallelic expression in the surrounding normal tissue (Kovalev *et al.*, 1998; Mai *et al.*, 1998b). These latter observations, taken together with the observation that p73 mRNA levels are augmented in these tumours relative to the surrounding tissue, would seem to be at odds with a role for p73 as a tumour suppressor protein. Further evidence against p73 as a tumour suppressor protein may be taken from the observation that the viral oncoproteins, SV40 T-antigen, human papillomavirus E6 and the adenovirus E1B 55K protein, which effectively abrogate p53 function during viral infection, do not similarly target p73 function (Marin *et al.*, 1998). It would therefore appear that

aberrant cell cycle progression, through expression of viral and cellular oncogenes, does not lead to increased p73-dependent apoptosis, as has been observed in the case of p53.

It is paradoxical that p73 should activate p53-inducible promoters and induce both growth arrest and apoptosis, whilst mRNA expression and allelic studies in tumours should imply that p73 expression may correlate with increased cell proliferation. Likewise, p51 appears to be necessary for proliferation of basal cells in the epidermis. Perhaps the answer to this conundrum lies in the relative expression of the different splice isoforms. It is possible, perhaps, that whilst p73 $\alpha/\beta/\delta$ and p51A/B induce cell cycle arrest and apoptosis in certain cell types, the other isoforms, being transcriptionally-deficient, may function in a dominant-negative fashion when their expression is predominant. In future research, it will be important to correlate these tissue specific differences with the p73 and p51 isoforms expressed in these tissues and their derived tumours. Additionally, the observation that p73 can participate in the DNA damage response without, apparently, possessing tumour suppressor activity forces us to re-evaluate our view of p53 as a protector against genetic abnormality. It will be necessary to define the differences between p53 and p73 in this regard. It is possible that p73 is simply responsive to fewer signals, expressed in fewer cell types, or indeed less potent in the induction of apoptosis in general. We must await the analysis of the p73-null mice in order to define the differences between the functions of the p53 family proteins *in vivo*.

The p53 status and cancer therapy

P53 AND TUMOUR SENSITIVITY TO CONVENTIONAL THERAPY

Modern chemotherapeutic and radiotherapeutic strategies for the treatment of cancer rely upon the fact that tumour tissue appears to be more sensitive to chemical and physical DNA damaging agents than the surrounding normal tissues. Clearly, this distinction is important to the success of these treatments. However, the reason for this increased sensitivity has remained obscure. Currently, there seems to be a vague correlation between the rapidity of cell division and the sensitivity to drug-induced cell death, the most rapidly dividing tumour cells being the most sensitive. Since each of these agents cause, or at least imitate, DNA damage, apoptosis in response to these agents is generally mediated through p53. One would, therefore, predict that loss of the wild-type p53 function during tumourigenesis should be indicative of failure of the resulting tumours to respond to chemotherapy and radiotherapy. However, since the inactivation of p53 allows for the accelerated accumulation of genetic aberrations, perturbation of wild-type p53 function is a very frequent event in tumourigenesis. The restoration of p53 expression in p53-negative tumour cells has been much discussed in recent years as a possible gene therapy for cancer. Evidently, effective use of both existing therapies and the development of new gene therapy strategies will be dependent upon a thorough understanding of the effect of p53 expression upon tumour growth *in vivo*.

The role of p53 in response to chemotherapy has been approached by both the study of p53 status in chemoresistant versus chemosensitive tumours *in vivo* and, additionally, by examining DNA damage-induced apoptosis in wild-type and p53-null cell

lines in culture. Generally, cancer types which are highly sensitive to chemotherapy also tend to retain wild-type p53 function, whilst cancer types such as lung cancers, which have a high frequency of p53 mutation, are more resistant to chemotherapy (Wada *et al.*, 1993; Greenblatt *et al.*, 1994; Chresta *et al.*, 1996; Wilson *et al.*, 1997). Despite this, not all p53-positive tumours respond to chemotherapy, whilst some p53-negative tumours are chemosensitive, suggesting that the relationship is not simply dependent upon wild-type p53 expression. There is currently some evidence that the p53 status within a subgroup of cancers may be used to determine how a given patient will respond to a particular treatment, for example, in patients suffering from ovarian cancer (Righetti *et al.*, 1996; Buttitta *et al.*, 1997). However, the emerging theme from the recent publications on this subject is that the effect of p53 expression on chemo- and radiosensitivity is dependent upon cell context. This is perhaps less surprising when we consider the numerous levels at which p53 activity can be controlled. One of the major shortcomings of p53 expression screening as a diagnostic procedure is that it does not take the possible presence of other cell death suppressive mutations into consideration. Many p53-positive cell lines express very high levels of p53 protein, at levels high enough to induce apoptosis in normal cells. This would seem to suggest the presence of other mutations in the apoptotic pathways, perhaps including the loss of bax expression, which would otherwise be induced by p53, or overexpression of the anti-apoptotic factor bcl-2. Alternatively, as has been observed to occur during the development of gliomas, overexpression of the *mdm-2* oncogene may also bypass wild-type p53 (Biernat *et al.*, 1997).

In terms of current assessment of what constitutes an active p53 allele, the use of transcriptional activity as a property which determines whether a given p53 allele is active may be flawed, for several reasons. The p53-mutant 175 proline, for example, is transcriptionally active and able to induce G1/S arrest but is unable to elicit an apoptotic response. Conversely, the transcriptionally inactive mutant 22/23, which is not generally associated with tumour development, can activate apoptosis in some cell types (Caelles *et al.*, 1994; Haupt *et al.*, 1995). If p53-induced apoptosis is vital to its role in drug-sensitivity, these two mutant alleles are somewhat deceiving. It is important to remember that some of the dominant negative p53 alleles, which block transcriptional trans-activation by the wild-type protein, may also possess gain of function properties. Differences in the types of tumours observed in p53-null mice versus mice which express a dominant negative p53 transgene, and the observation that dominant negative p53 increases the tumourigenicity of p53-negative cells, together support the notion that tumour-derived dominant negative alleles of p53 have other functions above and beyond the inactivation of the wild-type protein (Dittmer *et al.*, 1993). Furthermore, the dominant negative R175H allele has been shown to disrupt a checkpoint that presides over the formation of the mitotic spindle, endowing the protein with the ability to contribute directly to genetic instability in addition to the negation of wild-type p53 activity (Gualberto *et al.*, 1998). Finally, a mutant allele which disrupts oligomerization of p53 has been isolated from a Li-Fraumeni family, despite the fact that oligomerization is not required for either trans-activation or growth suppression in cultured cells. These data suggest that the criteria by which active and non-active p53 alleles are classified need to be modified.

Tissue culture studies have revealed that transformed cell lines lacking p53 activity are generally more chemosensitive than those expressing the wild-type protein (Fan

et al., 1994; Chresta *et al.*, 1996), whilst the correlation between p53 and radio-sensitivity appears to differ between cell types (Brachman *et al.*, 1993; McIlwrath *et al.*, 1994; Siles *et al.*, 1996). In one study, disruption of p53 function in a p53-positive tumour line through the introduction of the viral oncogene HPV16 E6 resulted in enhanced resistance to irradiation and to most chemotherapeutic agents (Fan *et al.*, 1995), with the exception of cisplatin. These studies, of course, are subject to the same caution as the studies *in vivo*, namely that they do not take other apoptosis-inhibitory mutations into account. To avoid this possibility, researchers have studied the effects of p53 status on the DNA damage response in primary tissues.

Surprisingly, data from several laboratories demonstrated that disruption of p53 function in normal diploid fibroblasts actually sensitized the cells to apoptosis following treatment with DNA damaging agents (Petty *et al.*, 1994; Hawkins *et al.*, 1996). Moreover, MEF obtained from p53-deficient mice were also found to be more sensitive to these chemicals than wild-type MEF. Some clues to resolving this paradox are evident from the work of Lowe *et al.* (1993b) who demonstrated, in contrast to the above results, that primary MEF expressing the adenoviral E1A protein were made resistant to drug treatment by disruption of p53. The E1A protein targets the pRB pathway and thus prevents p53-induced cell cycle arrest whilst sensitizing the cells to p53-induced apoptosis. This has lead to the hypothesis that primary cells are protected from DNA damage-induced apoptosis via the p53-induced G1 arrest pathway, whilst loss of p53 expression abrogates this pathway and thus the cells become susceptible to p53-independent apoptosis. Since many chemotherapeutic drugs target the DNA replication machinery directly, one can simply envisage that halting the cell cycle under these conditions would minimize DNA breakage and thus prevent apoptosis (Hanawalt, 1994). Evidence for a p53-independent mechanism of apoptosis in response to DNA damage comes from the observation that the intestinal epithelium of irradiated mice suffers a wave of p53-independent apoptosis late after the initial burst of p53-induced apoptosis (Clarke *et al.*, 1997). This simple balance would be disturbed by the presence of a transforming oncogene that abrogates the G1 arrest pathway. In these cells, activation of p53 can only induce apoptosis and therefore the loss of p53 becomes beneficial to cell survival. The enhanced cell death observed in cells that overexpress oncogenes may be due to priming of the p53 response through activation of the ARF pathway. There is one observation which apparently contradicts this hypothesis, that both radioresistance and chemoresistance are conferred on primary thymocytes and haematopoietic cells upon the loss of p53 function (Clarke *et al.*, 1993; Lee and Bernstein, 1993; Lowe *et al.*, 1993a). However, the biology of these cell types differs to other cells in that they more readily undergo apoptosis than establish a G1 phase arrest, a factor that could explain why these cells behave differently.

PROSPECTS FOR P53 IN GENE THERAPY

Following on from the studies that assess the influence of p53 status on the effectiveness of conventional therapeutic agents, researchers have endeavoured to evaluate the feasibility of re-introducing p53 into tumours as a means of gene therapy for patients with p53-null malignancies. Results of pilot studies have been promising. For example, introduction of p53 into a panel of cancer cell lines, using adenovirus as a

vector for the wild-type p53 allele, induced p21$^{WAF1/CIP1}$ expression and growth arrest, followed by apoptosis (Blagosklonny and El-Deiry, 1996). Interestingly, in this latter study, exogenous addition of p53 to p53-positive cell lines induced G1 arrest but reduced apoptosis, confirming the suspicion that these cells must contain downstream mutations in the apoptotic pathways. Encouraging results from Fujiwara *et al.* (1994) and Gallardo *et al.* (1996) demonstrated that similar transfer of p53 to human cancer cells increased the drug and radiation sensitivity of derived tumours when the cells were subcutaneously injected into mice. Unfortunately, the complexity of p53 regulation, as described above, complicates this seemingly simple strategy. The emerging message from these and related studies is that, in the event of the development of a suitable strategy for a p53-based gene therapy, it will be essential to exercise caution over the types of tumour to which it is applied. Prior to treatment, it will be important to establish whether p53 expression in a particular cell type will lead to apoptosis rather than cell cycle arrest, the latter phenotype being associated with resistance to chemotherapy. A wealth of recent reports indicates that the outcome of exogenous p53 expression appears to be both cell type and drug specific. However, the combination of gene therapy with chemotherapy and radiotherapy is still an intriguing and feasible prospect. Success depends upon exhaustive cell culture data being correlated with results obtained from animal models, thus allowing a tailored combination of drugs and targeted p53 expression to give the most effective results against each tumour type.

Conclusions

In this review we have attempted to present a broad overview of what is currently one of the most active fields of research in molecular biology. *TP53* was initially cloned twenty years ago, but here we have concentrated on the topics that have developed most rapidly during the last three years. Recent advances in the areas of p53 activation and stabilization; the identification of a p53 gene family and studies using transgenic and 'knockout' mice have broadened our knowledge of a gene that is crucial to our understanding of the mechanisms of carcinogenesis. In the coming years, we expect to see more interesting developments in this field. The role of the p53-related genes in development will be a focus of intense study. The recent development of nanotechnology will greatly aid p53 research, as well as research into other transcriptional activators and repressors. We can also hope to see some of the preliminary data generated from the study of animal models of p53-based gene therapy. It is clear that p53 can no longer be considered simply as 'active' or 'inactive'. One of the greatest challenges will be to correlate all of the data obtained from different mutants in primary and in tumour-derived tissues to construct a working model of p53 function in different contexts. Such information will be essential if p53-mediated gene therapy is to be used successfully.

Acknowledgements

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