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MECHANISMS INVOLVED IN p53 REGULATION

by

Supriya Vishwaraj Gaitonde

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF CANCER BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

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entitled Mechanisms Involved in p53 Regulation

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Jesse Martinez — Date 11/7/00

G. Tim Bowden — Date 11/7/00

Robert Gillies — Date 11-7-00

Ronald Heimark — Date 11/7/00

Luke Whitesell — Date 11/7/00

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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DEDICATION

I would like to dedicate my Ph.D. degree to my husband Vish Gaitonde and my parents Sumant and Suman Khanolkar. Their support and patience contributed immensely.

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ABSTRACT

Inactivation of the tumor suppressor protein p53 is a very important and common step in the process of carcinogenesis. The overall purpose of this project was to gain a better understanding of the mechanisms involved in the regulation of p53 function. To gain insight into these mechanisms, we chemically mutagenized A1-5 cells expressing high levels of temperature sensitive $p53^{val135}$ (tsp53) and selected for clones that were capable of growth at the permissive temperature for p53 activation. The clones generated, called ALTR (for A1-5 Low Temperature Resistant), could grow at the permissive temperature. Using the ALTR cell system and the parent A1-5 cells, we determined that nuclear translocation of p53 could result in a change in the conformation from mutant to wild-type but that these may be two separable events. We also investigated, in depth, the mechanism by which p53 was inactivated in one ALTR cell line, ALTR 9. We identified calpain mediated degradation of p53 as a partial mechanism of p53 inactivation in these cells. Our results suggest that degradation of p53 by calpain can lead to the functional inactivation of p53 and that this degradation can be regulated by genomic stress. To gain insight into the significance of cytoplasmically sequestered p53 protein in tumors, we chose a neuroblastoma derived cell line, SK-N-SH, that expresses a wild-type but cytoplasmically sequestered p53 protein We report here, that down regulation of p53 by HPV-16 E6 resulted in the morphological conversion of SK-N-SH cells to substrate-adherent fibroblast-like S-type cells. The morphologic conversion was accompanied by a loss of neurofilament expression, a marker for the

neuronal N-type cells, an increase in the expression of vimentin, a lack of responsiveness to RA induced neuronal differentiation, and loss of anchorage independent growth. These results suggest that p53 is required for the maintenance of the neuroblastic tumorigenic phenotype. Both the ALTR cell system and the SK-N-SH cells provided us with insight into the mechanisms involved in p53 inactivation resulting in tumor formation.

CHAPTER I

Background Information

Carcinogenesis is a multi-step process that culminates in a clinically observable tumor due to the accumulation of multiple genetic mutations that disrupt the functioning of a variety of genes that regulate cell growth, differentiation, and death. An important class of genes that are mutated in human cancers are the tumor suppressors and, of these, the most frequently mutated is p53.

Discovery of p53 as an oncogene

In 1979, two different types of studies led to the discovery of the p53 protein. The first used a virologic approach by using SV40-transformed cells. In these studies, a 55 kDa protein was observed to coprecipitate with the large T-antigen of the SV40 virus in SV40-transformed rodent and human cells (Crawford et al., 1980; Linzer & Levine, 1979; Linzer et al., 1979). In these studies, it was also determined that this 55 kDa protein was of cellular origin and related to the transformation process since it was overexpressed in SV40 transformed cells and since transformation with SV40 resulted in increased synthesis or stability of this protein (Linzer & Levine, 1979; Linzer et al., 1979).

The second approach was the lesser known serological one. Using this approach, antibodies were detected in mice injected with methylcholanthrene-induced tumor cells against a 53 kDa protein (DeLeo et al., 1979). Other groups showed this antibody

response by immunoprecipitating a 53 kDa protein in animals with several different types of tumors (Kress et al., 1979; Rotter et al., 1980). Later, Crawford et al (Crawford et al., 1982) also demonstrated an antibody response to a 53 kDa protein in the serum of patients with breast tumors.

Initial experiments with this 53 kDa protein suggested that it functioned as an oncogene since it was found elevated only in tumor and virus transformed cells and found at very low levels in normal cells. In the early experiments several codon changes noted in murine cDNA clones of p53 were thought initially to be polymorphisms. However, comparison of the differences in the sequences of these murine cDNA clones with p53 from lower species indicated that some of these differences occurred in highly conserved regions of the protein (Soussi et al., 1990; Soussi et al., 1987). This feature not being common to polymorphisms in fact suggested that the different cDNA clones were probably expressing mutant p53 protein.

This initial discovery of p53 as an oncogene, a gene that actively promotes tumor growth, was not as exciting to the scientific field since several other oncogenes had been isolated that seemed more promising than p53. When p53 was discovered as a tumor suppressor gene instead of an oncogene, which the mutant form represented, it gained the importance in the field and has since been the most extensively studied tumor suppressor gene. The fact that mutations in p53 are 'doubly dangerous' is evident since the mutant form not only deprives the cell of the wild-type's beneficial effects in cell cycle control but can also spur abnormal cell growth and behave as an oncogene.

The functional role of p53

p53 is a transcription factor

Since the late 1980s p53 has been shown to play the role of a tumor suppressor protein (Levine, 1997; Levine et al., 1994). It acts as a tumor suppressor mainly through its function as a transcription factor. The p53 gene product is a 53 kDa protein consisting of 393 amino acids and consists of 5 domains conserved through evolution (Prives, 1994). The p53 gene is located on the short arm of chromosome 17 at 17p13.105-p12 (Levine et al., 1991). The p53 gene product is expressed from the second of the 11 exons present in the genomic DNA. The protein has been divided into three major domains, a highly acidic amino terminus which is also responsible for the protein's transactivation abilities, the middle DNA binding domain which binds directly to DNA and also consists of 4 of the evolutionarily conserved domains (domains 2-5); and a highly basic carboxy domain which is important in oligomerization of the protein and in regulating the protein's ability to interact with DNA (Figure 1).

Downstream targets of p53 and their role in cell cycle progression

The p53 tumor suppressor protein is thought to transactivate approximately 100 genes but the consequences of this gene activation are not completely understood (Amundson et al., 1999; Zambetti et al., 1992a). Transactivation of genes by p53 is dependent on sequence-specific interactions with DNA containing the sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', typically in the context of two such sequence motifs separated by 0 to 13 bps (el-Deiry et al., 1992; Kern et al., 1992). In addition to transactivation, p53 has also been shown to be an active repressor of some genes

(Ginsberg et al., 1991; Haldar et al., 1994; Miyashita et al., 1994a; Miyashita et al., 1994b).

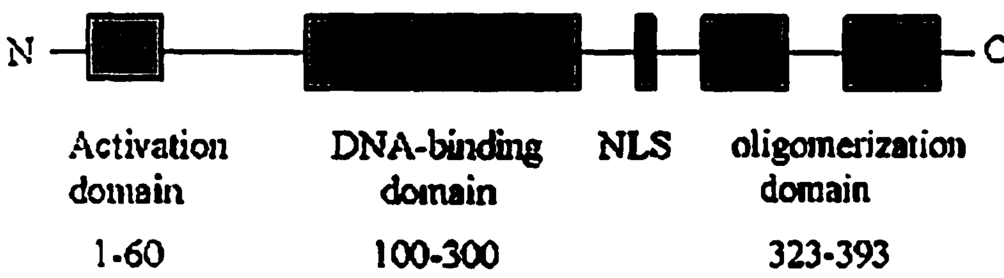


Figure 1. Structure of p53 showing the major domains. Abbreviations: Nuclear Localization Sequence (NLS)

p53 and the cell cycle checkpoints

A complex interaction of molecules regulates the cell cycle. Coordination of these complex processes is thought to be achieved by proteins called cyclins and their associated enzymes, cyclin-dependent kinases (CDKs) (Hartwell & Kastan, 1994). The cyclin-CDK complexes undergo changes in the kinase and cyclin components that are believed to drive the cell from one stage of the cell cycle to the next. This passage from one stage of the cell cycle to the next is tightly regulated by controls that act on the transcription of cyclin genes, the degradation of cyclin proteins and the modification of the kinase subunits by phoshorylation. Cell cycle progression can be modulated by negative controls which can play a role during development, differentiation, senescence and cell death. Such negative controls are important in preventing tumorigenesis and are called cell cycle checkpoints. Cell cycle checkpoints are signal-transduction pathways whose effectors interact with cyclin/CDK complexes and block the cell cycle. Such 'checkpoints' exist to monitor the integrity and replication status of DNA before cells commit to either replicate their DNA in S phase or to segregate chromosomes during mitosis.

The need to block cells during the cell cycle arises when the intergrity of the genome has been compromised, and failure to arrest cells could lead to cells with highly unstable genomes that could potentially evolve into tumor cells. Two distinct checkpoints have been described to block cell cycle progression in the event of DNA damage. One of the checkpoints exists at the G1/S boundary and the second at the G2/M transition. The tumor suppressor p53 is an important component of the G1 checkpoint

and has been shown to play a role in the maintenance of the G2 checkpoint as well. Cells null for p53 are not only unable to induce a G1 checkpoint but have a reduced capacity to maintain the G2 checkpoint as well (Bunz et al., 1998; Chan et al., 1999; Paules et al.. 1995). Implicating a role for p53 in both, G1 and G2 cell cycle checkpoints, suggests that this protein has an integral role in maintaining the normal status of the cell and therefore, inactivation of it either by mutation or through other mechanisms is an important event in the cell's transition to tumorigenesis. Therefore, it is not surprising that the p53 tumor suppressor gene is one of the most commonly mutated genes in human cancers (Hollstein et al., 1991; Nigro et al., 1989). During multi-stage carcinogenesis, it is widely assumed that functional inactivation of p53 is a common and requisite step towards tumorigenesis (Sherr, 1996).

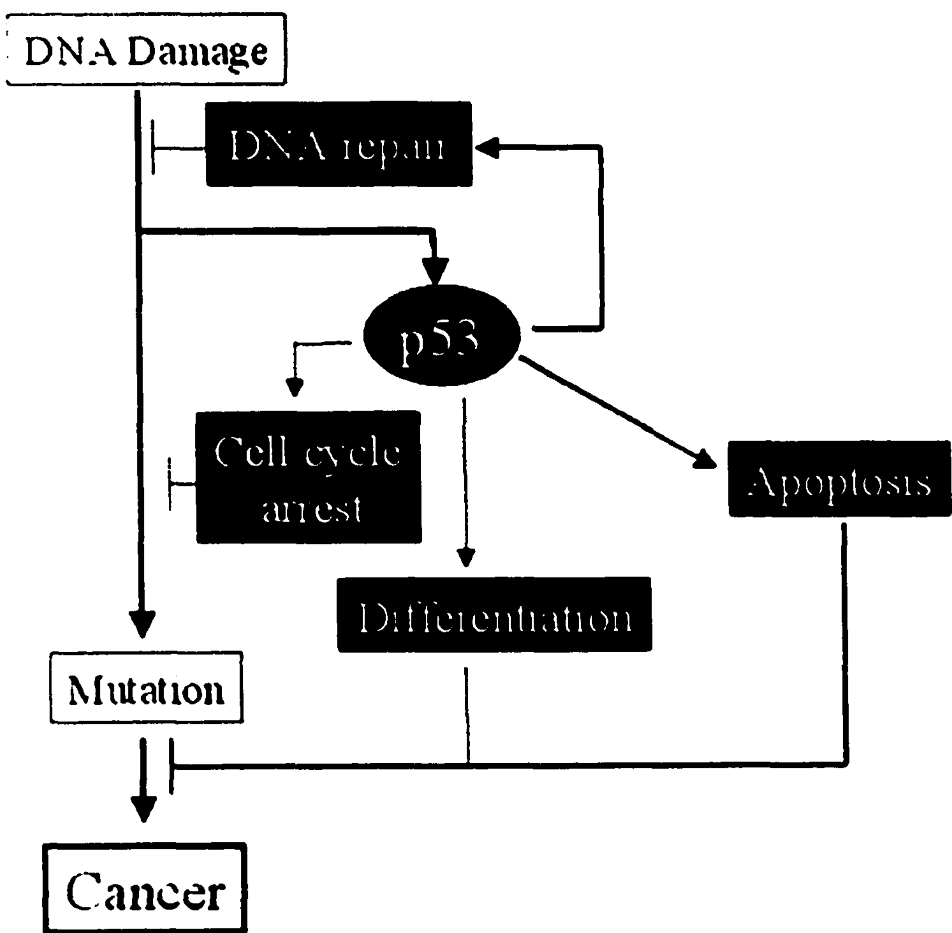


Figure 2. Schematic model describing the downstream functions of p53.

Role of p53 in G1 and G2 cell cycle arrest:

One of the most well understood targets downstream of p53 transactivation is the gene product of p21/WAF1 (el-Deiry et al., 1993) (Figure 2). The p21 protein induced by p53 in response to genotoxic stress is a potent inhibitor of G1 cyclin dependent kinases, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression. p21 is a member of the p27 and p57 KIP family of universal cell cycle inhibitors (Gorospe et al., 1999). p21 can also bind to proliferating cell nuclear antigen (PCNA) and inhibit processivity of DNA replication (Boulaire et al., 2000: Rousseau et al., 1999). Induction of p21 by p53 causes cell cycle arrest at the G1/S border of the cell cycle in response to stress signals such as ionizing radiation (I.R.) (Dulic et al., 1994). This arrest in the G1 phase of the cell cycle in response to genotoxic stress is thought to be important since it also allows p53-induced DNA repair activity to occur. Such DNA repair is necessary for cell survival since it is believed that DNA damage left unrepaired will result in an apoptotic response in response to genotoxic stress. Another down stream target of p53, 14-3-3 σ has been implicated in p53 dependent G2 arrest of the cell cycle in response to genotoxic stress such as IR (Chan et al., 1999). It is believed that upregulation of 14-3-3 by p53 in response to stress allows cdc25c to become bound to it and sequesters it into the cytoplasm thus altering cdc25c's phosphatase activity from targeting cyclin B/cdc2 present in the nucleus and blocking the cell in the G2 phase of the cell cycle (Hermeking et al., 1997).

P53's role in DNA repair

The p53 gene product has been shown to play a role in DNA repair through several different pathways. In response to damage induced by ultraviolet light, p53 has been shown to be directly involved in nucleotide excision repair (NER) by transactivating the GADD45 gene which plays a role in NER based on recent evidence that demonstrated GADD45's ability to bind to UV-damaged chromatin thus affecting lesion accessibility (Smith et al., 1995; Smith et al., 2000; Therrien et al., 1999). The ribonucleotide reductase gene, p53R2 is also involved in DNA repair and is directly induced by p53 in response to IR, UV and adriamycin treatment, suggesting its integral role in various types of DNA repair (Nakano et al., 2000; Tanaka et al., 2000).

Role of p53 in the induction of apoptosis

P53 also plays a role in the induction of apoptosis in response to genotoxic stress. Apoptosis or programmed cell death is a mechanism of 'cell suicide' that cells undergo in the event of high levels of DNA damage. The mechanism by which p53 decides to induce growth arrest or apoptosis is not clearly understood but it is widely believed that induction of apoptosis by p53 is dependent on the extent of DNA damage. If the damage to DNA is excessive and efficient DNA repair is not feasible, p53 is capable of sensing the extent of DNA damage and induces apoptosis instead of cell arrest (Bates & Vousden, 1996; Kastan et al., 1995). This allows cells in which DNA is not completely repaired to undergo apoptosis instead of allowing it to enter the cell cycle and replicate damaged DNA thus accumulating genetic alterations over time. To determine the

mechanism of p53-induced apoptosis, Polyak et al (Polyak et al., 1997) examined in detail the transcripts induced by p53 expression before the onset of apoptosis. Of 7,202 transcripts identified, only 14 (0.19%) were found to be markedly increased in p53-expressing cells compared with controls. Strikingly, many of these genes were predicted to encode proteins that could generate or respond to oxidative stress. Additional biochemical and pharmacologic experiments suggested that p53 results in apoptosis through a 3-step process: (1) the transcriptional induction of redox-related genes such as bax; (2) the formation of reactive oxygen species; and (3) the oxidative degradation of mitochondrial components, culminating in cell death. Specifically, p53 has been shown to induce several apoptosis related genes such as bax, killer, and PIGs (Kastan et al., 1995).

In addition to transactivation, p53 has been shown to play a role in apoptosis regulation through transactivation independent mechanisms. Such mechanisms have also been shown to involve the generation of reactive oxygen species and abrogation of the membrane potential in mitochondria (Johnson et al., 1996; Li et al., 1999). In addition, transport of the fas receptors from the Golgi complex to the cell surface has also been linked to transactivation-independent p53 mediated apoptosis (Bennett et al., 1998).

Role of p53 in cell differentiation

The p53 gene product has also been shown to play a role in differentiation. Several reports have shown that interference of p53 activity correlates with an inhibition of differentiation in hematopoietic as well as muscle differentiation (Cerone et al., 2000;

Rizzo et al., 1998; Soddu et al., 1994; Soddu et al., 1996). A direct role for p53 in the differentiation process was observed by its ability to transactivate muscle creatine kinase, a protein involved in muscle differentiation (Tamir & Bengal, 1998). Poluha et al (Poluha et al., 1997) indicated a role for p53 in the neuronal differentiation of PC12 cells, a pheochromocytoma cell line by Nerve Growth Factor (NGF) while Kokunai et al (Kokunai et al., 1998; Kokunai et al., 1997) indicated that p53 was required for the differentiation of glioma cells. Transfection of wild-type but not mutant p53 in HL60 cells, which are null for p53, restored their ability to differentiate into monocytes (Banerjee et al., 1995). P53 may also play a role in spermatogenesis since high levels of the protein were observed in adult mice testes (Rotter et al., 1993). The generation of p53 knockout mice is, however, indicative of a lack in the requirement of p53 for normal mammalian development but these results can be explained by the existence of other proteins such as p63 and p73, members of the p53-group of proteins, which display overlapping functions (Donehower et al., 1992; Kaelin, 1999a; Kaelin, 1999b). A schematic diagram of the downstream target genes of p53 and their role in tumor development has been shown in Figure 2.

P53 regulates itself

Finally, p53 is involved in the transactivation of the cellular proto-oncogene mdm2, which is responsible for proteasomal degradation of p53 and its maintenance at low levels in the cell. Mdm2 (mouse double minute) acts as a ubiquitin ligase and targets p53 for proteasomal degradation. Lack of mdm2 expression causes cells to undergo

apoptosis (de Rozieres et al., 2000) that is p53 dependent. Mdm2 knockout mice are embryonic lethal but can be rescued if p53 is also knocked out suggesting the importance of mdm2 in p53 regulation and this will be discussed in a later section (Lozano & Liu. 1998).

Regulation of p53 function

The tumor suppressor protein p53 is not required for normal mammalian development based on the development of p53 knockout mice (Donehower et al.. 1992). However. in response to several different types of responses such as DNA damage by IR, p53 gets induced and prevents the proliferation of cells in which DNA damage has occurred. In addition to radiation by IR and UV light, p53 is also induced in response to chemical mutagens. hypoxia, temperature stress, nucleotide pool depletion. generation of free radicals. blockage of RNA polymerase II and by oncogene expression (Ljungman. 2000) (Figure 3).

p53 is regarded as a protein that is regulated primarily by post-translational events (Hansen & Oren, 1997). However, stimulation of p53 transcription from a novel p53 promoter element has been observed in response to genotoxic stress (Sun et al., 1995). In addition. there is some evidence to suggest that p53 can also be regulated at the translational stage. Fu et al (Fu et al., 1996) demonstrated repression of p53 mRNA at the transcriptional level in unstimulated cells by the 3' untranslated region. Exposure to DNA damage has been shown to relieve this translational repression. Mosner et al demonstrated that p53 could bind to its own mRNA and inhibit translation which is

potentially relieved if p53 is induced to translocate into the nucleus in response to DNA damage (Mosner et al., 1995). However, p53 regulation is still considered to be most critical at the level of post-translational modifications.

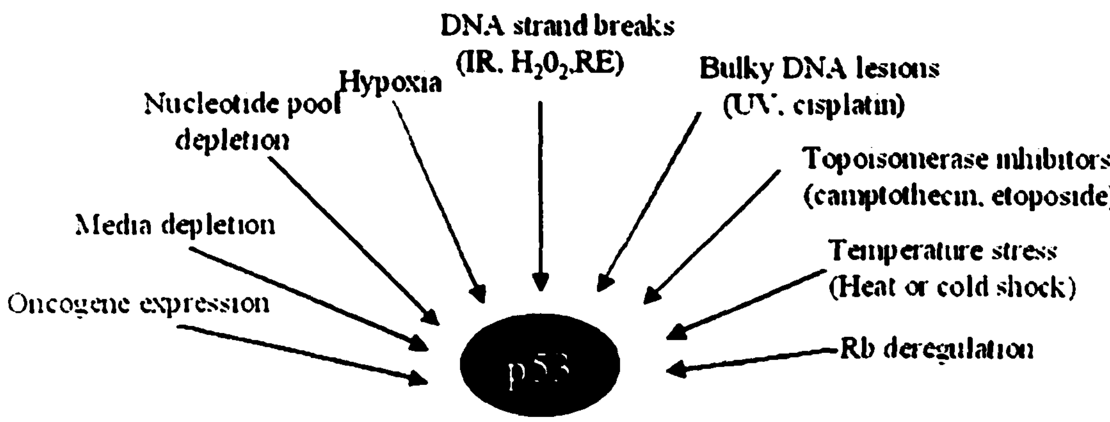


Figure 3. Many different stresses trigger a p53 response. Some stresses do not cause DNA damage such as temperature stress. Abbreviations: Ionizing radiation (IR), Restriction Enzyme (RE), and Ultraviolet light (UV)

Steps involved in p53 regulation

In response to a DNA damaging event such as exposure to IR, p53 has been found to undergo several different post-translational events that have been associated with a specific step in the activation of p53. These steps include the stabilization of p53 by resisting interactions with mdm2, translocation of p53 into the nucleus, changes in p53 conformation, and the tetramerization of p53, all of which lead to its activation for DNA binding and transactivation (Figure 4). Whether these events happen in succession or simultaneously is not known at this time, but is indicative of the tight regulation of p53 to protect the cell from its inappropriate activation.

P53 activity is regulated mainly through post-translational modifications

Phosphorylation of p53

Stabilization of p53 is thought to mainly be associated with abrogating the interaction of p53 with the cellular protein mdm2 (Ashcroft et al., 1999; Ashcroft & Vousden, 1999). As mentioned earlier, mdm2 targets p53 for proteasome mediated degradation by functioning as a ubiquitin ligase specific for p53 (Honda et al., 1997). For mdm2 to degrade p53 effectively, it must transport p53 out of the nucleus through its association with another protein CRM1, which is involved in the nuclear export of several different proteins (Freedman & Levine, 1998; Henderson & Eleftheriou, 2000). In response to genotoxic stress, p53 undergoes phosphorylation at several different residues, some of which interfere with p53's ability to complex with mdm2 (Ashcroft et al., 1999). Specifically phosphorylation of p53 at Serine 15, Serine 20

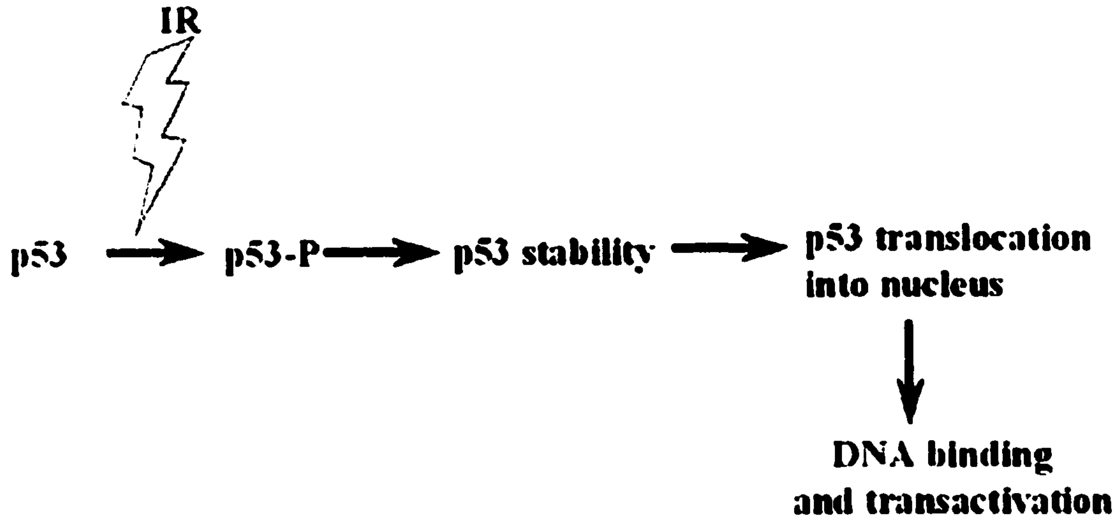


Figure 4. Steps involved in the activation of p53 function.

and Serine 37 have been shown to affect p53-mdm2 complex formation (Blasina et al., 1997; Canman et al., 1998; Sakaguchi et al., 1998). Phosphorylation of p53 at Serine 15 and Serine 37 has been demonstrated by ATM kinase, ATR kinase, and DNA-PK. ATM kinase phosphorylates p53 in response to exposure to IR, ATR kinase in response to IR and UV exposure and DNA-PK is activated by DNA double strand breaks and therefore can also be activated by IR (Ljungman, 2000). Phosphorylation of p53 at Serine 20 can be induced by either CHK1 or CHK2, where CHK1 phosphorylation is ATR induced and phosphorylation of p53 by CHK2 is induced by ATM phosphorylation (Chehab et al., 2000; Chehab et al., 1999). Another kinase, JNK, can also phosphorylate p53 at Serine 37 in response to IR and abrogate the interaction between p53 and mdm2 (Fuchs et al., 1998c; Hu et al., 1997; Milne et al., 1995). Interestingly, in the absence of a stress response JNK has been shown to target p53 for degradation by its direct ubiquitination (Fuchs et al., 1998b). Site-directed mutagenesis of these phosphorylation sites in p53 still allowed the resulting protein to be stabilized in response to IR and UV exposure suggesting that events other than phosphorylation of p53 at these sites were also important in stabilizing p53 (Blattner et al., 1999). Phosphorylation of mdm2 resulting in destabilization of the p53-mdm2 complex has also been proposed as a mechanism by which p53 is stabilized. Specifically, phosphorylation of p53 by DNA-PK at Serine 17 has been shown to decrease p53-mdm2 association (Mayo et al., 1997). In addition, association of mdm2 with p14/ARF1 protein has been shown to stabilize p53 levels and it is believed that this is due to the sequestration of mdm2 in the nucleolus by ARF1 thus making it unavailable for interactions with p53 (Pomerantz et al., 1998; Weber et al.,

1999). Thus, dissociation of p53 from mdm2 is an important step in the activation of p53.

Nuclear translocation of p53 is also an important step in its regulation as evidenced by the existence of some tumor types in which this step is compromised leading to a cytoplasmically sequestered p53 protein (Moll et al., 1995; Moll et al., 1992). Nuclear import of p53 is a process regulated by an importin complex consisting of importin alpha which associates with p53 through its nuclear localization signal (NLS) and facilitates p53 translocation into the nucleus (Kim et al., 2000; Liang & Clarke, 1999). The importin complex brings proteins close to the nuclear envelope and translocation of proteins through the nuclear pores is brought about by Ran, a small GTPase protein. Nuclear import of p53 can also be blocked by the anti-apoptosis gene bcl-2 by an unknown mechanism and thus regulate p53 function (Beham et al., 1997). Post-translational modifications such as phosphorylation and acetylation can occur on the NLS or adjacent to it which can directly or indirectly hide the NLS sequence and thus regulate p53's ability to translocate into the nucleus (Vandromme et al., 1996). Other modifications can alter p53's interactions with other proteins or itself and therefore regulate the subcellular localization of p53. Phosphorylation of p53 at Serine 392 by casein kinase II stimulates tetramerization of p53, which in turn leads to the masking of p53's NES (nuclear export signal) and permits accumulation of p53 in the nucleus (Sakaguchi et al., 1997a; Sakaguchi et al., 1997b). These results suggest that it is important for p53 to be appropriately modified in order to translocate into the nucleus and

also modified such that it stays in the nucleus where it can perform its function as a transcription factor.

Phosphorylation of p53 at serine 392 is not only important for tetramerization of p53 but also for sequence specific binding to DNA (Hupp, 1999; Meek et al., 1990; Sakaguchi et al., 1997a; Sakaguchi et al., 1997b). Other post-translational events that have been shown to increase sequence-specific binding of p53 to DNA include phosphorylation of p53 at Serine 371, 376, and 378 by both CAK (Cdk-activating kinase) and PKCs and (Gu & Roeder, 1997; Gu et al., 1997; Liu et al., 1999; Sakaguchi et al., 1998).

<u>Other post-translational modifications and their relationship to p53 function</u>

Acetylation of p53 at Lysine 320 and 382 by PCAF(p300/CBP-associated factor) and p300 respectively, both of which are histone acetyl transferases has been shown to be involved in p53 functional regulation (Lill et al., 1997; Sakaguchi et al., 1998). CBP/p300 and PCAF are thought to activate sequence-specific binding of p53 in response to DNA damage in part due to acetylation of p53 at the carboxy-terminus. Another post-translational modification recently identified to modulate p53 function is SUMO-1 modification. SUMO-1 is a small ubiquitin-like protein that can be covalently linked to several different proteins by the SUMO-1 activating enzyme. Rodriguez et al (Rodriguez et al., 1999) demonstrated SUMO-1 modification of p53 at Lysine 386 which led to an increase in the transcriptional activity of p53. Ribosylation of p53 within the central core domain and the carboxy-domain has been demonstrated by PARP (poly(ADP-ribose)polymerase) in response to exposure by IR (Vaziri et al., 1997; Wang et al., 1998;

Wesierska-Gadek et al., 1996; Wesierska-Gadek et al., 1999). This ribosylation of p53 was shown to increase p53 stability and also increase the transactivation ability of p53 for its target genes.

The abundance of post-translational modifications that p53 has been shown to undergo indicate the multiple levels at which this protein is regulated. All the observed modifications have been shown to have an impact on the functional regulation of p53 but some occur only in response to specific types of stresses, e.g., phosphorylation of p53 at Serine 392 has been shown to occur only in response to UV light exposure and not in response to IR. The downstream response of p53 is also different depending on the type of stress and the cellular background suggesting that the several post-translational modifications may regulate p53 function such that p53 responds specifically to the type of stress to which the cell is subjected.

Inactivation of p53 in tumors

High incidence of p53 mutations

Mutations in p53 are different from those observed in other tumor suppressor genes such as Rb, which in the latter case are missense mutations that lead to truncations or instability of the protein. In the case of p53, over 90% of the mutations are missense mutations that lead to a change in a single amino acid. Such changes in a single amino acid can alter the conformation of the protein and therefore, increase the stability of p53.

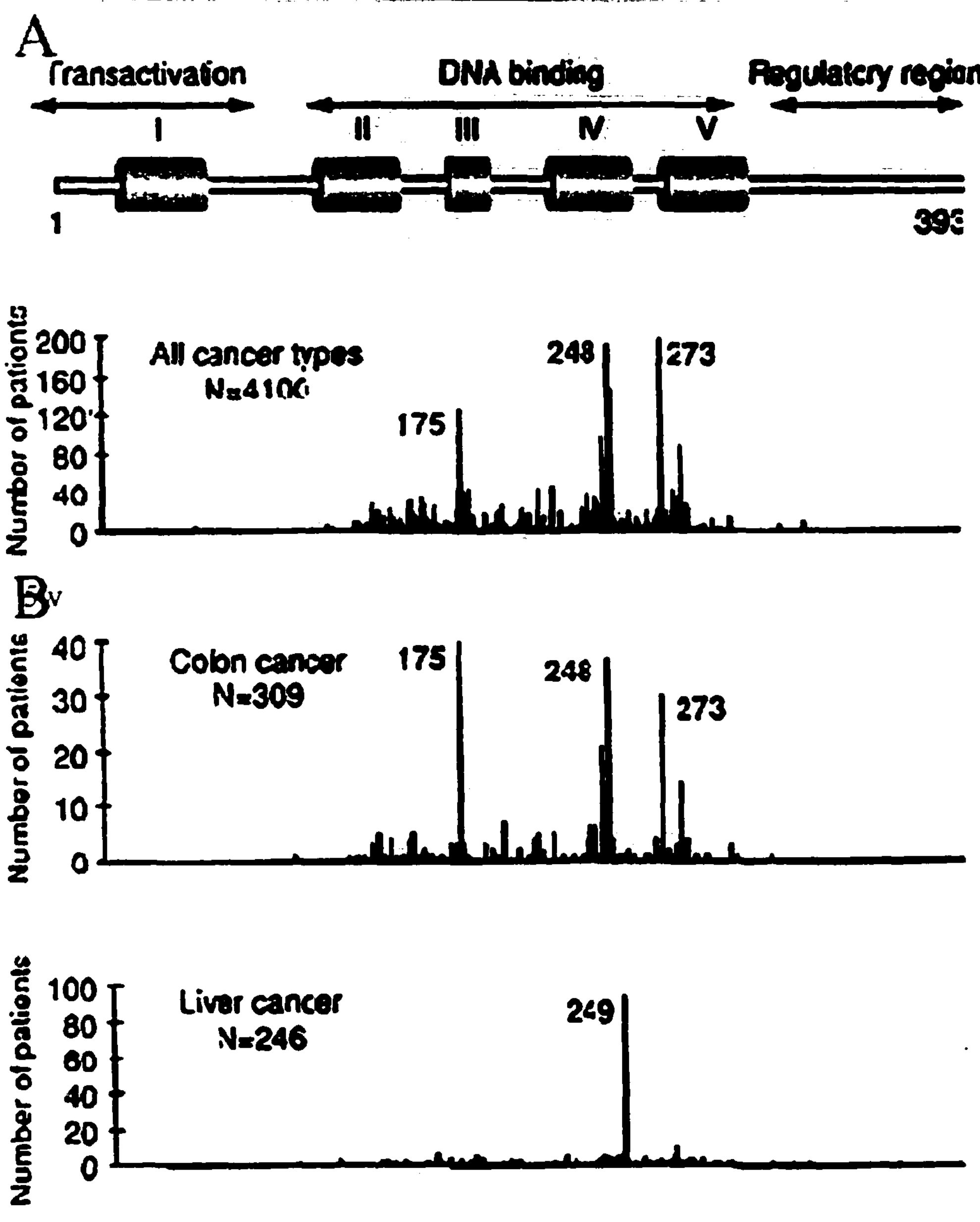


Figure 5. Types of mutations in p53 and their locations in two different types of tumors. Panel A represents the different domains of p53 and the location within p53 where most of the mutations are observed. Panel B shows the hotspot mutations for colon and liver cancer. Abbreviations: N is the number of samples studied.

Such mutations also alter the sequence-specific DNA binding ability of p53 and its ability to transactivate (Vogelstein & Kinzler, 1992). The majority of mutations in p53 occur in the DNA binding domain. The transcription factor function of p53 is very sensitive to conformational changes in this region that arise from amino acid substitutions due to mutations, thus directly affecting p53 function. In addition, changes to the conformation of p53 can also disrupt its association with other cellular proteins affecting its ability to transactivate.

The p53 protein has been divided into 3 major domains, a highly acidic amino terminus which is also responsible for the protein's transactivation abilities, the middle DNA binding domain which binds directly to DNA and also consists of 4 of the evolutionarily conserved domains (domains 2-5); and finally, a highly basic carboxy domain which is important in oligomerization of the protein and in regulating the protein's ability to interact with DNA (Figure 1). Cho et al succeeded in co-crystallizing the core domain of p53 bound to DNA (Cho et al., 1994). The structure of p53 consists of a large beta-sandwich that acts as a scaffold for 3 loop-based elements. The sandwich is composed of 2 anti-parallel beta-sheets containing 4 and 5 beta-strands, respectively. The first loop binds to DNA within the major groove, the second loop binds to DNA within the minor groove, and the third loop packs against the second loop to stabilize it. The DNA binding domain of p53 is the most highly conserved in all the species expressing p53 and consists of four out of five evolutionarily conserved p53 domains. It is also the region most susceptible to mutations suggesting that p53's role as a transcription factor is most important in its ability to function as a tumor suppressor.

However, transcription independent functions of p53 have also been shown to be important in tumor suppression (Canman & Kastan, 1997; Zhou et al., 1999). Figure 5 shows a schematic illustration of the p53 protein and the most common sites for mutations.

Transgenic mice carrying a mutant p53 gene develop many types of cancer, with a high proportion of sarcomas (Lavigueur et al., 1989). This observation led to the study of patients with Li-Fraumeni syndrome. This syndrome is associated with a broad spectrum of cancers including osteosarcomas, breast cancer, soft tissue sarcoma and leukemias, appearing at a very early age. Statistical analysis predicts that 50 % of these individuals will have a tumor before the age of 30, and 90 % before the age of 70. Germ-line mutations in the p53 gene were found in several families with this syndrome (Malkin et al., 1990; Srivastava et al., 1990). However, tumors present in these patients with Li-Fraumeni syndrome are homozygous for mutant p53.

Frequency of mutation in p53 varies across tumor types but on average 50% of tumors will have acquired a mutation in the p53 gene (Beroud & Soussi, 1998) (Figure 6). Mutations in p53 mainly involve point mutations leading to single amino acid substitutions in the central DNA binding region of p53 (Prives, 1994). Mutations residing in this central region of p53 severely impair protein function. In colorectal cancers, however, a high incidence of heterozygous loss of the short arm of chromosome 17, and point mutation of the remaining allele has been determined as the mechanism of p53 inactivation (Baker et al., 1989; Vogelstein et al., 1988). Similar observations have been made in the case of lung cancer (Takahashi et al., 1989).

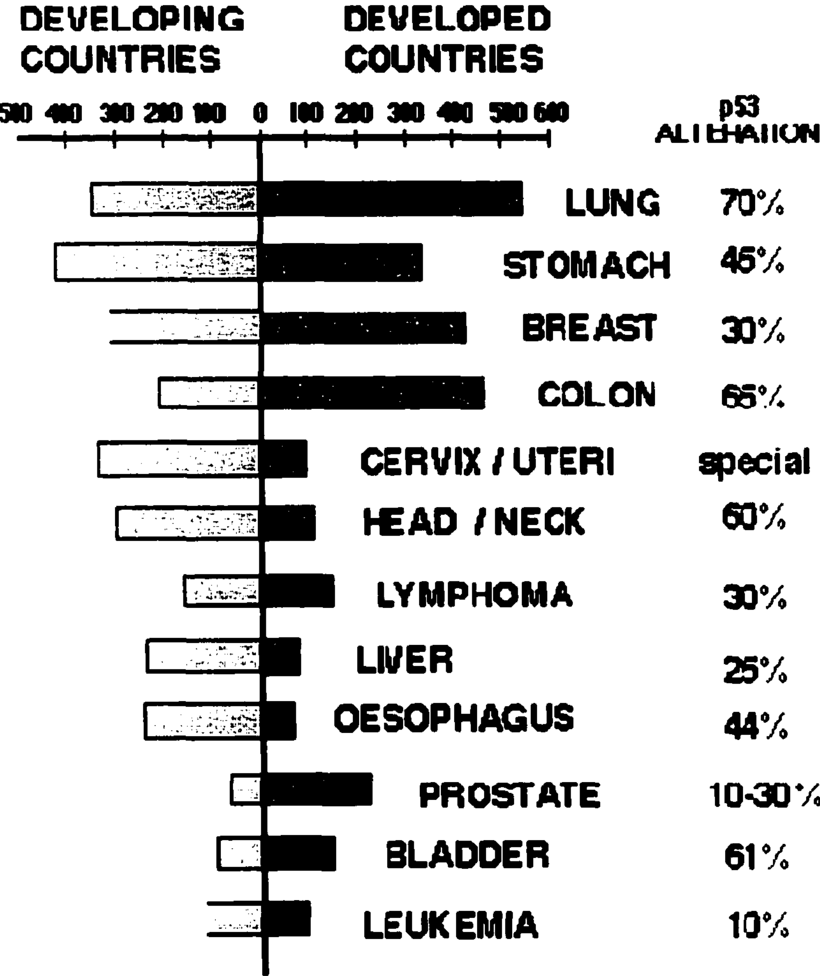


Figure 6. Worldwide distribution of cancers and the incidence of p53 mutation in these cancers. Cancer of the cervix /uteri is regarded as special since p53 inactivation occurs by infection with Human Papilloma Virus even though the p53 protein is wild-type.

Analysis of the mutational events that target the p53 gene has revealed evidence for both exogenous and endogenous mutational mechanisms. For example, the p53 mutational spectrum reveals evidence for a direct causal effect of ultraviolet radiation in skin cancer, of aflatoxin B1 in liver cancer and of tobacco smoke in lung cancer (Dogliotti et al., 1998).

Point mutations in the p53 gene have been found in most human cancers. More than 10,000 different tumors from various types have been analyzed for p53 alterations and have led to the identification of mutations in almost all cancer types (Greenblatt et al., 1994). The frequency of p53 mutations is relatively high, and observed in approximately 50% of all types of cancer (Figure 3). In general, these mutations are associated with a loss of the second allele of the gene.

Mutations in p53 have been shown to predominantly reside in the DNA binding domain of the protein. This is the same region that consists of four of the five evolutionarily conserved domains present in p53 (Zambetti & Levine, 1993). Several sites within the DNA binding domain have mutations at high frequency and are referred to as hot spot mutations and these include codons 143, 175, 248, 273 and 281. A bias in the site of mutation and the tissue type has been observed, e.g., there is an increased incidence of mutation at amino acid 175 in colon carcinomas but rarely observed in lung cancers (Levine et al., 1991). These differences in frequency of mutation at a specific site suggest that they may be a result of the specificity of a specific mutagen that is encountered by that organ.

Mutant p53 as an oncogene

Wild-type p53 acts as a tumor suppressor but there is evidence to support the notion that mutant p53 acts as an oncogene, and promotes tumorigenesis (Zambetti & Levine, 1993). In the years following the discovery of p53, researchers believed that the protein they were studying was a potential oncogene and that the differences in the p53 sequence between the clones that they were studying were polymorphisms instead of mutations in the protein. Since increased expression of p53 was observed only in tumors and vira transformed cells while low levels were detected in normal cells, a phenotype specific for oncogenes, their reasoning for p53's role in tumor formation as an oncogene was justified. Closer examination however, indicated a high incidence of these 'polymorphisms' in highly conserved regions of the protein a characteristic that is uncommon in polymorphisms and instead indicated that these were in fact mutations in the various clones (Soussi et al., 1990).

Besides increased levels of mutant p53, there are several other lines of investigation that also support a role for mutant p53 as an oncogene. Mutant p53 is capable of cooperating with activated ras to promote transformation of normal cells (Hinds et al., 1990) while wild-type p53 inhibits this transformation process (Eliyahu et al., 1989; Finlay et al., 1989). The mechanism by which mutant p53 cooperates with ras in cell transformation is not completely understood but it may be through p53's function as a dominant negative gene by complexing with wild-type p53. Such hetero-oligomers

between wild-type and mutant p53 can inactivate wild-type p53 function in a trans-dominant negative fashion (Hinds et al., 1989).

A gain of function phenotype has also been associated with the oncogenic phenotype of mutant p53. This was determined when introduction of mutant p53 into p53 null cells resulted in increased transformation and tumorigenic potential of these cells (Dittmer et al., 1993; Shaulsky et al., 1991c). These results clearly demonstrated a function for mutant p53 other than its trans-dominant effect over wild-type p53.

The mechanism by which mutant p53 exerts its transdominant effects is not well understood but it may be through the direct transactivation or suppression of certain genes or through its association with other proteins in the cell. Mutant p53 has been shown to directly transactivate the expression of the gene that codes for multi-drug resistance (MDR) (Chin et al., 1992). In transient transfection assays using an MDR promoter-CAT reporter gene construct, transactivation of the reporter was observed after introduction of the mutant p53-175H expression plasmid in cells wild-type or mutant for p53 status. Other mutant p53 proteins have also been shown to transactivate the MDR gene (Dittmer et al., 1993). Similarly, mutant p53 has been shown to transactivate PCNA gene expression in p53-null cells (Deb et al., 1992). These genes are involved in increasing tumorigenic potential as well as in the progression of the cell cycle, suggesting a significant role for mutant p53 in contributing to an oncogenic phenotype. Mutant p53 is incapable of transactivating the genes that wild-type p53 transactivates.

Mutant p53 has been shown to associate with the chaperone proteins hsc70 and hsp90 (Hinds et al., 1987; Whitesell et al., 1998). This association has not been observed in cells expressing wild-type p53. Whether this association is important to the oncogenic potential of mutant p53 is still unclear and it is possible that this association exists only because the mutant protein is not appropriately folded in the cell. However, it does indicate that the mutant protein is capable of interacting with proteins other than those associated with wild-type p53 and it is possible that some of these interactions are important for mediating mutant p53's oncogenic function.

Finally, mutant p53 is found in a conformation different from that observed by wild-type p53 in the cell (Milner, 1994). Immunoprecipitation analysis using conformation specific antibodies demonstrated that wild-type p53 had affinities for specific antibodies that were different from mutant p53. However, there is some evidence that suggests that wild-type p53 protein can assume a mutant conformation in the cell as part of its functional regulation, therefore the significance of the different conformations is unclear in mutant p53's role as an oncogene.

Inactivation of p53 by mechanisms other than mutation

Physical sequestration of p53 in the cytoplasm

In certain types of cancer, p53 can be inactivated by mechanisms other than mutation. Since p53 functions as a tumor suppressor mainly through its role as a transcription factor, exclusion from the nucleus is a powerful mechanism of its

inactivation. Such cytoplasmic sequestration of p53 is commonly observed in neuroblastoma derived tumors as well as in some breast and colon cancers (Moll et al., 1995; Moll et al., 1992). In tumors in which p53 is cytoplasmically sequestered, the p53 is also wild-type by sequencing (Davidoff et al., 1992) and is found over-expressed (Zaika et al., 1999a). A specific mechanism for nuclear exclusion of p53 due to the truncation of importin alpha, a protein involved in the import of proteins in the nucleus has been recently identified in a breast cancer cell line (Kim et al., 2000).

Enhanced degradation of p53 by cellular and viral proteins

Another mechanism of p53 inactivation is its increased degradation resulting in its inability to be stabilized in response to genotoxic stress. Mdm2, a downstream target gene of p53 functions as a ubiquitin ligase and targets p53 for ubiquitination and subsequently proteasome mediated degradation in normal cells (Honda et al., 1997; Kubbutat et al., 1997; Kubbutat & Vousden, 1998). This protects the cell from inappropriate stabilization of p53 and its deleterious effects on the cell which could result in either cell cycle arrest or apoptosis. However, overexpression of mdm2 by gene amplification has been observed in approximately 20% of soft tissue sarcomas and osteosarcomas while the overall gene amplification frequency of mdm2 in tumors is only about 7% (Momand et al., 1998).

Infection with the Human Papilloma Virus 16 or 18 DNA tumor virus is a major risk factor for cervical cancer development. The early gene products E6 and E7 of HPV 16 or 18 are known to be involved in the transformation process by this virus (Ishiji,

2000). In cervical carcinomas, the frequency of p53 gene mutation is very low. It is thought that p53 is inactivated due to its association with the oncogenic HPV 16 or 18 E6 protein which also targets p53 for degradation similarly to mdm2 mediated p53 degradation (Mietz et al., 1992). The E6 oncoprotein behaves as a ubiquitin ligase and ubiquitinates p53 hence targeting it for proteasome mediated degradation. This E6 mediated degradation of p53 is dependent on a cellular protein called E6-AP (E6 associated protein) which is also important in the ubiquitination process (Huibregtse & Beaudenon, 1996).

Statement of purpose for dissertation project:

Development of the ALTR model system and its rationale

In spite of the overwhelming amount of research performed in the field of p53 research and the advances made in this field, there are still gaps in our understanding of how p53 is regulated. Our overall goal for this project was to understand novel mechanisms involved in the regulation of p53. Multiple steps are involved in the activation of p53 as discussed earlier in this section. The purpose of these studies was to examine each step involved in p53 activation very closely and to have a better understanding of how these steps were regulated.

We decided to use a genetic approach to address the complexity of p53 regulation. We chose A1-5 cells to generate our genetic screen. A1-5, a rat embryonic fibroblast cell line has been transformed by the introduction of activated Ha-ras and mouse temperature-

sensitive p53 (Martinez et al., 1991). This temperature sensitive p53 protein has a single amino acid substitution from Valine to Alanine at residue 135. The phenotype of this mutant p53 dictates that at 39°C the protein is cytoplasmically sequestered and in a mutant conformation allowing for the propagation of these cells. However, at the permissive temperature of 32°C, p53 translocates into the nucleus, assumes a wild-type conformation and causes cell cycle arrest by inducing the cyclin-dependent kinase inhibitor p21 (Figure 7). The A1-5 cells produce copious amounts of p53 protein that is either inactive or active depending on the temperature at which the cells were maintained. It is not possible to overexpress wild-type p53 protein since its transfection into cells is not tolerated well and normal cells express wild-type p53 at very low levels making it a difficult proposition to study using the techniques available to us today. Since A1-5 cells express large amounts of p53, it can be easily studied using biochemical techniques.

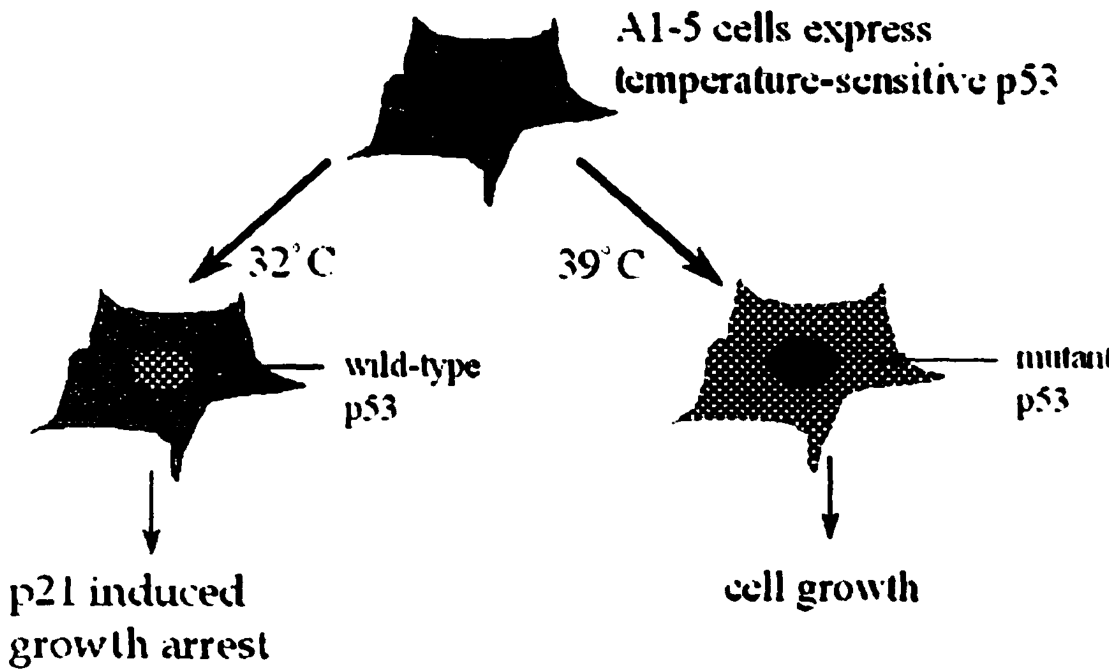


Figure 7. Schematic representation of the localization and function of p53 in A1-5 cells dependent on the temperature.

In order to develop a genetic screen in which p53 was functionally inactivated, the multiple copies of p53 expressed in A1-5 cells was also important. Based on previous data it was believed that A1-5 cells expressed multiple copies of the plasmid expressing temperature sensitive p53 protein (unpublished data). Inactivation of p53 function by mutation in these cells would not be feasible since mutation in one of the copies of the p53 expressing plasmid could be overcome by the rest of the copies in which mutations should not have occurred. Inactivation of a protein responsible for the activation of p53, on the other hand, would affect all the copies of p53 in the cell and allow for its complete inactivation. Since mutation is the most common mechanism of p53 inactivation, the presence of multiple copies of p53 would overcome this mechanism of p53 inactivation and allow other mechanisms to occur at a higher rate in our system.

We developed our genetic screen by mutagenizing A1-5 cells with a low concentration of EMS at 39°C and transferred the cells to 32°C (Gaitonde et al., 2000) (Figure 8). At this temperature, the p53 in A1-5 cells is activated for DNA binding and transactivation and induces p21 mediated growth arrest. If mutations occurred in the pathway leading to the activation of p53 or downstream of p53 activation due to mutagenesis of these cells, then these cells could grow at 32°C and form colonies. If, on the other hand, no mutations arose in the p53 pathway, the cells would growth arrest at this temperature and no colony formation would be observed. Generation of such variant cell lines that were capable of growth at 32°C was observed using this procedure. These cells were called ALTR for A1-5 Low Temperature Resistant cells. Out of 40 colonies

that were originally isolated during this selection process, 28 have been successfully maintained in our laboratory and most have been characterized to some extent for different properties.

My research goals were to study the regulation of p53 function with a focus on subcellular localization.

My specific aims were

1. To investigate the regulation of p53 translocation into the nucleus
2. To determine if cytoplasmically sequestered p53 was capable of functional activation in response to genotoxic stress.
3. To determine the defect observed in one of the ALTR cell lines generated in our panel which involved the stabilization of p53

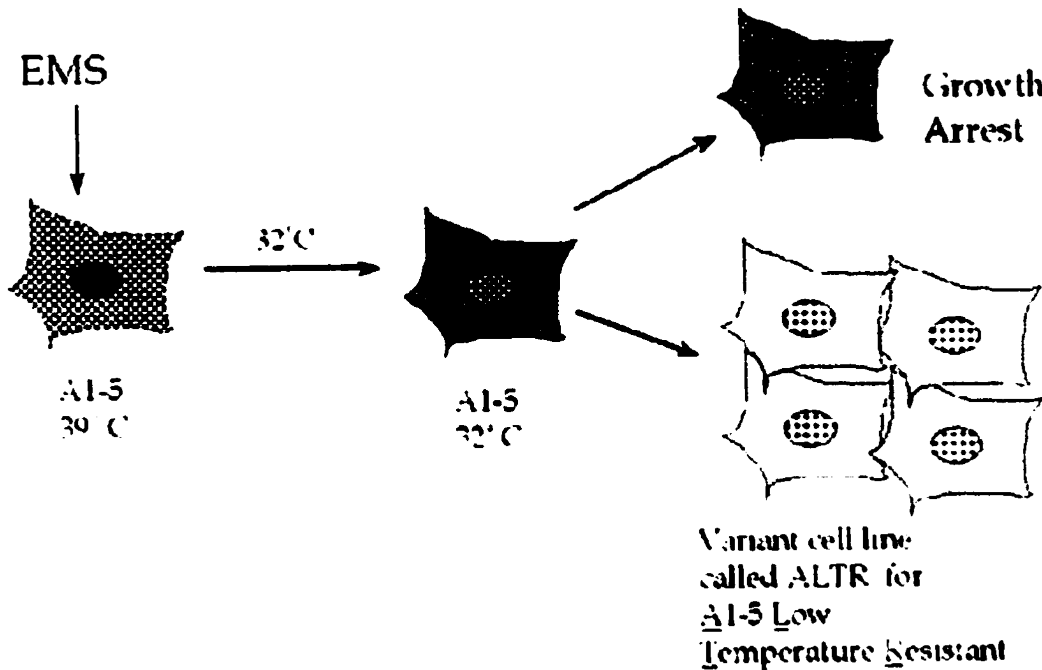


Figure 8. Schematic representation of the development of the ALTR cell system.

CHAPTER 2.

Conformational phenotype of p53 is linked to nuclear translocation.

Introduction:

The p53 tumor suppressor plays a crucial role in the maintenance of genomic stability by transcriptionally activating genes involved in DNA repair, growth arrest and apoptosis (Levine et al., 1994; Levine et al., 1991). P53 is activated by genotoxic agents and although the events involved in the activation of p53 are not well understood, the process is thought to be a complicated one, involving the stabilization of the protein (Kubbutat & Vousden, 1998), its translocation into the nucleus (Shaulsky et al., 1991b; Shaulsky et al., 1991d), and tetramerization (McLure & Lee, 1998), all of which are important steps in the process that lead to its activation for DNA binding and induction of target gene expression. p53 function is lost in over 50% of all human cancers which leads to genetic instability and neoplastic progression (Nigro et al., 1989). P53 is inactivated primarily as a consequence of mutations in the p53 gene itself. However, other mechanisms have been described which can also nullify the activity of the p53 protein. These include interaction with viral antigens (Scheffner et al., 1990) and cellular proteins which lead to enhanced degradation of p53 (Brown & Pagano, 1997; Haupt et al., 1997)], and by physically sequestering the protein in the cytoplasmic compartment of cells (Goldman et al., 1996; Moll et al., 1996). In cells where this latter mechanism is operative, p53 is unable to reach the nucleus to induce the expression of critical downstream target genes. Cytoplasmic sequestration of p53 has been observed in

neuroblastomas, breast and colon cancers suggesting that this mechanism may be important in the development of these tumors (Bosari et al., 1995; Moll et al., 1995; Moll et al., 1992).

At the biochemical level, one feature that is critical for normal function is a wild-type conformation of the p53 protein (Wolkowicz et al., 1998). Mutations in the p53 gene frequently lead to the expression of an inactive protein with a mutant conformation that can be detected by mutant protein specific antibodies such as PAb 240 (Gannon et al., 1990; Stephen & Lane, 1992). Hainaut et al demonstrated that metal chelators such as 1,10-phenanthroline could perturb the wild-type conformation of p53 (246+) to a mutant (240+) conformation, which also led to the inhibition of its ability to bind DNA in electrophoretic mobility shift assays (Hainaut & Milner, 1993). Recent data suggests that tumor growth can be inhibited by pharmacological agents that reverse the mutant conformation of p53 to the wild-type functional conformation (Foster et al., 1999). Nevertheless, there is evidence that the wild-type protein can also undergo changes in conformation during normal cell function (Milner, 1991a; Milner, 1991b; Milner, 1994; Milner & Watson, 1990; Sabapathy et al., 1997; Shaulsky et al., 1991a). For example, genotypically wild-type p53 found in a mutant conformation promotes cell growth, and only behaves as a tumor suppressor when present in the wild-type conformation (Milner & Watson, 1990; Sabapathy et al., 1997). In addition, wild-type p53 bound to DNA has been shown to be recognizable by both PAb 240 and 246 since binding to DNA exposes p53's hydrophobic core domain within which resides the PAb 240 epitope (Halazonetis et al., 1993; McLure & Lee, 1996).

To study the process of activation of p53 in more detail, we adopted a genetic approach designed to select for mutations in the pathways that lead to p53 activation. Cells containing a high copy number of the temperature sensitive $p53^{val135}$ (tsp53) gene and expressing large quantities of the protein were mutagenized. Cells that were resistant to the growth inhibitory properties of wild-type p53 induced by incubating at the permissive temperature of 32°C ,were selected and characterized. Interestingly, we found that the most frequent change in the mutagenized cells was cytoplasmic sequestration of the tsp53 protein. Moreover, we determined that conformation and localization of p53 in the ALTR cells at 32°C was strongly correlated. Our data suggest that the subcellular location of wild-type p53 and its conformation are closely linked events involved in the activation of p53.

Materials and Methods

Cell Culture, Reagents and Irradiation :

A1-5 cells are rat fibroblast cell lines transfected with multiple copies of the temperature-sensitive murine p53val^{135} gene (Martinez et al., 1991). A1-5 and ALTR cell lines were maintained in DMEM medium, containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD) with 5% CO_2. A1-5 cells were incubated at 37°C unless otherwise noted, and ALTR cell lines at 32.5°C. SK-N-SH cells were grown in DMEM medium containing 15% heat inactivated fetal bovine serum, 2 mM L-Glutamine, 4 mM sodium pyruvate and 100 U/ml penicillin, and 100 μg/ml streptomycin) (Gibco BRL, Gaithersburg, MD). SK-N-SH cells were maintained at 37°C, in 5% CO_2. Antibodies specific for p53 were obtained from the following: PAb 1620, 1801 (Calbiochem), PAb 421, 240, 246 and 419 (kindly provided by Dr. Arnold Levine). Cells were exposed to 5 Gy Ionizing radiation using a ^{60}Co source at an average dose rate of 66 cGy/Min.

Selection of low temperature resistant cell lines:

Variant A1-5 cell lines capable of growing at 32.5°C were derived by mutagenesis with ethyl methane sulfonate and selection at 32.5°C. Briefly, two 150 cm^2 flasks containing A1-5 cells (passage 9) at 90% confluence were mutagenized by incubating with 500 μg/mL of ethyl methane sulfonate (Sigma Chemical Co.) per milliliter of complete medium for 13 hours at 37°C. After mutagenesis was complete, the cell monolayers were rinsed 3X with DMEM and the cells incubated for an additional 24 hours at 37°C in

fresh complete medium. Subsequently, the cells were trypsinized from the flasks and replated onto twenty 10 cm dishes and incubated at 37°C for an additional 16 hours. Following this, the cultures were transferred to a 32.5°C incubator and incubation at this temperature continued until colonies began to appear (approximately 3 weeks). Forty resistant colonies were selected, 22 of which were successfully expanded into stable cell lines.

RT-PCR for p53:

Reverse transcription of 2 μg total RNA from A1-5 and ALTR cells was carried out using 0.5 μg Oligo$(dT)_{12-18}$, 2 mM dNTP mix, 1 mM DTT, 0.5 mM $MgCl_2$, 10X RT buffer and 50 units Superscript II RT in a 20 μL reaction (Gibco BRL). Full length P53 was amplified by dividing it into three part, the first 1/3rd NH2 part, the middle DNA binding domain region, and the remaining 1/3rd COOH domain. The NH2-domain of p53 was amplified by PCR using 5'ATGGAGGAGTCACAGTCGGATA3' as the upstream primer and 5'ATAACAGACTTGGCTGTCCCAG3' as the reverse primer, to generate a PCR product of 366bp. The DNA binding domain of p53 was amplified by PCR with 2 μL of the above reaction. The upstream primer 5'-GCCAAGTCTGTTATGTGCA-3', and the reverse primer 5'-GAGGCGCTTGTGCAGGTGG-3' were used to specifically amplify 590 bp of the p53 cDNA which maps to the DNA binding domain. The final 1/3rd region was amplified using 5'AGAAAATTTCCGCAAAAAGGAA3' as the forward primer and 5'CCCCACTTTCTTGACCATTGTT3' as the reverse primer, to give a PCR product of 330bp. All regions were overlapping to ensure full sequence

information. PCR conditions were : denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 55°C for 30 sec and 72°C for 1 min. The PCR products was separated on a 1% agarose gel, excised and extracted from the gel using the QIAQuick gel extraction kit (Qiagen). The product was then sequenced in both directions on an automated sequencer using the same primers.

Indirect Immunofluorescence and Image Processing:

Cells were plated onto coverslips at an approximate concentration of $2X10^5$ cells per 60 mm^2 plate. The plates were then incubated at the appropriate temperature for 12-16 hours. Coverslips with attached cells were rinsed three times in cold PBS (containing 3 mM KCl, 1 mM KH_2PO_4, 0.2 mM $MgCl_2$, 137 mM NaCl, and 8 mM Na_2HPO_4, pH 7.5), and cells fixed to the coverslips using methanol:acetone (1:1) for 5 minutes, at –20°C. Sample coverslips were allowed to air dry, stored at -20°C and rehydrated using cold PBS when ready to be stained. For A1-5 and ALTR cells, undiluted PAb 421 antibody supernatent (approx. 200 μL) was applied and the coverslips were incubated at room temperature for one hour, after which, they were rinsed three times with cold PBS. Goat anti-mouse antibody conjugated to FITC (Sigma Chemical Co.) at a 1:500 dilution was then applied for an incubation time of 30 minutes, at room temperature. Samples were washed three times with cold PBS, and mounted on standard glass slides using Mowiol mounting medium (Calbiochem). PAb 421 (Calbiochem) and Goat-anti-mouse Cy-3 conjugate at 1:50 dilution (KPL) were used as the primary and secondary antibodies respectively for indirect immunfluorescent staining of SK-N-SH cells. A Zeiss LSM-10

model confocal microscope was used for all observations. Digital images were collected on each series using identical brightness and contrast settings within each cell line..

Immunoprecipitation :

Cells grown on 10 cm^2 plates to 90% confluency at the appropriate temperature were used to immunoprecipitate p53 using antibodies specific for its conformation. Briefly, cells were labeled with 200 μCi ^{35}S-Methionine (New England Nuclear, Boston, MA) per plate in DMEM containing 10% FBS for 1 hour after starving for 1 hour with DMEM (Methionine-free) containing 10% dialyzed fetal bovine serum. Cells were lysed on ice for 10 min in 1mL Lysis buffer (50 mM Tris-Cl (pH 8.0), 5mM EDTA, 150 mM NaCl, 0.5% NP-40, 1mM PMSF, and 1 mM aprotinin, leupeptin and pepstatin A) (Sigma Chemical Co.). The supernatant was retained and 30 μL of protein A-sephadex beads (Gibco BRL) was added and samples rotated for 30 min at 4°C to eliminate non-specific binding. After quantitation using a scintillation counter, 2 to 6 X 10^6 cpm of cell lysate were incubated with 100 μL of antibody (approx. 1 μg) and 30 μL ProA-Sephadex and the tubes rotated at 4°C overnight. The beads were washed three times with 1 mL SNNTE buffer (50mM Tris, 5 mM EDTA, 5% sucrose, 1% NP-40 and 0.5 M NaCl, pH 7.4) and once with 1 mL RIPA buffer (1% Sodium deoxycholate, 0.1% SDS in lysis buffer). Samples were electrophoresed on a 10% denaturing polyacrylamide gel. Vacuum dried gels were exposed to film (Kodak X-OMAT AR) for approximately 48 H before developing. Quantitation of ^{35}S-labeled p53 protein was done using either

densitometry analysis using Scion Image software or by phosphoimager analysis using ImageQuant software.

In experiments where cells were exposed to Ionizing radiation, cells were not starved in methionine-free medium, instead, labeling was carried out for 2 hours for A1-5 and ALTR cells and 3 hours for SK-N-SH cells immediately after exposure to IR.

Results:

Selection of mutant A1-5 cells capable of growth at 32°C:

We selected A1-5 cells for mutagenesis because they have been extensively characterized previously and they contain a large number of copies of the tsp53^{val135} gene which is constitutively expressed (Martinez et al., 1991). At 32°C, the p53 in A1-5 cells assumes a wildtype conformation and is translocated into the nucleus, where it binds DNA and causes growth arrest by inducing p21. However, at 39°C, the restrictive temperature for p53 function, the p53 is sequestered in the cytoplasm in a mutant conformation, thus allowing for the proliferation of A1-5 cells at this temperature. Since A1-5 cells have multiple copies of the temperature sensitive p53 gene transfected into them, they were an ideal system in which we could force mutations in genes involved in the activation of p53 rather than p53 itself. To derive such variant cell lines that were incapable of activating p53, A1-5 cells were chemically mutagenized with EMS and clones capable of growth at 32°C, the permissive temperature for p53 activation in A1-5 cells, were selected. Twenty eight of 40 cell lines picked were expanded and labeled ALTR 1 through 28, for A1-5 Low Temperature Resistant, of which 22 cell lines have been successfully maintained at 32°C. Unlike the A1-5 parent cells, most of the ALTR cell lines were capable of vigorous growth at 32°C, the permissive temperature for p53 activation. Moreover, this phenotype was stable since ALTR cells could be grown at 39° C for an extended period of time, then returned to 32°C, and still retained their ability for growth at this lower temperature. To confirm that the p53 in the ALTR cells had not acquired mutations during the selection process, RT-PCR and automated sequence

analysis of full length p53 was performed. No mutations were found in ALTRs 1, 17 and 25.

Localization of p53 in ALTR cells

The localization of p53 in A1-5 cells has been shown previously to follow a strict adherence to temperature sensitivity. At 32°C, the p53 translocates into the nucleus while at 39°C, the p53 is cytoplasmically sequestered. It was important to determine if the p53 in ALTR cells also maintained the same localization characteristics as the parent A1-5 cells at 32°C. To determine the subcellular localization of p53 we performed indirect immunofluorescence, using an antibody specific for p53 (PAb 421), on ALTR cells maintained at either 32 or 39°C (Table 1). Similar to parental A1-5 cells at 39°C, most of the ALTR cell lines had cytoplasmically localized p53 (Table 1 and Figure 9). However, at 32°C, all but four ALTR cell lines (ALTRs 9, 10, 17 and 18) still exhibited predominantly cytoplasmic p53 localization. Based on this subcellular localization data, we observed two classes of ALTR cells at 32°C. Those with p53 predominantly sequestered in the cytoplasm, and those with predominantly nuclear p53.

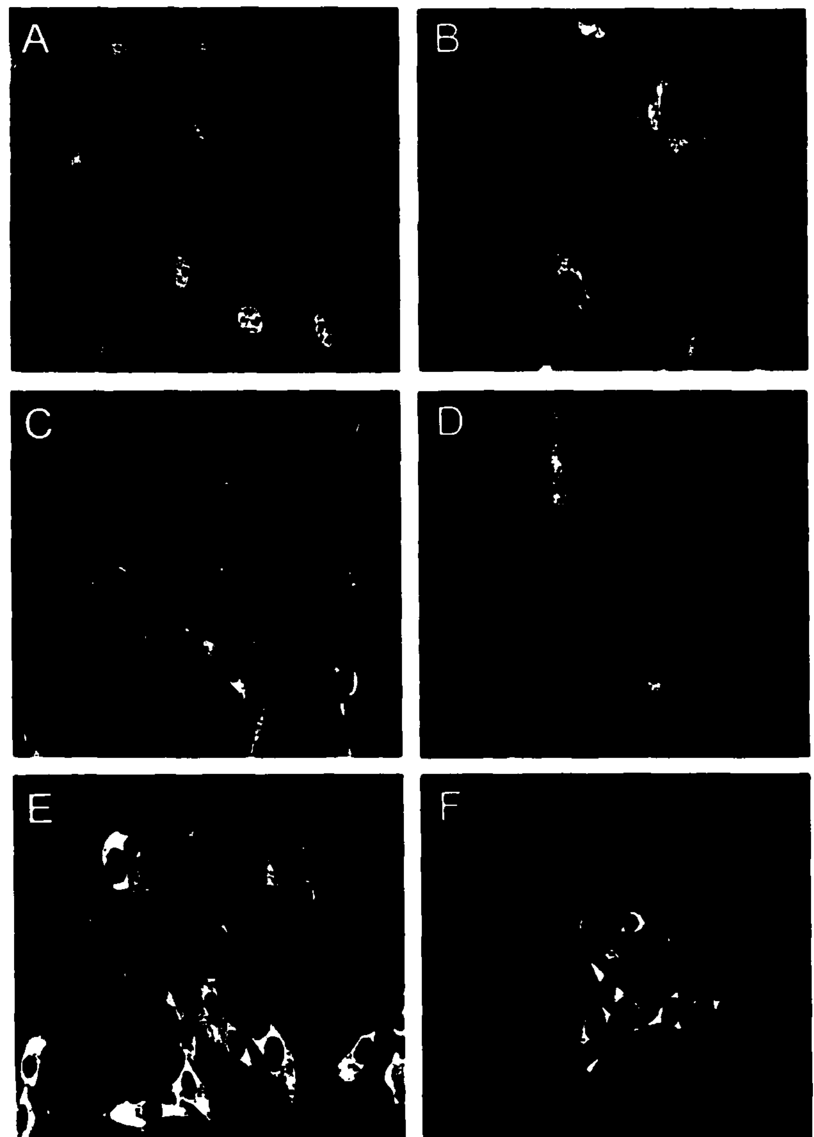
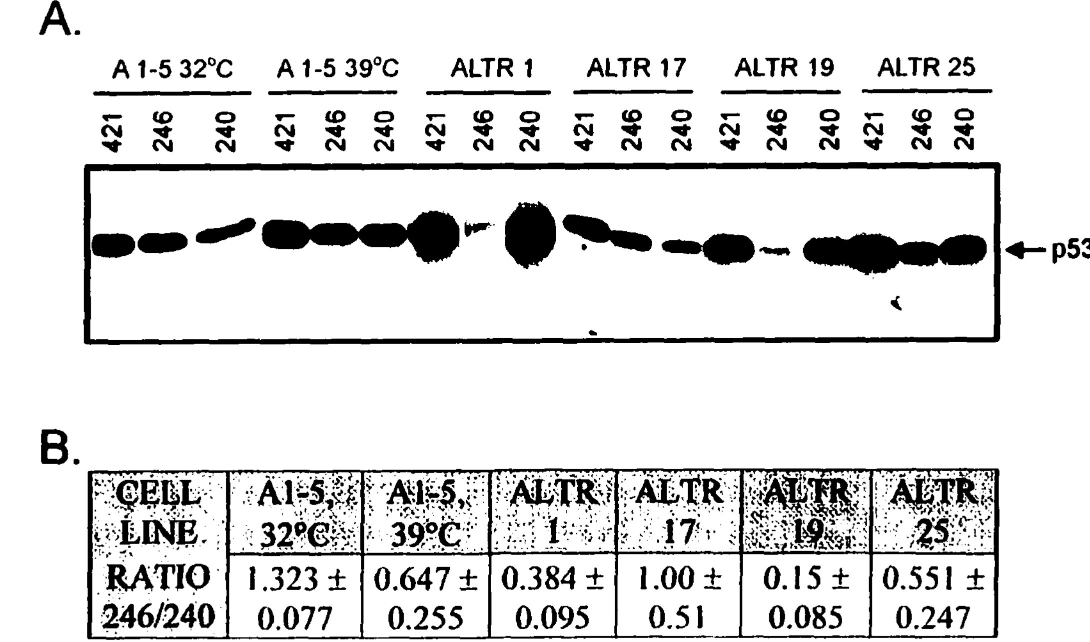


Figure 9. Subcellular Localization of p53 in A1-5 and ALTR cells. A1-5 and ALTR cells were grown on coverslips at the appropriate temperature for 48 hours and indirect immunofluorescence using PAb 421 was used to determine the subcellular location of p53. **A** and **B** represent confocal images of A1-5 cells maintained at 32° and 39°C respectively. **C, D, E** and **F** represent confocal images of ALTR cell lines 1, 17, 19 and 25 respectively, all of which were maintained at 32°C. All photographs were taken using a 40X objective lens.

Conformation of p53 in ALTR cells is varied

Ability to bind DNA in a stable manner has been shown to be dependent on the conformation of p53 (McLure & Lee, 1999; Wolkowicz et al., 1998). Wild-type p53 tetramers can bind DNA with enhanced stability compared to tetramers that have a mixture of wildtype and mutant p53 (McLure & Lee, 1998; McLure & Lee, 1999). The p53 in A1-5 cells is conformationally flexible depending on the temperature and at 32°C it is predominantly in a wild-type conformation as recognized by PAb 246 (Figure 10). Our results with the localization of p53 in ALTR cells indicated a temperature independent subcellular localization pattern. To determine if the conformation of p53 in these cells was also temperature independent, we performed immunoprecipitation analysis using antibodies that recognize specific conformations of p53. Conformation of the p53 protein was examined in ALTR cells maintained at 32°C by immunoprecipitation (IP) analysis using the conformation-specific antibodies PAb 246 (wild-type specific), PAb 240 (mutant specific) and PAb 421 (non-conformation specific) (Gamble & Milner, 1988; Gannon et al., 1990; Milner et al., 1987). We compared the mutant and wild-type IP profile observed by A1-5 cells at 39° and 32°C with that obtained for ALTR cells at 32 °C. The ratio of p53 immunoprecipitated by PAb 246 (wild-type specific) to PAb 240 (mutant specific) was used to determine the conformation of p53 in A1-5 and ALTR cells. A ratio of 1 or higher indicated a wild-type p53 conformation, consistent with p53 IP profiles obtained in A1-5 cells at 32 °C. Figure 10 A represents the immunoprecipitation profiles obtained for ALTRs 1, 17, 19 and 25 and Figure 2 B shows the ratio of p53 immunoprecipitated by PAb 246 to 240.

A.



B.

CELL LINE	A1-5, 32°C	A1-5, 39°C	ALTR 1	ALTR 17	ALTR 19	ALTR 25
RATIO 246/240	1.323 ± 0.077	0.647 ± 0.255	0.384 ± 0.095	1.00 ± 0.51	0.15 ± 0.085	0.551 ± 0.247

Figure 10. Conformation analysis of tsp53 in A1-5 and ALTR cells. Conformation of p53 in A1-5 and ALTR cells was determined by examining reactivity with conformation specific antibodies. After [^{35}S]-methionine labeling, cells were harvested, and p53 immunoprecipitated using either PAb 421 (for total p53), PAb 246 (wild-type) or PAb 240 (mutant). The immunoprecipitated protein was resolved by SDS-PAGE analysis on 10% polyacrylamide gels (see Materials and Methods). **Panel A** represents an autoradiograph of the [^{35}S]-methionine labeled p53 immunoprecipitated by the different antibodies. **Panel B**. Densitometry analysis was performed to determine the quantity of p53 immunoprecipitated by each antibody and the ratio of protein precipitated by PAb 246 to PAb 240 determined.

Table 1. Correlation between subcellular localization of p53 and conformational phenotype.

ALTR Cell #	Localization of p53 by Indirect Immunofluorescence		p53 conformation by IP
	32°C	39°C	
1	Cytoplasmic	Cytoplasmic	Mutant
3	Cytoplasmic	Cytoplasmic	Mutant
4A	Cytoplasmic	Cytoplasmic	Mutant
4B	Predom*. Cytoplasmic	Cytoplasmic	Mutant
6	Predom. Cytoplasmic	Cytoplasmic	Mutant
7	Cytoplasmic	Cytoplasmic	Mutant
8	Cytoplasmic	Cytoplasmic	Mutant
9	Nuclear	Predom. Cytoplasmic	Wild Type
10	Both	Predom. Cytoplasmic	Wild Type
11	Predom. Cytoplasmic	Predom. Cytoplasmic	Mutant
15	Cytoplasmic	Cytoplasmic	Mutant
17	Nuclear	Cytoplasmic	Wild Type
18	Nuclear	Cytoplasmic	Wild Type
19	Cytoplasmic	Cytoplasmic	Mutant
25	Cytoplasmic	Cytoplasmic	Mutant
28	Cytoplasmic	Cytoplasmic	N.D.

N.D. -Not Done

* -Predominantly

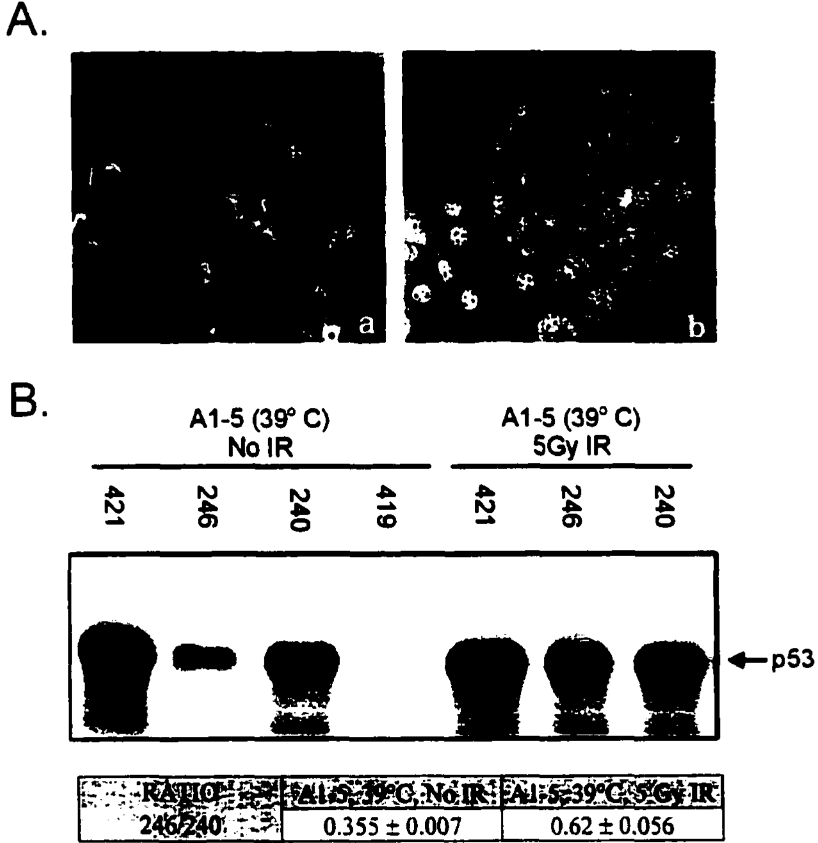
To our surprise, we found that the p53 in only four ALTR cell lines (ALTRs 9, 10, 17 and 18) was present in a wild-type conformation at 32°C. The p53 protein in the remaining ALTR cell lines displayed a mutant phenotype, i.e., the p53 immunoprecipitated preferentially with the PAb 240 antibody similar to that observed with A1-5 cells grown at 39°C. Co-immunoprecipitation of proteins such as hsc70 and mdm-2 was also observed to occur in a conformation-dependent manner, i.e., hsc 70 co-precipitated with the mutant conformation of p53 (Zambetti & Levine, 1993) and mdm-2 with the wild-type conformation of p53 (Chen et al., 1993; Momand et al., 1992) (data not shown).

Table 1 summarizes our results for the conformational analyses of p53 in 16 ALTR cell lines tested and their subcellular localization. As can be seen from this summary, there is a strong correlation in ALTR cells between the conformation of the p53 protein and its subcellular location. When p53 is in the cytoplasm, even at 32°C, it is present in a mutant conformation, while nuclear localized p53 at the same temperature is found in a wild-type conformation. This is interesting since it suggests that the conformation of tsp53 in the ALTR cells is not dictated by temperature alone. These data lead us to believe that subcellular localization of p53 may play an important role in determining the conformation of p53.

Conformation of p53 changes in response to IR in A1-5 and SK-N-SH cells:

Based on the correlation observed in ALTR cells indicating that the conformation of p53 was specific to its subcellular localization, we wanted to determine if changes in

localization of p53 could cause a corresponding change in the conformation of p53 independent of a temperature shift. To determine if the p53 in A1-5 cells could undergo such a change in conformation in response to a stimulus other than temperature, we exposed A1-5 cells to 5 Gy ionizing radiation (IR). A1-5 cells were maintained at 39°C for this experiment. We observed translocation of p53 into the nucleus at 2 H after exposure to IR by indirect immunofluorescence (Figure 11 A and B). This translocation was transient since it was not detectable 3 and 6 hours post irradiation. In the absence of IR, at 39°C, the p53 in these cells was in a predominantly mutant. However, in response to IR, there was a 50% (approximate) increase in the 246+ conformation of p53 (Figure 11 C).



RATIO 246/240	A1-5, 39°C, No IR	A1-5, 39°C, 5Gy IR
	0.355 ± 0.007	0.62 ± 0.056

Figure 11. Subcellular localization and conformation of tsp53 in A1-5 cells in response to genotoxic stress. **Panel A.** A1-5 cells maintained at 39°C (**a**) were exposed to 5 Gy IR (**b**) and the location of p53 determined using indirect immunofluorescence with PAb 421. **Panel B.** Conformation analysis of tsp53 in A1-5 cells after exposure to 5 Gy IR by immunoprecipitation with PAb 421 (total p53), and the conformation specific antibodies PAb 246 (wild-type) and PAb 240 (mutant) as described in Figure 2. PAb 419 is specific for the large T-antigen of the SV-40 virus and serves as an isotype control antibody After exposure to IR, cells were labeled with [^{35}S]-Methionine containing medium for 2 hours, followed by cell lysis and immunoprecipitation. Immunoprecipitated protein was resolved by SDS-PAGE, and the dried gel exposed to film for 48 hours. Phosphoimager analysis was performed to determine the ratio of ^{35}-S labeled p53 immunoprecipitated by PAb 246 to PAb 240.

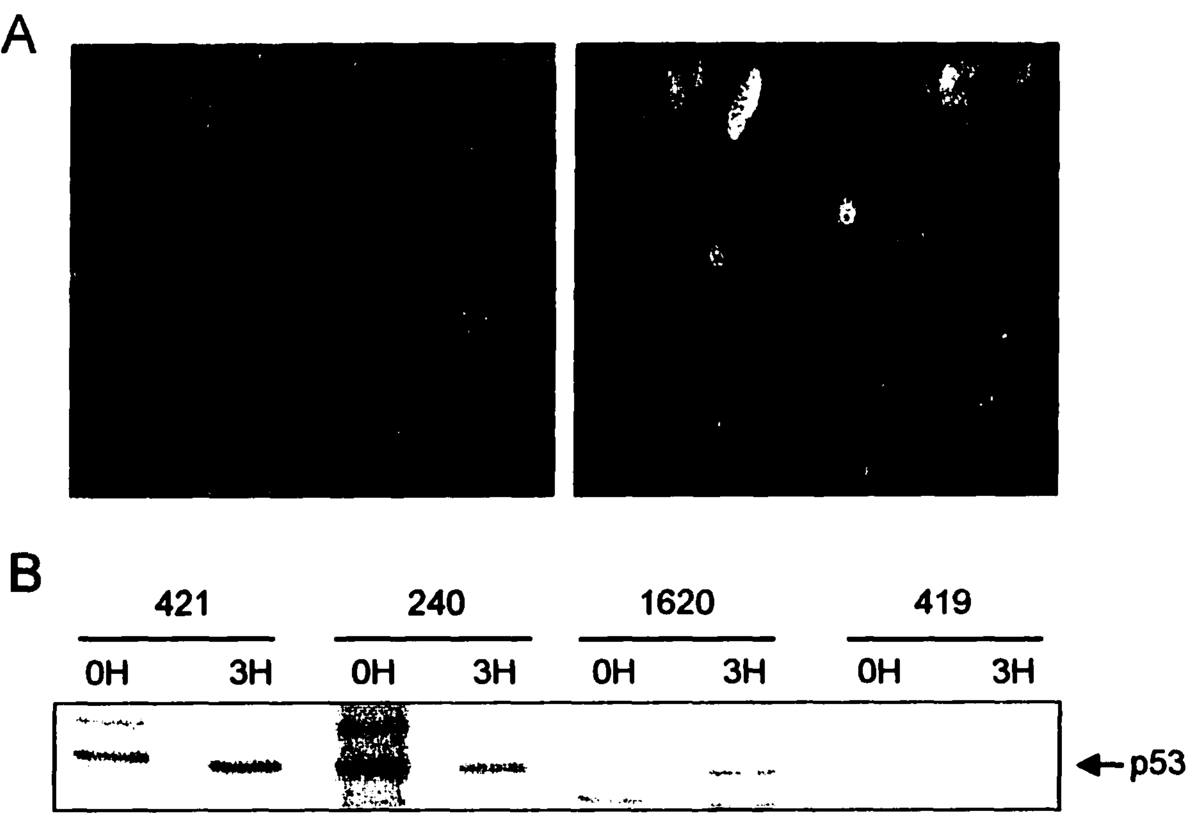

A
B
421
240
1620
419
0H
3H
0H
3H
0H
3H
0H
3H
p53

Figure 12. Subcellular localization and conformation of p53 in SK-N-SH cells after genotoxic stress. **Panel A.** The nuclear translocation of p53 in SK-N-SH cells was examined by indirect immunofluorescence using PAb 421, an antibody specific p53. **A a** and **A b** indicate the subcellular localization of p53 before and after exposure to 5 Gy IR respectively, as determined by indirect immunofluorescence using the monoclonal antibody PAb 421. **Panel B**. Conformation analysis of [^{35}S]-labeled p53 protein was performed by immunoprecipitation using PAb 421 (total p53), PAb 1620 (wild-type specific), PAb 240 (mutant specific) and PAb 419 (isotype control) antibodies. SK-N-SH cells were exposed to 5 Gy IR followed by [^{35}S]-methionine labeling for 3 hours. The labeled protein extracts were used for immunoprecipitation. Immunoprecipitated protein was resolved by SDS-PAGE, and the dried gel exposed to film for 48 hours.

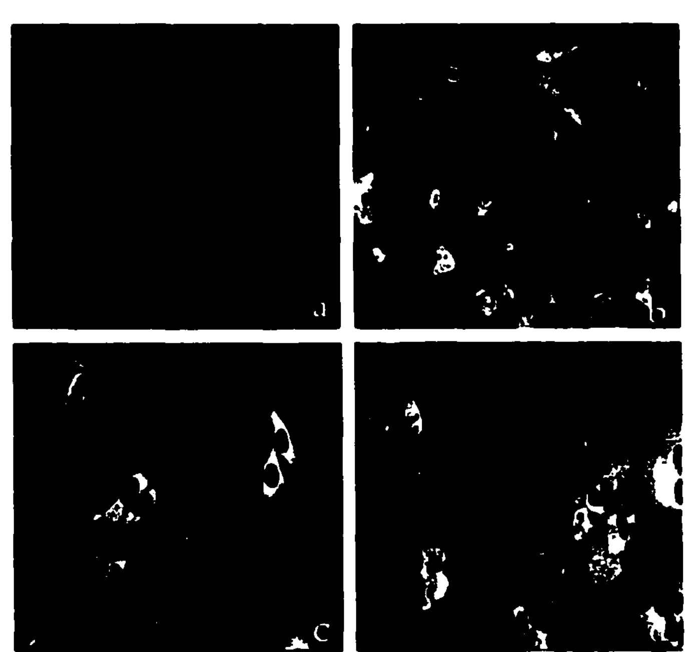
Since the p53 in A1-5 and ALTR cells does have a point mutation at residue 135, it could affect the integrity of the protein and therefore alter the conformation of the protein as well. If in fact, the correlation between localization and conformation was true, we argued, we would be able to see it in a cell line expressing wild-type p53. However, in most cells expressing wild-type p53, the level of protein is undetectable due to a rapid turnover rate. Therefore, we decided to use a cell line, such as SK-N-SH, a neuroblastoma derived cell line, which expresses wild-type p53 determined by sequencing (Davidoff et al., 1992) which is cytoplasmically sequestered and more stable (Moll et al., 1996; Zaika et al., 1999a). We exposed SK-N-SH cells to 5 Gy IR and observed strong translocation of p53 into the nucleus within 3 hours by indirect immunofluorescence using PAb 421 (Figure 12 A and B). To determine the conformation of the p53 in these cells, we immunoprecipitated p53 using PAb 421, 240, 1620, and 419. PAb 421 recognizes both mutant and wild-type conformations of p53. PAb 240 is specific for the mutant conformation of p53 while PAb 1620 is specific for wild-type p53. PAb 419 is specific for large T-antigen of the SV-40 virus and was used as the isotype control antibody. In the absence of IR, p53 was observed by immunoprecipitation predominantly with the 421 and 240 antibodies (Figure 12 C). However, after exposure to IR all three antibodies were able to immunoprecipitate p53. These results indicate that in the absence of IR, the p53 is in a predominantly mutant conformation, correlating with the cytoplasmic localization of p53 in these cells. However, upon exposure to IR, the p53 translocates into the nucleus where it assumes a wild-type conformation, which can then be immunoprecipitated by PAb 1620 antibody.

Conformation and localization are related but independent events:

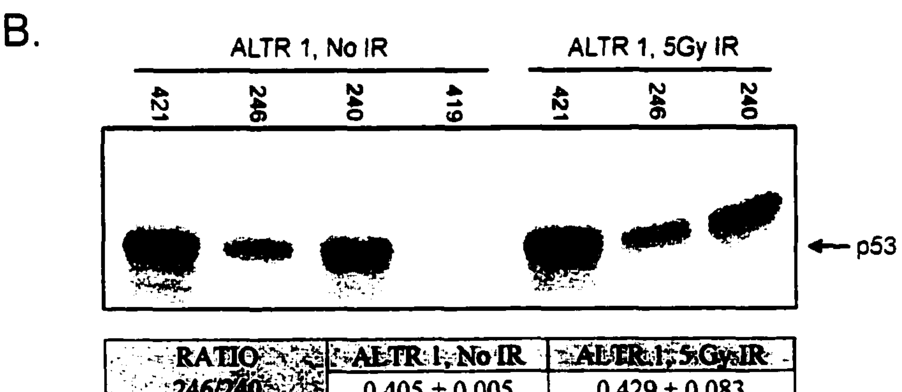
The ALTR cells were selected to have mutations in pathways that activate p53. To determine if conformation or localization was compromised in these cells in response to IR, we tested two cytoplasmically sequestered ALTR cell lines, ALTR 1 and 25 to determine their response. Both cells have cytoplasmically localized p53 at 32°C in a predominantly mutant conformation. Upon exposure to IR, strong translocation of p53 was observed into the nucleus in ALTR 1 cells (Figure 13 A and B). However, by immunoprecipitation analysis, we did not observe a significant increase in the 246+ conformation of the protein in these cells (Figure 13 C and D). This inability to undergo a change in the conformation of p53 despite nuclear localization of the protein may, in fact, be an additional defect in the ALTR 1 cells that leads to the inactivation of p53. Transcriptional induction by p53 of the down-stream target gene p21 was also not observed in ALTR 1 cells following IR exposure (data not shown).

Interestingly, the p53 in ALTR 25 cells did not translocate into the nucleus in response to IR. Consistent with this, we did not observe a corresponding increase in the wild-type conformation of the p53 protein. These results suggest that an integral component of the pathway responsible for the translocation of p53 into the nucleus has been affected in the ALTR 25 cells. Together, these results with ALTR 1 and 25 cells also suggest that translocation of p53 into the nucleus and the corresponding change to a wild-type conformation are independent events that p53 must undergo in order to be functionally active.

A.

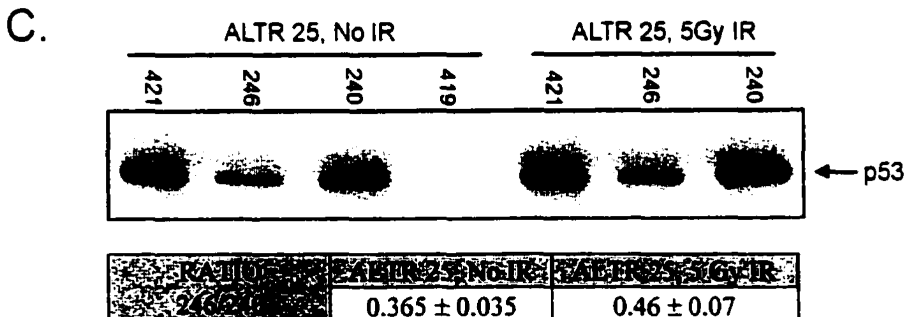


B.



RATIO 246/240	ALTR 1, No IR	ALTR 1, 5Gy IR
	0.405 ± 0.005	0.429 ± 0.083

C.



RATIO 246/240	ALTR 25, No IR	ALTR 25, 5Gy IR
	0.365 ± 0.035	0.46 ± 0.07

Figure 13. Nuclear translocation and conformation of tsp53 in ALTR cells exposed to genotoxic stress. Panel A. Indirect immunofluorescence using PAb 421 was performed to determine the subcellular localization of p53 in response to exposure to IR. Cells plated on coverslips and incubated for 48 hours at 32°C were exposed to 5 Gy IR and harvested 2 hours later for staining. **A a** and **A b** represent subcellular localization of p53 in ALTR 1 cells before and after exposure to IR. Panel B represents the autoradiograph of [^{35}S]-labeled p53 immunoprecipitated from ALTR 1 cells using PAb 421 (total p53), PAb 246 (wild-type), PAb 240 (mutant) and PAb 419 (isotype control) antibodies. ALTR 1 cells were exposed to 5 Gy IR followed by [^{35}S]-methionine labeling for 2 hours. The labeled protein extracts were used for immunoprecipitation. The ratio of tsp53 immunoprecipitated by PAb 246 to PAb 240 was determined using ImageQuant Phosphoimager Analysis. Immunoprecipitated protein was resolved by SDS-PAGE, and the dried gel exposed to film for 48 hours. **A c** and **A d** are representative of the subcellular location of p53 in ALTR 25 cells before and after exposure to IR respectively. Panel C represents an autoradiograph of [^{35}S]-labeled tsp53 immunoprecipitated from ALTR 25 cells using PAb 421 (total p53), PAb 246 (wild-type), PAb 240 (mutant) and PAb 419 (isotype control) antibodies immunoprecipitation profile of tsp53 in response to IR in ALTR 25 cells. The ratio of tsp53 immunoprecipitated by PAb 246 to PAb 240 was determined as mentioned above.

Discussion:

Elimination of a functional p53 is a critical step in tumor development. Inactivation can occur either at the level of the gene, through mutation, or at the level of the protein, by interacting with viral antigens or other cellular proteins or by physical sequestration in an inappropriate subcellular compartment (Levine, 1997; Livingstone et al., 1992; Moll et al., 1996; Momand et al., 1992; Scheffner et al., 1990). Although the mutations that occur in the p53 gene have been well documented and the proteins which bind to p53 and inactivate it have been described, little is known about the dysfunction that leads to cytoplasmic sequestration and inactivation of p53 function by physical means.

Translocation of p53 into the nucleus is an integral event in the functional activation of p53. In the absence of its nuclear localization sequence (NLS), wild-type p53 is unable to translocate into the nucleus and is functionally inactive as a tumor suppressor (Shaulsky et al., 1990b; Shaulsky et al., 1991b; Shaulsky et al., 1991d).

To gain insight into the relationship between nuclear localization and p53 function, we generated variant ALTR cell lines that contained high levels of the temperature sensitive $p53^{val135}$ protein, capable of growth at 32°C, the permissive temperature for p53 activation. On determining the subcellular localization of p53 in these cell lines, we observed that most of the ALTR cell lines generated had cytoplasmically sequestered p53 even at 32°C. Only four of 16 ALTR lines tested displayed nuclear localization of p53 at 32°C, the characteristic pattern of localization observed in parent A1-5 cells from which these ALTR cell lines were derived. This

predominance of ALTR cells with cytoplasmically sequestered p53 at 32°C suggests that an important mechanism by which p53 is inactivated in these cells is by physically suppressing p53 from interacting with its target site, the genomic DNA in the nucleus. Even though cytoplasmic sequestration has been previously identified as a mechanism by which wild-type p53 is inactivated in tumors, the mechanism by which this inactivation occurs in still largely unknown.

The conformation of p53 has been shown to change in a cell cycle specific manner (Milner, 1991a; Milner, 1991b; Milner & Watson, 1990; Sabapathy et al., 1997; Shaulsky et al., 1991a). In addition, spatial regulation of p53 during the cell cycle has also been reported (Shaulsky et al., 1990a). The authors found an accumulation of p53 in the nucleus at the beginning of the S phase, after which it was maintained in the cytoplasmic compartment of the cell. This and other evidence indicates that wild-type p53 is involved in the regulation of the cell cycle, even in the absence of genotoxic stress. On further characterization of the ALTR cell lines we also found a strong correlation between the subcellular localization of p53 and its subsequent conformation.

Wild-type p53 can exist in two different conformations, mutant and wild-type, based on reactivity with conformation specific antibodies (Milner, 1991a). The wild-type conformation of p53 correlates with growth suppression while the mutant conformation promotes cell proliferation in genotypically wild-type p53 expressing cells (Milner & Watson, 1990). We observed two main classes of ALTR cell lines based on their relationship between the localization of p53 and its conformation. In the first class, ALTR cells with predominantly cytoplasmically sequestered tsp53 at 32°C also exhibited

a predominantly mutant conformation of tsp53 as determined by the conformation specific antibody PAb 240. The second class included cell lines with tsp53 localized in the nucleus at 32°C in which the tsp53 was maintained in a wild-type conformation, as recognized by PAb 246. We were able to isolate only four ALTR cell lines, ALTRs 9, 10, 17, and 18 that displayed characteristics of this second class. This correlation between the localization of p53 protein and its conformation was interesting primarily since the temperature-sensitive $p53^{val135}$ present in these cells has been described previously to display a strong temperature-dependent phenotype (Milner & Medcalf, 1990). In vitro and in vivo studies indicate that $p53^{val135}$ folds into a wild-type conformation at 32°C, however, in the ALTR cells in which $p53^{val135}$ was cytoplasmically localized at 32°C, the p53 protein was still maintained in a predominantly mutant conformation. Only in cells with nuclear localized p53 was the $p53^{val135}$ also present in a wild-type conformation therefore suggesting that the subcellular location of p53 was an important determinant of its subsequent conformation.

The notion that conformation was determined by the subcellular localization was further supported by observing that a similar response could be observed to stimuli other than temperature in parental A1-5 cells. We found that the protein in A1-5 cells could, in fact, alter its conformation from mutant to wild-type in response to ionizing radiation when maintained at 39°C. This is a novel finding indicating that the conformation of $tsp53^{val135}$ in A1-5 cells was not dictated by temperature alone. When we examined the correlation between p53 localization and conformation in SK-N-SH cells, a neuroblastoma cell line known to express bona-fide wild-type p53, we were surprised to

find that in untreated cells, none of the p53 was recognizable by the wild-type conformation specific antibody PAb 1620. The p53 in these cells has been found by sequencing to be wild-type, cytoplasmically sequestered, and resistant to mdm-2 mediated degradation. Upon exposure to IR, we observed an increase in not only the total level of p53 in these cells, but also its detectability by PAb 1620, concurrent with the translocation of p53 into the nucleus. This is similar to what we observed in IR treated A1-5 cells and suggests that the existing p53 in these cells is cytoplasmically sequestered in the mutant conformation, and upon translocation into the nucleus p53 is induced to fold into the wild-type conformation.

Our results are consistent with the observations made by Zerrahn et al who previously showed a similar correlation between the subcellular localization of p53 and its conformation (Zerrahn et al., 1992). They found that cytoplasmically sequestered p53 was presented in a mutant conformation whereas p53 capable of translocating into the nucleus was folded in a wild-type conformation. However, in their system, the cytoplasmically localized mutant p53 was also found by sequencing to have mutations in the p53 gene. Their data suggest that genotypically mutant p53 is preferentially maintained in the cytoplasmic compartment of the cell. In contrast, our results suggest that wild-type p53 can have a 240+ conformation when in the cytoplasm and that genotoxic stress causes p53 to fold into the wild-type conformation and concentrate in the nucleus.

Although there is a strong correlation between translocation of p53 into the nucleus and a change in its conformation to wild-type, these events are independent of

each other based on results we obtained upon irradiation of the two ALTR cell lines, ALTR 1 and 25. In ALTR 1 cells no considerable increase in the wild-type conformation of p53 was observed in spite of almost all of the protein translocating into the nucleus. This suggests that translocation of p53 into the nucleus is not sufficient for mediating a change in the conformation of the protein. Changes in conformation of p53 may be mediated by post-translational mechanisms or by association with other proteins which allow p53 to fold appropriately, either of which may be mutated in the ALTR 1 cell line. Interestingly, in ALTR 25 cells, the p53 did not translocate into the nucleus in response to exposure to IR and also did not show a corresponding increase in the wild-type conformation of the p53 protein. These results indicate that these cells have a defect in either the translocation machinery for p53 or in the signaling pathway upstream of p53 which is responsible for triggering its nuclear translocation response.

Our results indicate that physical sequestration of p53 in the cytoplasm can be a potent mechanism for inactivating p53 and that the translocation process is closely linked to the protein's conformation. The panel of ALTR cells generated during this study will be important reagents for examining the mechanisms involved in regulating p53 subcellular localization. In particular, ALTR 1 and ALTR 25 cells will aid in dissecting the two closely linked processes of p53 activation which involve its translocation into the nucleus and its change in conformation from mutant to wild-type. In addition, cell lines such as ALTR 17 which have nuclear localized p53 in a wild-type conformation which is functionally inactive will prove valuable in understanding additional post-translational modifications that p53 must undergo to function as a transcription factor. We are

currently characterizing the defects in the ALTR cells that lead to the inactivation of p53 in an attempt to understand its regulation. We expect our panel of ALTR cells to provide us with a deeper understanding of how p53 is regulated and its role in several signal transduction pathways.

CHAPTER 3.

A novel mechanism of p53 inactivation by calpain mediated degradation

Introduction

The tumor suppressor p53 plays an important role in cell cycle progression. This is evident from the high percentage of tumors in which p53 is inactivated by gene mutation (Nigro et al., 1989). Other mechanisms also exist by which p53 can be inactivated. These include physical sequestration of p53 in the cytoplasmic compartment of the cell (Moll et al., 1996; Moll et al., 1992) and enhanced degradation by viral and cellular gene products such as Human Papilloma Virus 16 E6 protein and mdm2, a cellular oncogene which when overexpressed, enhance proteasome mediated degradation of p53 (Capoulade et al., 1998; Crook et al., 1991; Haupt et al., 1997; Huibregtse & Beaudenon, 1996). However, in normal cells, in the absence of stress signals, low levels of p53 are maintained through ubiquitin-dependent proteolysis by mdm2 which functions as an E3 ligase and targets p53 for ubiquitination and subsequent degradation by the proteasome (Honda et al., 1997). In response to stress signals, p53 accumulates in normal cells (Ashcroft et al., 1999; Colman et al., 2000). This is thought to occur as a result of post-translational modifications such as phosphorylation that both p53 and mdm2 undergo which result in their inability to associate with each other (Ashcroft et al., 1999; Chehab et al., 1999; Colman et al., 2000; Ljungman, 2000; Shieh et al., 1997; Shieh et al., 1999). Phosphorylation of p53 at serine residues in the amino terminus of the protein by kinases such as ATM (Ataxia Telangiectasia Mutated) and DNA-PK

(DNA activated protein kinase) have been shown to phosphorylate p53 at Serine 15, and Serine 37 (human p53), resulting in the dissociation of p53 and mdm2 and the consequent accumulation of p53 and its functional activation (Canman et al., 1998; Lees-Miller et al., 1992; Shieh et al., 1997; Shieh et al., 1999). Phosphorylation of p53 by Chk2 at Serine 20 (human p53) has also been implicated in the stability of p53 by abrogating its association with mdm2 (Chehab et al., 2000; Chehab et al., 1999; Craig et al., 1999).

Recently, four separate research groups have reported that p53 can also be regulated by calpain. Calpains are neutral proteinases belonging to the family of cysteine proteases that depend on Ca2+ for their activity (Sorimachi et al., 1997). They are present in two forms in cells (μ- and m-calpain) and are distiniguished by the concentration of calcium needed for their functional activation *in vitro* (Sorimachi et al., 1997). A role for calpains has been found in brain function, apoptosis, structural organization, muscular dystrophy and signal transduction. Calpains have also been shown to target p53 for degradation *in vitro* (Dietrich et al., 1996; Gonen et al., 1997; Kubbutat & Vousden, 1997; Pariat et al., 1997; Piechaczyk, 2000). Kubbutat and Vousden showed that calpain cleaves p53 *in vitro*, and that calpain inhibitors I and II could enhance endogenous p53 levels in MCF-7 breast carcinoma and RKO colon cancer derived cells (Kubbutat & Vousden, 1997). Pariat and colleagues showed that the tertiary structure of p53 is essential for its recognition by calpain and interestingly, also identified some naturally occurring mutant human and mouse p53 proteins that were more sensitive to calpain mediated degradation than wild-type p53 itself (Pariat et al., 1997). Calpain may be a natural regulator of p53 function, as suggested by a recent report by Atencio et

al demonstrating p53 dependent apoptosis in tumor cells expressing wild-type p53 after treatment with calpain inhibitor I (Atencio et al., 2000). Treatment with the inhibitor caused an increase in p21 protein levels, a downstream target gene of p53, and caspase activation in a p53-dependent manner.

In this study, we report calpain mediated degradation as a potential mechanism by which p53 can be inactivated. We have previously described a panel of cell lines, called ALTR for A1-5 Low Temperature Resistant that are capable of growth at 32°C (Gaitonde et al., 2000). These cell lines have been generated from A1-5 cells (transfected with temperature sensitive mouse p53, Val → Ala at residue 135), and can grow at 32°C, the permissive temperature for p53 activation in parent A1-5 cells. We investigated the mechanism by which p53 was inactivated in one such ALTR cell line, ALTR 9, and found that the p53 in these cells has a reduced half-life at 32°C relative to the parental cells at this temperature. On further investigation, we found that the p53 in these cells was subject to enhanced degradation partially by calpain. In addition, we observed that the p53 in ALTR 9 cells could be stabilized by exposing cells to ionizing radiation suggesting that calpain mediated degradation of p53 may be regulated by genotoxic stress.

Materials and Methods

Cell Culture, Reagents and Irradiation :

A1-5 cells and the ALTR 9 variant have been described previously (Gaitonde et al., 2000). A1-5 and ALTR cell lines were maintained in DMEM medium, containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MD) with 5% CO_2. A1-5 cells were grown at 37°C and the temperature shifted to 32° or 39°C twenty four hours prior to the experiment, and ALTR 9 cells were maintained at 32°C for all experiments unless noted otherwise. Antibody against p53, Pab 421, was kindly provided by Dr. Arnold Levine. Antibody used to determine phosphorylation of p53 at Serine 18 was obtained from New England Biolabs. Antibody used to determine phosphorylation of p53 at Serine 23 was kindly donated by Dr. Yoichi Taya. Other antibodies used include anti-mdm2 (smp14, Santacruz Research) and anti-p21 (Ab-6, Oncogene Science). Calpain Inhibitor I was obtained from Sigma, and Lactacystin was obtained from Calbiochem. Cells were exposed to 5 Gy Ionizing radiation using a ^{60}Co source at an average dose rate of 66 cGy/Min.

RT-PCR for p53:

Reverse transcription of 2 µg total RNA from A1-5 and ALTR cells was carried out using 0.5 µg Oligo$(dT)_{12-18}$, 2 mM dNTP mix, 1 mM DTT, 0.5 mM $MgCl_2$, 10X RT buffer and 50 units Superscript II RT in a 20 µL reaction (Gibco BRL). The full length p53 coding

sequence was amplified by dividing it into three separate reactions, the first 1/3rd NH_2 part, the middle DNA binding domain region, and the remaining 1/3rd COOH domain. The NH_2-domain of p53 was amplified by PCR using 5'ATGGAGGAGTCACAGTCGGATA3' as the upstream primer and 5'ATAACAGACTTGGCTGTCCCAG3' as the reverse primer, to generate a PCR product of 366bp. The DNA binding domain of p53 was amplified by PCR with 2 μL of the above reaction. The upstream primer 5'-GCCAAGTCTGTTATGTGCA-3', and the reverse primer 5'-GAGGCGCTTGTGCAGGTGG-3' were used to specifically amplify 590 bp of the p53 cDNA which maps to the DNA binding domain. The final 1/3rd region was amplified using 5'AGAAAATTTCCGCAAAAAGGAA3' as the forward primer and 5'CCCCACTTTCTTGACCATTGTT3' as the reverse primer, to give a PCR product of 330 bp. All regions were overlapping to ensure full sequence information. PCR conditions were: denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 55°C for 30 sec and 72°C for 1 min. The PCR products was separated on a 1% agarose gel, excised and extracted from the gel using the QIAQuick gel extraction kit (Qiagen). The product was then sequenced in both directions on an automated sequencer using the same primers.

Indirect Immunofluorescence and Image Processing:

Cells were plated onto coverslips at an approximate concentration of $2X10^5$ cells per 60 mm^2 plate. The plates were then incubated at the appropriate temperature for 12-16

hours. Coverslips with attached cells were rinsed three times in cold PBS (containing 3 mM KCl, 1 mM KH_2PO_4, 0.2 mM $MgCl_2$, 137 mM NaCl, and 8 mM Na_2HPO_4, pH 7.5), and cells fixed to the coverslips using methanol:acetone (1:1) for 5 minutes, at –20°C. Sample coverslips were allowed to air dry, stored at -20°C and rehydrated using cold PBS prior to staining. For A1-5 and ALTR cells, undiluted PAb 421 antibody supernatent (approx. 200 μL) was applied and the coverslips were incubated at room temperature for one hour, after which, they were rinsed three times with cold PBS. Goat anti-mouse antibody conjugated to FITC (Sigma Chemical Co.) at a 1:500 dilution was then applied for an incubation time of 30 minutes, at room temperature. Samples were washed three times with cold PBS, and mounted on standard glass slides using Mowiol mounting medium (Calbiochem). A Zeiss LSM-10 model confocal microscope was used for all observations. Digital images were collected on each series using identical brightness and contrast settings for each cell line.

Western blot Analysis:

Protein expression was determined by Western Blot analysis using the Laemmli method. Briefly, cells were lysed in Lysis buffer (50 mM Tris-Cl (pH 8.0), 5mM EDTA, 150 mM NaCl, .5% NP-40, 1mM PMSF, and 1 mM aprotinin, leupeptin and pepstatin A) (Sigma Chemical Co.) and protein concentration determined using DC Protein Assay kit (BioRad). Fifty μg protein was loaded per lane and resolved on a 10% acrylamide gel followed by transfer onto Nitrocellulose membrane (Pall Gelman Corp.). The membrane was blocked overnight in Blotto (Tris, 20mM, NaCl 500mM, 0.05% Tween-20 and 5%

Blotting Grade Blocker non-fat dry milk (BioRad Corp)). Primary antibody reaction was carried out at room temperature for 1.5 H for p53, mdm2 and p21 reactions or overnight at 4°C for phospho-p53 reactions, and secondary antibody reactions were incubated for 1 H. Chemiluminescence was observed using the ECL reagent kit (Amersham Inc.).

Results

Subcellular localization of p53 and protein stability in ALTR 9 cells:

The ALTR cell system has been described previously (Gaitonde et al., 2000). A1-5 cells, a rat fibroblast cell line expressing temperature sensitive mutant p53 (Val→Ala at residue 135), undergo growth arrest at 32°C. In order to identify proteins that regulate p53 activity, A1-5 cells were mutagenized with EMS and colonies capable of growth at 32°C were selected for further characterization. The rationale for this system was based on selecting cell lines in which p53 function was inactivated, either by mutations in p53 itself or by mutations in pathways upstream of p53 that could also abrogate p53 function. We generated 22 cell lines called ALTR for A1-5 Low Temperature Resistant. These ALTR cell lines are capable of growth at 32°C and are maintained at this temperature for routine culturing. In our previous study we determined the subcellular localization of the p53 in all ALTR cell lines at both 32° and 39°C (Gaitonde et al., 2000). One of these ALTR cell lines, ALTR 9, showed a subcellular distribution pattern of p53 similar to that observed in A1-5 cells at 32° and 39°C (Figure 14) suggesting that inappropriate subcellular localization of p53 was not the mechanism by which p53 had been inactivated in these cells. The p53 in A1-5 cells is functional at 32°C while that in ALTR 9 cells is not, as determined by growth of these cells at 32°C and transient transfections using pG13-LUC, a reporter construct containing thirteen p53 response elements linked to a luciferase reporter gene (el-Deiry et al., 1993) (data not shown). In addition, we also determined the level of p21 induction in the ALTR 9 cells compared to that in A1-5 cells at 32° and 39°C and found high levels of

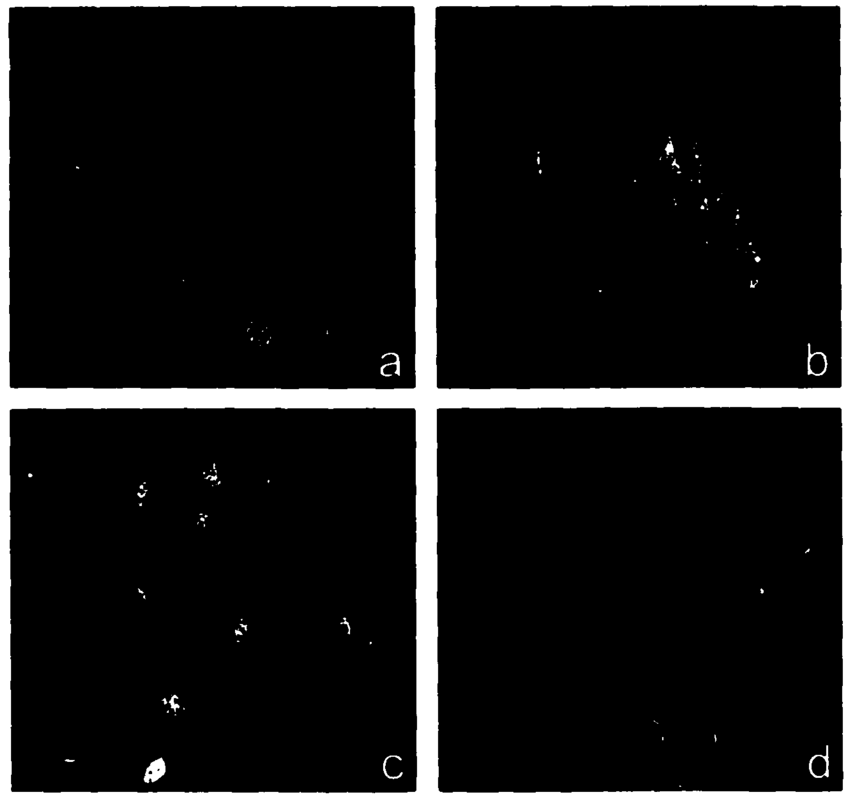


Figure 14. Subcellular localization of p53 in A1-5 and ALTR 9 cells.
Indirect Immunofluorescence was used to determine the subcellular localization of p53 in A1-5 and ALTR 9 cells. Cells were grown on coverslips and maintained at either 32° or 39°C for 24 hours. The cells were stained with a primary antibody specific for p53, Pab 421, followed by staining with a fluorescein isothiocyanate-conjugated secondary antibody. **Figure 1 a** and **b** represent p53 staining in A1-5 cells at 32° and 39°C respectively and **c** and **d** represent ALTR 9 cells at 32° and 39°C respectively. All images are at 400X magnification.

Table 2. Half-life of p53 in A1-5 and ALTR 9 cells, determined by pulse-chase experiments using ^{35}S-labeled p53 protein immunoprecipitated by PAb 421

Cell Line (32°C)	Half-life of p53
A1-5	10.5 ± 0.5 H
ALTR 9	5.5 ± 0.5 H

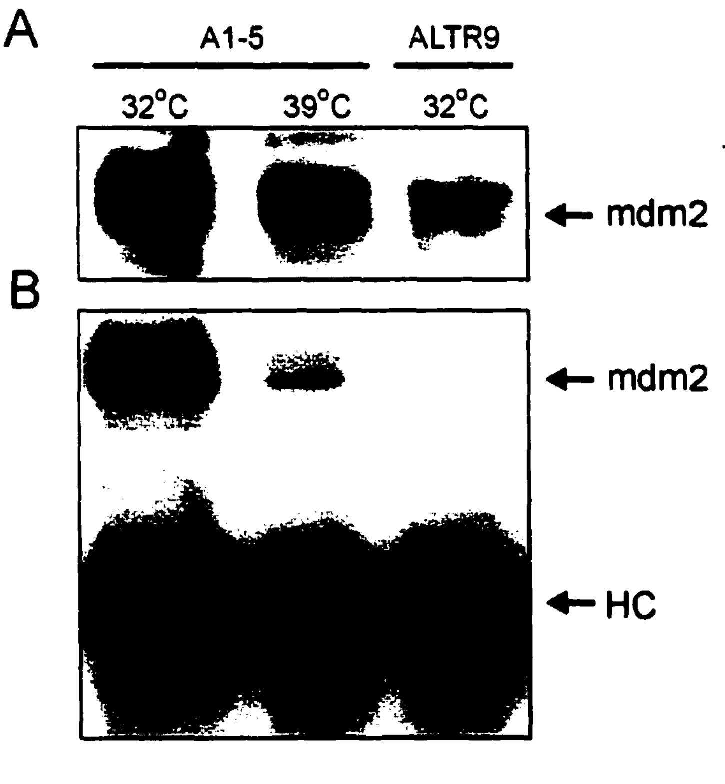
p21 expression in A1-5 cells at 32°C compared to that observed in A1-5 cells at 39°C and ALTR 9 cells at 32°C (data not shown). RT-PCR analysis of the temperature sensitive mouse p53 cDNA in ALTR 9 cells, however, indicated that the protein had acquired a single point mutation in the DNA binding domain of p53 at residue 186 (Ser → Pro). Although this could account for the inactivity of the p53 in these cells at 32°C, a closer examination of these ALTR 9 cells suggested that a more interesting mechanism for p53 regulation had been selected.

As part of the characterization of the ALTR cells, the half-life of the p53 was also examined in ALTR 9 cells at 32°C, by pulse chase experiments using ^{35}S-labeled p53 protein, and compared it with that observed in A1-5 cells at 32°C. We observed the p53 in ALTR 9 cells had a markedly reduced half-life; about 4 hours compared to that in A1-5 cells at 32°C, which was about 10.5 hours (Table 2). These results suggested that the p53 protein in ALTR 9 cells was less stable than that observed in the parental A1-5 cells at the same temperature.

P53 in ALTR 9 cells is partially degraded by calpain:

Previous reports have described p53 degradation by the proteasome via association with mdm2 (Honda et al., 1997). To determine whether this was the mechanism by which p53 was being degraded in ALTR 9 cells, we examined the level of mdm2 expression in these cells and compared it with that found in A1-5 cells at 32° and 39°C. No elevation in the levels of mdm2 expression was observed in the ALTR 9 cells compared to that in A1-5 cells (Figure 15A). We reasoned that the level of mdm2

expressed in these cells may not reflect the level of association of this protein with p53 which may be enhanced and so also examined the association between p53 and mdm2 in ALTR 9 cells by immunoprecipitation analysis and compared it with that observed in A1-5 cells at 32° and 39°C (Figure 15B). A strong association between p53 and mdm2 in A1-5 cells at 32°C, and a reduced association in A1-5 cells at 39°C was observed. However, little to no association was observed between p53 and mdm2 in ALTR 9 cells at 32°C. To further investigate if proteasome mediated degradation was the mechanism by which p53 was being degraded in ALTR 9 cells we used an inhibitor of the proteasome, lactacystin, to determine if it could stabilize the level of p53 in these cells (Figure 15C). No accumulation of p53 was observed in these cells after treatment with 5 μM lactacystin indicating again that the p53 in these cells is not degraded by the proteasome through association with mdm2. This concentration of lactacystin was capable of stabilizing p53 in HCT 116 cells, a colon cancer derived cell line expressing wild-type p53 (data not shown).


A
A1-5
ALTR9
32°C
39°C
32°C
mdm2
B
mdm2
HC

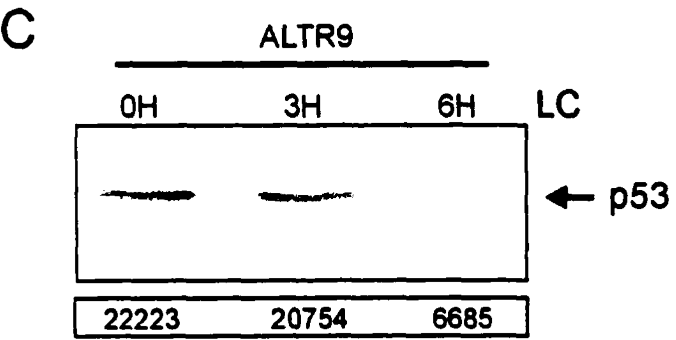

C
ALTR9
0H
3H
6H
LC
p53
22223
20754
6685

Figure 15. Analysis of mdm2 mediated degradation of p53.
A. Mdm2 protein expression was determined in A1-5 cells at 32° and 39°C and in ALTR 9 cells at 32°C by Western Blot analysis using an antibody specific for mdm2 (smp14). B. Association of mdm2 with p53 in A1-5 cells at 32° and 39°C and in ALTR 9 cells at 32°C was determined by immunoprecipitation of mdm2 using an antibody specific for p53 (Pab 421) followed by Western blot using an mdm2 specific antibody (smp14) of the immunoprecipitated protein. C. ALTR 9 cells maintained at 32°C were treated with the proteasome inhibitor Lactacystin (5 μM), and the levels of p53 protein determined over time by Western blot analysis using Pab 421, and antibody specific for p53. Densitometry analysis was used to quantitate the level of p53 protein expression, and the numbers are listed below the figure.

Previous reports have identified p53 as a substrate for calpain mediated cleavage *in vitro* (Kubbutat & Vousden, 1997; Pariat et al., 1997; Sorimachi et al., 1997). To determine whether the p53 in ALTR 9 cells was targeted for degradation by calpain cleavage, we used 20 μM Calpain Inhibitor I (ALLN), an inhibitor specific for calpains, and determined if p53 could be stabilized after treatment (Figure 16A). The concentration of calpain inhibitor I (ALLN) used was low enough to be a specific inhibitor of calpains and not other proteases. We did, in fact, observe a moderate accumulation of p53 in ALTR 9 cells within 3 hours of treatment with ALLN suggesting that calpain cleavage was responsible for p53 degradation in these cells. We also determined if p21 could be induced in these cells as a result of p53 stabilization by calpain inhibitor I (Figure 16B). In ALTR 9 cells, we observed induction of p21 concomitant with increased levels of p53. This increase in p21 induction was p53 dependent since we did not observe it in A1-5 cells at 39°C which also did not respond to ALLN treatment by p53 accumulation (data not shown). These results suggest that in ALTR 9 cells p53 is being partially degraded by calpain and blocking calpain activity by using calpain inhibitor I could result in induction of p21 by p53.

Levels of p53 can be stabilized in ALTR 9 cells by exposure to IR:

Genotoxic damage has been shown previously to stabilize p53 protein and cause its accumulation in cells. To determine if the p53 in ALTR 9 cells was responsive to a signal other than a temperature- shift which could cause its accumulation, we exposed the cells to 5 Gy IR and quantitated the level of p53 protein over time (Figure 17A). We

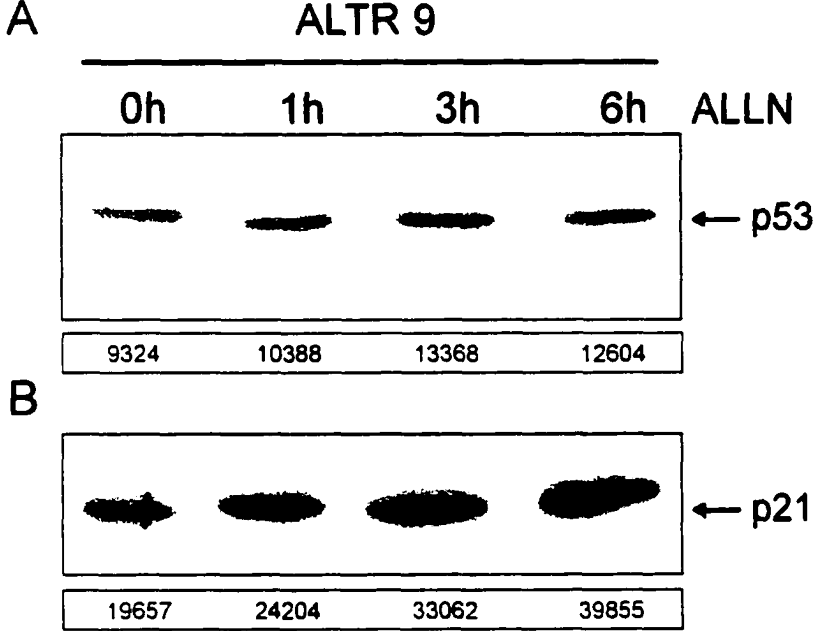


Figure 16. Effect of Calpain Inhibitor I treatment on p53 and p21 levels in ALTR 9 cells.
A. ALTR 9 cells maintained at 32°C were treated with 20 μM Calpain Inhibitor I and the total levels of p53 protein determined over time by Western blot analysis using the p53 specific antibody Pab 421. Quantitation of the p53 levels in the Western blot in Panel A was performed by densitometry analysis using the Stratagene Eagle Eye II and the values are listed below the figure. **B.** Total level of p21 protein was also determined in ALTR 9 cells after treatment with calpain Inhibitor I, by Western blot analysis. Densitometry analysis of the p21 levels in the Western blot shown in Panel B are listed below the figure.

observed that the level of p53 in these cells became elevated within an hour after exposure to IR and that the quantity of protein peaked 3 hours after Irradiation. We also observed an increase in the level of p21 expression in these cells after Irradiation, and the increase was observed 6 hours after irradiation (Figure 17B).

Since part of the process that leads to the stabilization of p53 in reponse to IR is thought to be phosphorylation of the protein at key amino terminus serine residues Ser 18 and 23 (Ser 15 and 20 in human p53, respectively), we determined the phosphorylation status of the p53 in ALTR 9 cells at these residues and compared it to A1-5 cells at 32° and 39°C (Figure 18A). Phosphorylation of p53 at residue 18 was observed only in A1-5 cells at 32°C, but none in A1-5 cells at 39°C and ALTR 9 cells at 32°C. However, phosphorylation could be induced at the Serine 18 residue in response to IR at 0.5 and 1 hour in both A1-5 cells at 39°C and ALTR 9 cells at 32°C (Figure 18 B and C). Phosphorylation of Ser 23 (human Ser 20) residue was observed in all cell lines tested (data not shown) suggesting that phosphorylation of this residue was not important in our system for maintaining p53 protein stability. These results suggest that phosphorylation of p53 at Ser 18 by ionizing radiation may be important in resisting calpain mediated degradation of p53 in these cells.

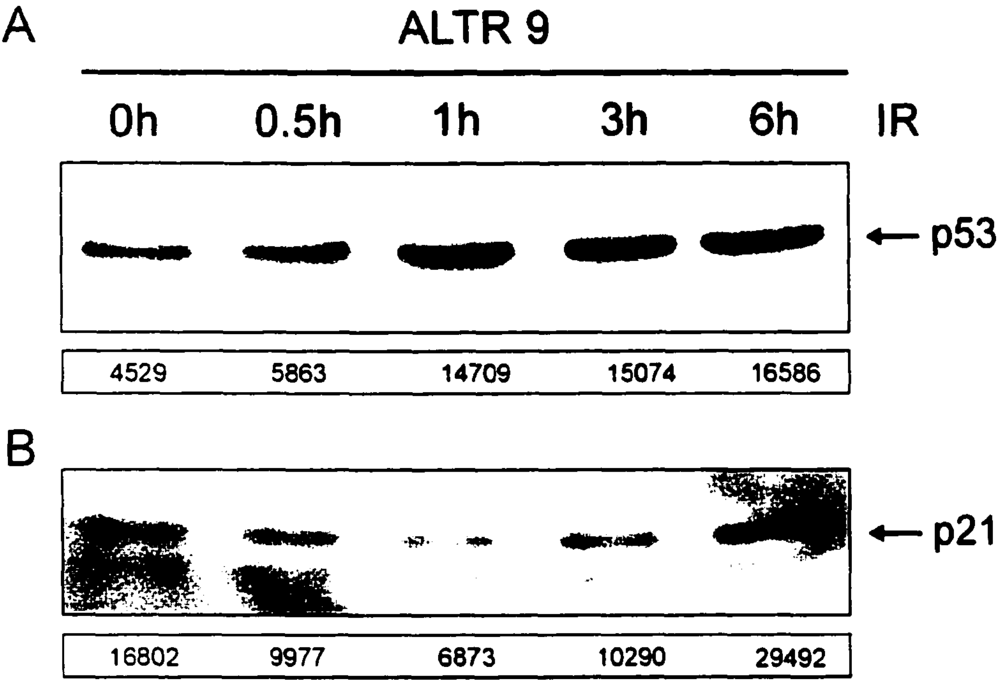


Figure 17. Effect of ionizing radiation on the total levels of p53 in ALTR 9 cells. **A.** ALTR 9 cells maintained at 32°C were exposed to Ionizing radiation (5 Gy) and the total levels of p53 determined over time by Western blot analysis using Pab 421, an antibody specific for p53. Densitometry analysis of the level of p53 expression in the Western blot in Panel A is shown below the figure. **B.** Total levels of p21 in ALTR 9 cells after exposure to IR was also determined by Western blot analysis using an antibody specific for p21 and densitometry analysis of the p21 levels are shown below the figure.

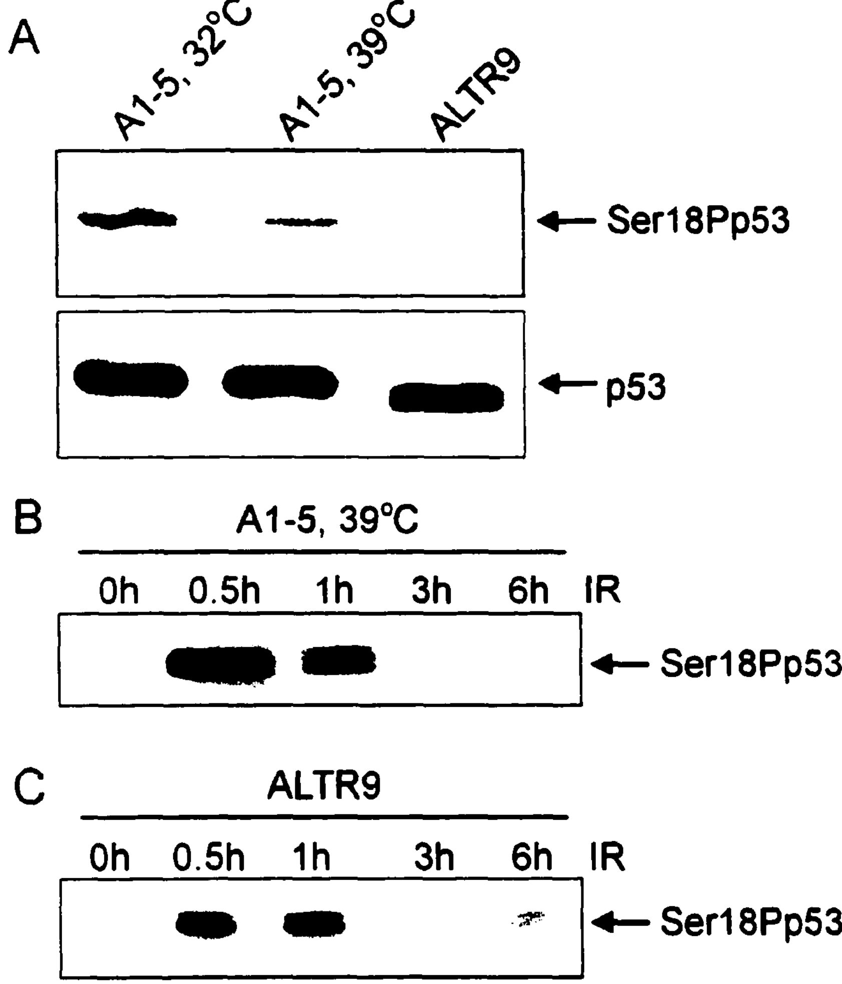


Figure 18. Phosphorylation of p53 at Ser18 in ALTR 9 cells compared to A1-5 cells at 32° and 39°C and in response to I.R.

A. Phosphorylation of p53 at Ser18 residue was determined by Western blot analysis using a phospho-specific antibody that recognizes p53 only when phosphorylated at this residue. The total levels of p53 were normalized such that equal amounts of p53 were loaded in all lanes, as shown in the lower panel.

Panel B. and C. demonstrate the phosphorylation status of p53 at Ser18 in A1-5 cells at 39°C and ALTR 9 cells at 32°C following exposure to 5 Gy I.R. Western blot analysis using an antibody that recognizes p53 only when phosphorylated at Ser18 was used to determine the phosphorylation status of p53 at this residue.

Discussion

In this report, we identify a novel mechanism by which p53 can be inactivated in cells. We show here, that p53 can be targeted for degradation partially by calpain cleavage resulting in a nonfunctional protein. Such enhanced degradation of p53 as a mechanism of its inactivation has only been described previously as mediated by viral oncogenes such as HPV-16 E6 and the cellular oncogene mdm2, both of which degrade p53 through ubiquitination and degradation via the proteasome pathway (Fuchs et al., 1998a Honda et al., 1997 Huibregtse & Beaudenon, 1996). In ALTR 9 cells, p53 associated very poorly with mdm2, and an inhibitor of the proteasome, lactacystin, had no effect on the total levels of p53 present. The concentration of lactacystin used was capable of inhibiting the proteasome since p53 accumulation was observed in other cell types tested (data not shown). In fact, in ALTR 9 cells, treatment with lactacystin caused reduction in p53 levels, without any cellular toxicity, suggestive of upregulation of other proteases in the cell potentially needed to balance the cell system. However, treatment with calpain inhibitor I or ALLN, an inhibitor of calpain, resulted in a moderate increase in the total level of p53 in ALTR 9 cells and also an upregulation of p21 protein suggesting that the p53 in these cells could be activated for transcription after its stabilization.

The p53 in ALTR 9 cells is found localized in the nucleus at 32°C. This is similar to the subcellular localization observed in A1-5 cells at the same temperature. However, the stability of the two proteins was markedly different in the two cell types suggesting

that the defect in the p53 in ALTR 9 cells was due to a shortened half-life. Association of p53 with mdm2 did not correlate with stability of p53 in A1-5 and ALTR 9 cells at 32°C. We observed a strong association between p53 and mdm2 in A1-5 cells at 32°C and yet the protein was found to be very stable. This may be due to the fact that p53 is retained in the nucleus at 32°C in these cells and degradation of p53 by the proteasome is contingent on its export out of the nucleus (Freedman & Levine, 1998). However, ALTR 9 p53 could be degraded in spite of it also being localized in the nucleus. Kubbutat and Vousden described calpain degradation of p53 in both nuclear and cytoplasmic fractions thereby indicating that export of p53 out of the nucleus was not required for calpain mediated p53 degradation (Kubbutat & Vousden, 1997).

Since stabilization of p53 in these cells also restored the ability of p53 to induce p21, the additional mutation (Serine 186 → Proline) did not appear to impact p53's ability to bind DNA or transactivate p21 and only affected its stability. Previous reports have identified different mutant p53 proteins that were still capable of binding DNA and transactivation suggesting a similarity to the mutant p53 in ALTR 9 cells (Di Como & Prives, 1998 Ory et al., 1994). However, this additional mutation in the p53 of ALTR 9 cells may be responsible for its increased sensitivity to calpain targeted degradation. Pariat et al tested several mutant human and mouse p53 proteins and observed that some of them were targeted for degradation by calpain more efficiently than wild-type p53 protein (Pariat et al., 1997). The tertiary structure of p53 was also shown to be important for calpain cleavage. In ALTR 9 cells, the p53 is expressed in a wild-type conformation,

as determined by immunoprecipitation analysis, despite its additional mutation suggesting that the protein folding is important in its recognition and cleavage by calpain.

The fact that the p53 in ALTR 9 cells could be stabilized in response to ionizing radiation may be due in part to the phosphorylation of p53 at Ser18 in these cells in response to irradiation (Canman et al., 1998 Lees-Miller et al., 1992 Shieh et al., 1997). At 32°C, the temperature at which p53 is phosphorylated at Serine 18 in A1-5 cells, no phosphorylation was observed at this residue in the p53 in ALTR 9 cells. When Kubbutat and Vousden deleted the mouse equivalent amino acids 13-22 of the human p53, the protein was completely resistant to calpain mediated degradation suggesting this to be the calpain cleavage site (Kubbutat & Vousden, 1997). It is conceivable that phosphorylation of p53 at Serine 18 (Serine 15 for human p53) could therefore, also render p53 resistant to calpain cleavage. When they replaced serine 15 residue of human p53 with Aspartate, they found no difference in the protein's susceptibility to calpain cleavage in their *in vitro* assay system. However, substitution to Aspartate does not fully mimic phosphorylation of p53 at a serine residue and therefore it is possible that resistance to calpain cleavage could not be observed. Our results are consistent with this notion and suggest that phosphorylation of p53 at this residue can abrogate calpain mediated cleavage. Since phosphorylation of p53 at Serine 18 is regulated by stress, our results also suggest that degradation of p53 by calpain is an important event that can also be regulated by stress responses. Since these cells were selected for growth at 32°C, the permissive temperature for p53 function, they survived by acquiring a mutation that resulted in elimination of p53 phosphorylation at Serine 18 normally induced by the shift

to 32°C. This lack of phosphorylation may have led to more efficient cleavage by calpain leading to the inactivation of p53. However, phosphorylation of p53 at the Serine 18 residue can be stimulated by alternative stress regulated signaling pathways such as IR and results in p53 protein that is resistant to calpain cleavage thus allowing for its functional activation.

CHAPTER 4.

Morphologic conversion of a neuroblastoma derived cell line by E6 mediated p53 degradation

Introduction

Neuroblastomas are one of the most common solid tumors found in children (Triche, 1990). In culture, human neuroblastoma cell lines are typically comprised of heterogeneous cellular subpopulations (Biedler et al., 1973; Ross et al., 1983). These include neuroblastic (N-type) and substrate-adherent/Schwannian (S-type) that can be distinguished by their characteristic morphologies and the expression of differentiation-associated antigens. Each of the two different cell types are capable of transdifferentiation or interconversion into the other type (Ross et al., 1983). The N-type cells have properties of noradrenergic or cholinergic neurons while the S-type cells have properties of embryonic Schwann/glial/melanocytic cells of the neural crest (Bernal et al., 1983; Ciccarone et al., 1989). Neuronal differentiation can be induced in vitro in neuroblastoma cells by exposure to a variety of agents, retinoic acid (RA) being the most commonly used compound (Sidell, 1982; Sidell et al., 1983; Sidell & Horn, 1985). Differentiation is associated with an increase in the number and length of neurite extensions as well as an increase in the expression of differentiation markers such as neurofilament (Sidell & Horn, 1985).

Inactivation of p53 protein is observed in over 50% of human tumors and the most common mechanism is gene mutation (Nigro et al., 1989). Other mechanisms of

p53 inactivation include its enhanced degradation by cellular proteins such as mdm2 and by viral proteins such as HPV-16 E6 (Haupt et al., 1997),(Scheffner et al., 1990). Surprisingly, in neuroblastoma-derived tumors, the incidence of p53 gene mutation as the mechanism of p53 inactivation is very low (Castresana et al., 1994; Davidoff et al., 1992; Hosoi et al., 1994). Instead, in these tumors, p53 is physically sequestered in the cytoplasmic compartment of the cell under normal growth conditions (Moll et al., 1995; Moll et al., 1996). The functional role of p53 as a transcription factor requires that it be capable of nuclear translocation therefore suggesting that cytoplasmic sequestration may be very effective in abrogating p53's function in spite of a genotypically wild-type protein. Reports have also shown that this wild-type p53 in neuroblastoma cells is over-expressed due to enhanced stability (Davidoff et al., 1992; Isaacs et al., 1998),(Zaika et al., 1999b). Moll et al (Moll et al., 1995) have correlated such increased levels of wild-type p53 protein with poorly differentiated neuroblastomas. These latter findings are in direct contrast to the known regulatory role p53 plays in the differentiation process in a broad range of cell lineages (Banerjee et al., 1995; Kokunai et al., 1997; Mazzaro et al., 1999; Moretti et al., 1997; Poluha et al., 1997; Soddu et al., 1994; Soddu et al., 1996). Increased expression of p53 protein can induce differentiation in such varied cell types such as glioma, thyroid, hematopoietic and muscle. However, in transgenic mice over-expressing wild-type p53 protein, defects in the differentiation of the ureteric bud were observed suggesting that p53 can also play a negative role in the differentiation process (Godley et al., 1996). That embryonic development is normal in p53 knockout mice suggests that p53 is not required for normal mammalian development. However, p53

function in these mice may be compensated for by the recently discovered p53-related proteins, p63 and p73, members of the p53 family of proteins, found to display overlapping functions (Donehower et al., 1992),(Chen, 1999; Levrero et al., 2000). Recent reports have identified a role for p73 in neuronal differentiation, and p63 in ectodermal differentiation (De Laurenzi et al., 2000; Mills et al., 1999).

In the present study, we demonstrate that p53 can directly influence the differentiation of SK-N-SH cells, a neuroblastoma-derived cell line which expresses wild-type p53 protein that is cytoplasmically sequestered under non-stressed conditions. Normally, these cells are present as a heterogeneous mix of N- and S-type cells. In response to retinoic acid, the N-type cells undergo neuronal differentiation associated with neurite extension and the increased expression of neurofilament protein, a marker specific for neuronal differentiation. Reduction of p53 protein levels by stable introduction of a plasmid encoding the Human Papilloma Virus (HPV)-16 E6 protein resulted in the conversion of SK-N-SH cells from a predominantly N-type cell line to an exclusively S-type cell line which was resistant to RA treatment. Our results suggest that expression of wild-type p53 protein is necessary for the transdifferentiation between N- and S-type cells.

Materials and Methods

Cell Cultures, antibodies used and treatments: The human neuroblastoma cell line SK-N-SH was obtained from the American Type Culture Collection. Cells were grown in DMEM medium containing 15% FBS, 4 mM Sodium Pyruvate, Non-Essential Amino Acids, 2 mM L-Glutamine, 100 U/mL Penicillin and 100 μg/mL Streptomycin. The SK-E6 and SK-mutE6 cell lines were generated in the laboratory, from SK-N-SH cells at passage 36, by transfecting the pCMV-Bam-Neo-E6 and pCMV-Bam-Neo-mutE6 plasmids respectively. Transfections were performed using Lipofectamine (Gibco BRL) and stable clones generated by G418 (500 μg/mL) selection were pooled together and maintained in medium containing 500 μg/mL G418.

Antibodies using for Western blotting and immunofluorescent analysis include: anti-p53 (PAb 421, provided by Dr. Levine); anti-p21 (Ab-6, Oncogene Science); anti-Neurofilament (M+H, Ventana Medical Systems); and anti-Vimentin (Dako Corp.).

SK-N-SH, SK-E6 and SK-mutE6 cells were plated in 60 mm^2 tissue culture plates at a cell density of 2×10^5 cells per plate. After 48 hours the cells were treated for 7 days (refed every 3rd day) with 5 μM all-trans retinoic acid (Sigma) and allowed to differentiate. Cells were exposed to 5 Gy Ionizing radiation using a ^{60}Co source (Atomic Energy of Canada) with an average dose of 60 cGy/min, and 25 μM Menadione (Sigma).

Generation of plasmids: The wild-type HPV-16 E6 (p1221) and mutant E6 (p2022) plasmids were obtained from P. M. Howley (Mietz et al., 1992). The mutant E6 plasmid

has amino acid substitutions at residues 7 (Arg → Ser), 8 (pro → Ala) and 9 (Arg → Thr). The full length E6 and mutant E6 inserts were amplified by PCR using 5'-ACGGATCCATGTTTCAGGACCCACAGGAG-3' as the forward primer and 5'-CGGATCCTTACAGCTGGGTTTCTCTACG-3' as the reverse primer and the products cloned into the pCMV-BAM-NEO vector. Appropriate cloning of both plasmids (pCMV-Neo-E6 and pCMV-Neo-mutE6), was confirmed by automated sequence analysis.

RT-PCR analysis: Reverse transcription of 2 μg total RNA from SK-N-SH, SK-E6 and SK-mutE6 cells was carried out using 0.5 g Oligo(dT)$_{12-18}$, 2 mM dNTP mix, 1 mM DTT, 0.5 mM $MgCl_2$, 10X RT buffer and 50 units Superscript II RT in a 20 μL reaction (Gibco BRL). Full length E6 or mutant E6 was amplified by using 5' - ACGGATCCATGTTTCAGGACCCACAGGAG-3' as the upstream primer and 5'-TCGGATCCTTACAGCTGGGTTTCTCTACG-3' as the reverse primer, to generate a PCR product of 450 bp. PCR conditions were: denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 55°C for 30 sec and 72°C for 1 min. The PCR product was separated on a 1% agarose gel and an image acquired using Stratagene EagleEye II.

Indirect Immunofluorescence: Cells were plated onto coverslips at a density of 1 X 10^5 cells per 35 mm^2 plate. Forty-eight hours after plating, 5 μM RA was added to the plates and cells incubated for 7 days. In untreated cells, the cells were harvested 48 H after

plating onto coverslips to ensure cell attachment. Coverslips were harvested by rinsing 2 X in PBS (containing 3 mM KCl, 1 mM KH_2PO_4, 0.2 mM $MgCl_2$, 137 mM NaCl, and 8 mM Na_2HPO_4, pH 7.5) and incubating in ice cold Methanol : Acetone (1:1) for 5 min before air drying. When coverslips were ready to be stained, cells were rehydrated in PBS for 10 min, and blocked in PBS containing 0.1% BSA for 10 min. Primary and secondary antibody reactions were done at room temperature for 1 hour and 30 min respectively and the antibodies were dissolved in PBS containing 0.1% BSA. After the secondary antibody was applied, nuclei were counterstained with DAPI for 5 min and the coverslips rinsed twice. Secondary antibodies used were Goat-antiMouse Cy3 and Goat-antiMouse FITC conjugates (Jackson Immunoresearch).

Western Blot Analysis: Protein expression was determined by Western Blot analysis using the Laemmli method. Briefly, cells were lysed in Lysis buffer (50 mM Tris-Cl (pH 8.0), 5mM EDTA, 150 mM NaCl, .5% NP-40, 1mM PMSF, and 1 mM aprotinin, leupeptin and pepstatin A) (Sigma Chemical Co.) and protein concentration determined using DC Protein Assay kit (BioRad). Fifty μg protein was loaded per lane and resolved on a 10% acrylamide gel followed by transfer onto Nitrocellulose membrane (Pall Gelman Corp.). The membrane was blocked overnight in Blotto (TBS containing 0.05% Tween-20 and 5% Milk (BioRad Corp). Primary antibody reaction was carried out at room temperature for 1.5 H and secondary antibody reaction for 45 min. Chemiluminescence was observed using the ECL reagent kit (Amersham Inc.).

Apoptosis Assay using fluorescent dye method: After treatment with apoptosis inducing agents, percent apoptosis was determined using a fluorescent dye staining protocol (-). Briefly, cells were collected from the plates by collecting the floating cells and removing the substrate adherent cells by trypsin. Cells were centrifuged at 2000 rpm for 5 min at 4°C and resuspended in a small volume of medium (approximately 1 ml). From this cell suspension, 10 μl was removed and mixed with 1 μl of fluorescent dye mix containing acridine orange and propidium iodine (Sigma). The acridine orange stains the nuclei green in viable cells and the propidium iodide is excluded. However, in apoptotic or necrotic cells, the nuclei stain red by the propidium iodide and the morphology of the nucleus easily determines if the cell is apoptotic or necrotic. A total of 200 cells were counted per time point and percent apoptosis determined.

Soft agar colony forming assay: A stock 1.2% LMP agarose (Gibco BRL) was autoclaved and maintained at 37°C overnight to allow the temperature to equilibrate. Two milliliters of a 1:1 solution of LMP agarose and DMEM medium (supplemented as described above) was poured into each well of a 6-well plate and the basal layer was allowed to solidify for 5 min at 4°C and equilibrated at room temperature for 30 min. The top layer was similar to the basal layer but contained 4000 cells per well. The top layer was allowed to solidify at room temperature for approximately 15 min and the plates transferred to a 37°C incubator with 5% CO_2. The following day, 1 ml of medium was added to each well, and the wells refed every 3-4 days for 2 weeks. Two sets of

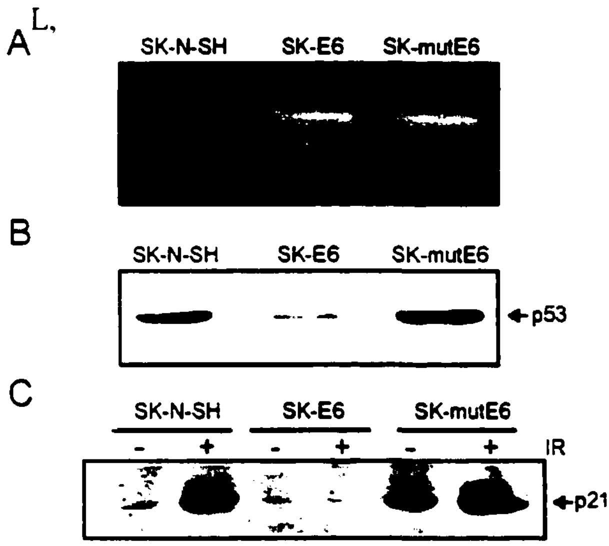
experiments were performed, each in triplicate. The total number of cells and colonies were counted and percent colony formation determined.

Results

Elimination of p53 protein by introduction of HPV-16 E6

A role for p53 in cell differentiation has been indicated in many different cell types. In SK-N-SH cells, the p53 has been previously sequenced and found to be wild-type (Davidoff et al., 1992). To determine if p53 is involved in the differentiation response of neuroblastoma cells, we inactivated it by enhancing its degradation by introducing the human Papilloma Virus–16 (HPV) E6 protein. SK-N-SH cells were stably transfected with either the HPV-E6 expressing plasmid shown previously to degrade wild-type p53 protein, or a mutant E6 plasmid that is similar to the wild-type E6 except for a 3 amino acid substitution (residues 7,8 and 9) in the conserved region of the E6 protein which makes it non-oncogenic by abrogating its ability to interact with p53 (Mietz et al., 1992). Colonies resulting from the transfection were pooled together and the entire pooled population was maintained in 500 μg/mL G418 to ensure Neomycin resistance. RT-PCR analysis (Figure 19 A), using primers specific for E6, was used to confirm the expression of E6 and mutant E6 in the transfected cell populations. The relative level of p53 protein was determined by Western blotting. As expected, E6 transfected SK-N-SH cells (SK-E6) had reduced levels of p53 protein compared with the parent SK-N-SH cells and the mutant E6 transfected cells (SK-mutE6) (Figure 19 B).

Previous reports have shown that neuroblastoma cells are capable of activating p53 in response to stress signals and inducing either growth arrest by p21 induction, an inhibitor of cyclin dependent kinase activity, or apoptosis in a p53-dependent manner


A
B
C
SK-N-SH
SK-E6
SK-mutE6
p53
IR
p21

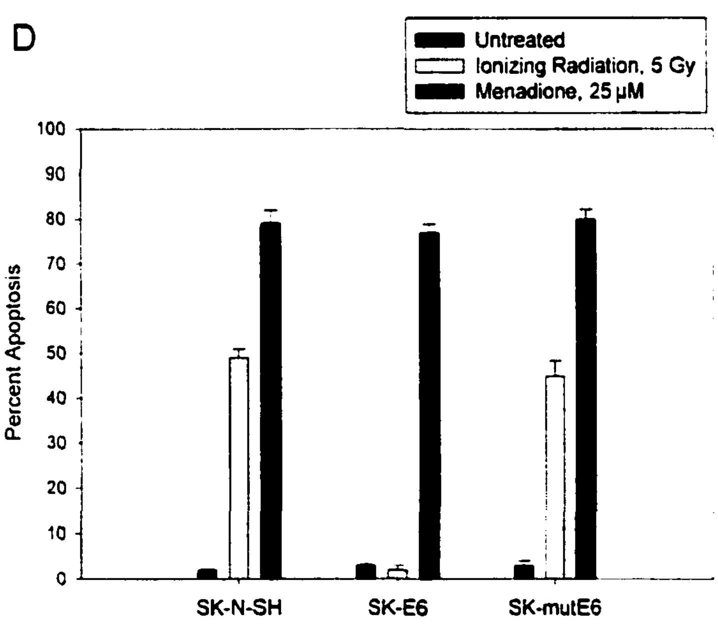

D
Untreated
Ionizing Radiation, 5 Gy
Menadione, 25 µM
Percent Apoptosis
100
90
80
70
60
50
40
30
20
10
0
SK-N-SH
SK-E6
SK-mutE6

Figure 19. Characterization of SK-N-SH cells transfected with wild-type or mutant HPV E6. SK-N-SH cells were transfected with either wild-type HPV E6 or mutant E6 expressing plasmids and stably transfected colonies were pooled together and maintained in G418 containing medium. **Panel A.** RT-PCR was performed on the pooled populations using primers specific for HPV E6 and the PCR products resolved on a 1% agarose gel containing ethidium bromide to stain DNA. **Panel B.** Expression of p53 protein was determined by Western blotting of 50 μg total cell protein extracts obtained from parent SK-N-SH, E6 transfected (SK-E6) and mutant E6 (SK-mutE6) transfected SK-N-SH cells with a p53-specific antibody (DO-1). **Panel C.** SK-N-SH, SK-E6 and SK-mutE6 cells were exposed to 5 Gy ionizing radiation (IR) and the level of p21 protein induction 12 hours after exposure was determined by Western analysis. **Panel D.** The apoptotic response of SK-N-SH, SK-E6 and SK-mutE6 cells to 5 Gy Ionizing radiation and 25 μM Menadione was determined 12 H after treatment. Apoptosis was quantitated using a fluorescent dye method, which stains viable cell nuclei green and apoptotic cell nuclei red. A total of 200 cells were counted for each time point and the experiment was repeated two times in duplicate.

(Goldman et al., 1996; McKenzie et al., 1999). To determine if the p53 protein in the E6 transfected cells could be functionally activated in response to genotoxic stress, we exposed the cells to 5 Gy ionizing radiation and compared the level of p21 induction and apoptosis in these cells to the parent SK-N-SH cells and the mutant E6 transfected cells. We observed a lack of p21 induction in the E6 transfected cells after exposure to Ionizing radiation as well as a complete abrogation of the apoptotic response in these cells (Figure 19 C and D). When exposed to Menadione, a vitamin K3 analogue which induces apoptosis in cells in a fas-dependent and p53-independent manner (Caricchio et al., 1999), all three cell lines responded by undergoing approximately 80% apoptosis within 12 hours indicating that the effects of E6 were specific to p53. Our data indicated that p53 protein levels as well as functionality had been successfully down regulated by E6 overexpression.

Reduction of p53 protein levels in SK-N-SH cells affects cell morphology

During the characterization of SK-N-SH cells transfected with the E6 plasmid, we noticed a complete change in the morphology of these cells. The E6 transfected cells (SK-E6), expressing low levels of p53 protein, were exclusively S-type as judged by morphology compared to the parent SK-N-SH cells and SK-mutE6 E6 cells both of which expressed approximately 90% N-type and 10% S-type cells in their population (Figure 20).

We further characterized this change in morphology by Western blot analysis as well as by indirect immunofluorescence using vimentin and neurofilament as specific

markers for the two cell types. As shown in Figure 21 A and B, we were unable to detect the expression of neurofilament protein in SK-E6 cells by both Western analysis as well as immunofluorescent staining. Interestingly, in the parent SK-N-SH cell population as well as the mutant E6 transfected cells, one can observe S-type cells in the light micrographs, which also do not express any neurofilament protein (Figure 21 B). Hence, consistent with previous reports, S-type cells in both SK-N-SH and SK-derived populations lack neurofilament expression. Vimentin protein expression was slightly increased in SK-E6 cells compared to the parent and SK-mutE6 transfected cells as judged by western analysis (Figure 21 C). Indirect immunofluorescence demonstrated that all the cells in the SK-E6 cell population expressed vimentin. However, in the parent SK-N-SH cells and the SK-mutE6 transfected cells, only a few of the N-type cells in the mixed population expressed vimentin and at low levels compared with the S-type cells of this population (Figure 21 D). Taken together, the Western analyses and immunofluorescent staining data demonstrate that both parent and SK-mutE6 cells are comprised of a mixed population of N- and S-type cells while SK-E6 cells are exclusively S-type in morphology. This phenotype of the E6 transfected cells has persisted up to passage #20.

Differential expression of p53 in N-type versus S-type SK-N-SH cells

To determine if there was differential expression of p53 in N- and S-type cells in the parent SK-N-SH cultures, these two phenotypes were separated based on their differences in substrate adhesion properties and p53 expression determined by Western

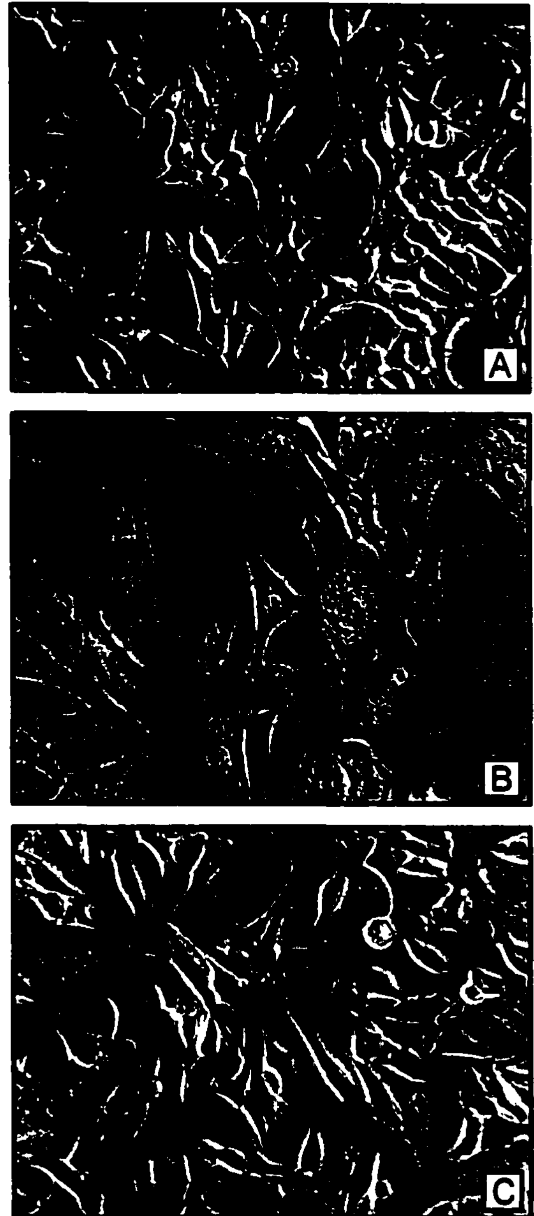


Figure 20. Morphology of parent SK-N-SH cells compared to wild-type E6 and mutant E6 transfected SK-N-SH cells. Panel A, B and C represent phase contrast images of SK-N-SH, SK-E6 and SK-mutE6 cells respectively. All images were acquired at the same magnification using a 10X objective lens.

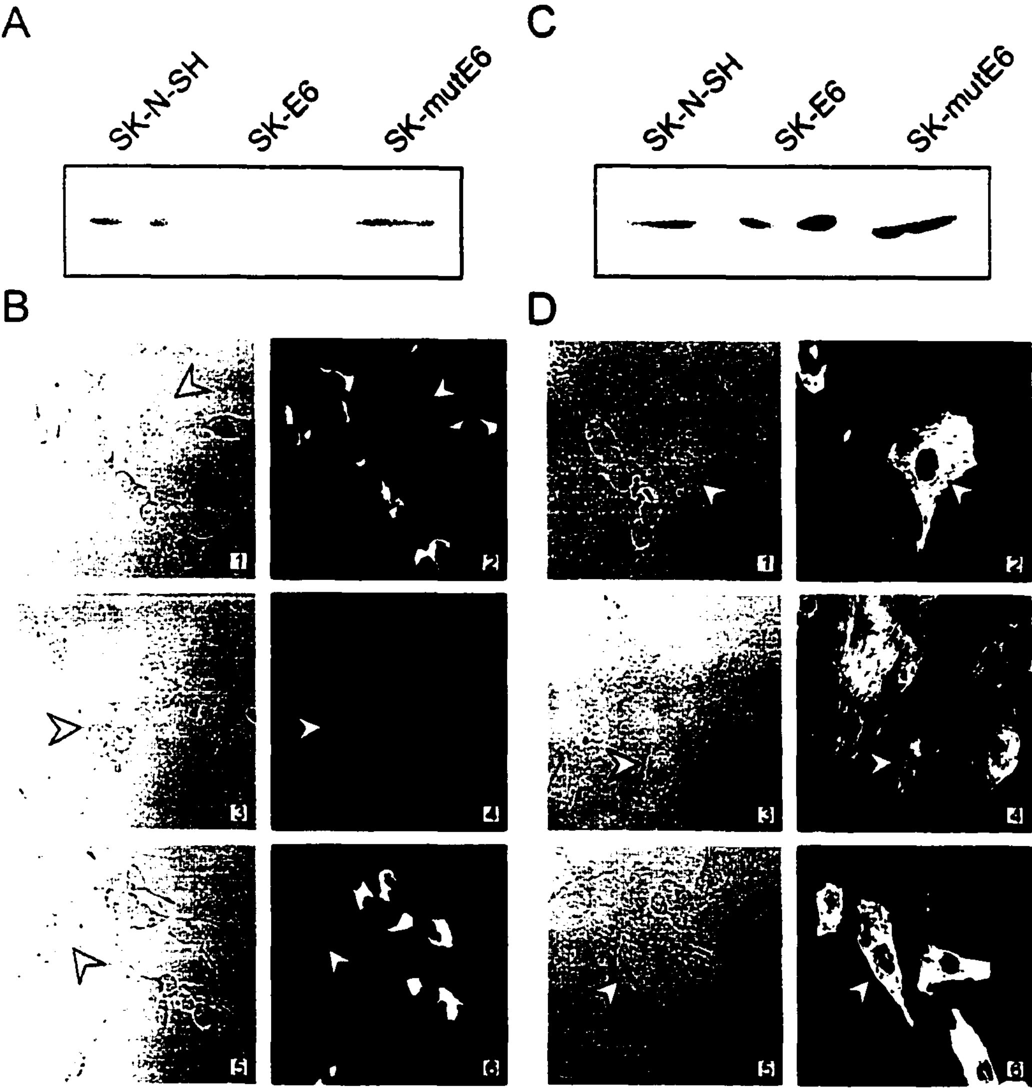

A
SK-N-SH
SK-E6
SK-mutE6
C
SK-N-SH
SK-E6
SK-mutE6
B
D
1
2
3
4
5
6
1
2
3
4
5
6

Figure 21. Characterization of E6 and mutant E6 transfected SK-N-SH cells. Cells were plated on 60 mm^2 dishes and 4 days later harvested and 50 μg of the protein extract was used for Western blot analysis. For immunofluorescence, cells were plated on to coverslips and harvested 48 hours later, fixed and used for staining. **Panel A.** Western blot analysis was performed to determine the expression of neurofilament protein in the total cell extracts obtained from SK-N-SH, SK-E6 and SK-mutE6 cells. **Panel B.** Indirect immunofluorescence was performed on SK-N-SH (**2**), SK-E6 (**4**) and SK-mutE6 (**6**) to determine the expression of neurofilament protein expression and compared with reflective light pictures (**1, 3 and 5** respectively) obtained of the same field by confocal microscopy. **Panel C.** Vimentin protein expression was determined in SK-N-SH, SK-E6 and SK-mutE6 cells by Western blot analysis. **Panel D.** Expression of vimentin in the two different cell types within each cell line was also determined by indirect immunofluorescence using an antibody specific for vimentin. Note N-type cells in SK-N-SH and SK-mutE6 populations also express vimentin. **1** and **2** are reflective and immunofluorescent images of SK-N-SH cells, **3** and **4** are of SK-E6 while **5** and **6** are images of SK-mutE6 cells respectively. All images were taken using a 40X objective and taken at the same brightness and contrast settings for each panel. The white arrow heads in panel B and D are pointed towards S-type cells.

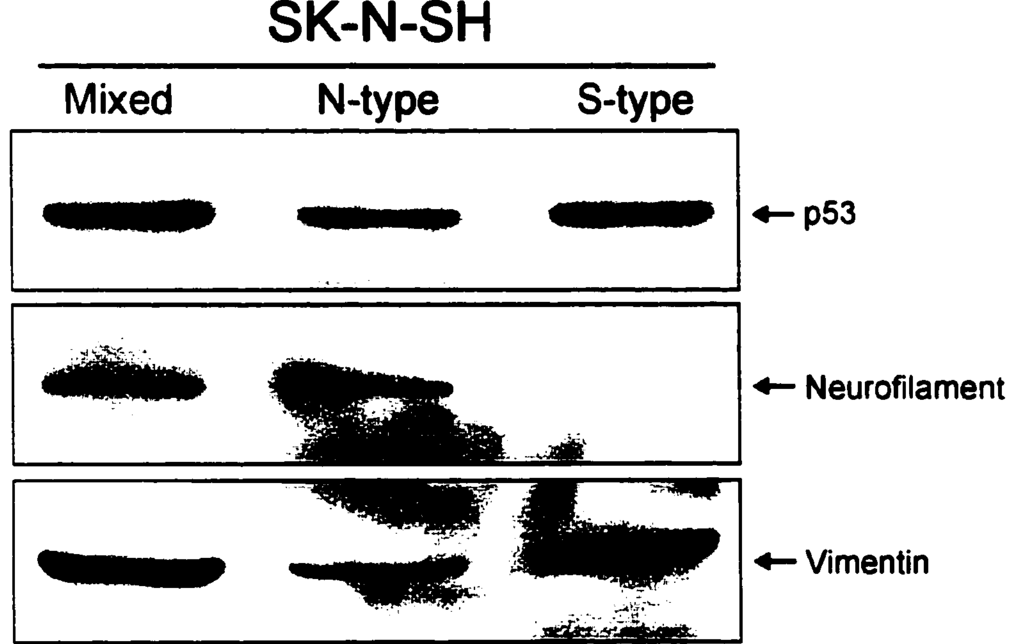


Figure 22. Expression of p53 protein levels between N- and S-type cells derived from SK-N-SH cells. Panel A. Enrichment of the two cell types was achieved by vigourous shaking off of the N-type cells from a mixed population of cells and subsequent culturing. When populations were approximately 99% pure for N-type and 95% pure for S-type, cells were lysed and 50 μg total cell protein was used for Western Blot analysis. The mixed population of cells used for these experiments contained approximately 80 % N-type and 20% S-type cells. Membranes were probed with DO-1, an antibody specific for p53. **Panels B** and **C**. Western analysis of the same extracts was also performed to determine the expression of neurofilament and vimentin respectively.

blotting. Enrichment of the two populations was accomplished by separately reculturing the cells that lost adherence following mild to moderate shaking (enriched for N-type) versus those cells that remained adherent following vigorous shaking (enriched for S-type). This technique was used a total of 3 times to yield cultures that were approximately 99% pure for N- and 95% pure for S-type cells as judged by their microscopic morphology. As shown in Figure 22, Western blotting for p53 indicated that there was an approximately two-fold increase in the level of p53 in the S-type cells compared to the N-type cells. P53 expression in the mixed population of parent SK-N-SH cells was similar to that found in S-type cells. To confirm our enrichment procedure for the cell types, we assessed the expression of two phenotype specific markers. To confirm the N-type morphology, we examined the expression of the neurofilament protein and for the S-type morphology we used vimentin expression as a marker. Figure 22 B and C shows increased expression of neurofilament in the N-type cultures while increased expression of vimentin was observed in cells from the S- versus N-type cultures. These results suggest that the p53 in the two cell types may be differentially regulated.

E6 transfected SK-N-SH cells are refractory to retinoic acid treatment

Previous reports have demonstrated that SK-N-SH neuroblastoma cells can undergo neuronal differentiation in response to treatment with RA as reflected by formation of neurite projections and increased expression of differentiation markers such as neurofilament expression. Figure 23 demonstrates the induced increase in neurite

projections when SK-N-SH cells were treated with 5 μM RA for 1 week. A marked increase in the number of neurite projections from cells as well as an overall increase in the length of these projections was observed, indicative of neuronal differentiation of these cells in response to RA treatment. However, the morphologic transformation was not complete since examination by microscopy revealed that S-type cells were still present in the RA-treated population. The number of S-type cells in the basal layer increased with time indicating that these cells were still growing, albeit slowly. Hence, RA appeared to be causing the N-type cells to differentiate and cease proliferation but had little or no effect on the morphology of the S-type cells.

To determine if SK-E6 cells were capable of responding to RA and transdifferentiate into N-type cells we exposed them to 5 μM RA for 1 week and characterized their morphological response as well as the expression of neurofilament protein. Light micrographs showed an increase in neurite extensions in the parent SK-N-SH cells and SK-mutE6 transfected cells along with an increase, by western blot analysis, in the expression of neurofilament protein in these cells in response to RA treatment (Figure 24 A and B). In contrast, no neurite extensions or expression of neurofilament protein was observed in the E6 transfected cells. No differences in the level of p53 protein expression were observed by Western analysis in SK-N-SH or SK-mutE6 transfected cells after RA treatment (data not shown). Over the course of the RA treatment, we observed an increase in overall the number of SK-E6 cells as well as an increase in the number of S-type cells growing below the N-type population in SK-N-SH

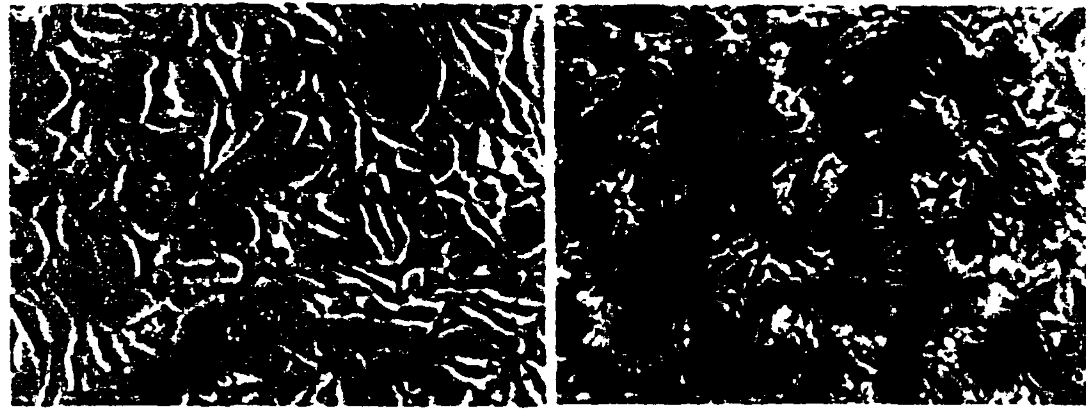
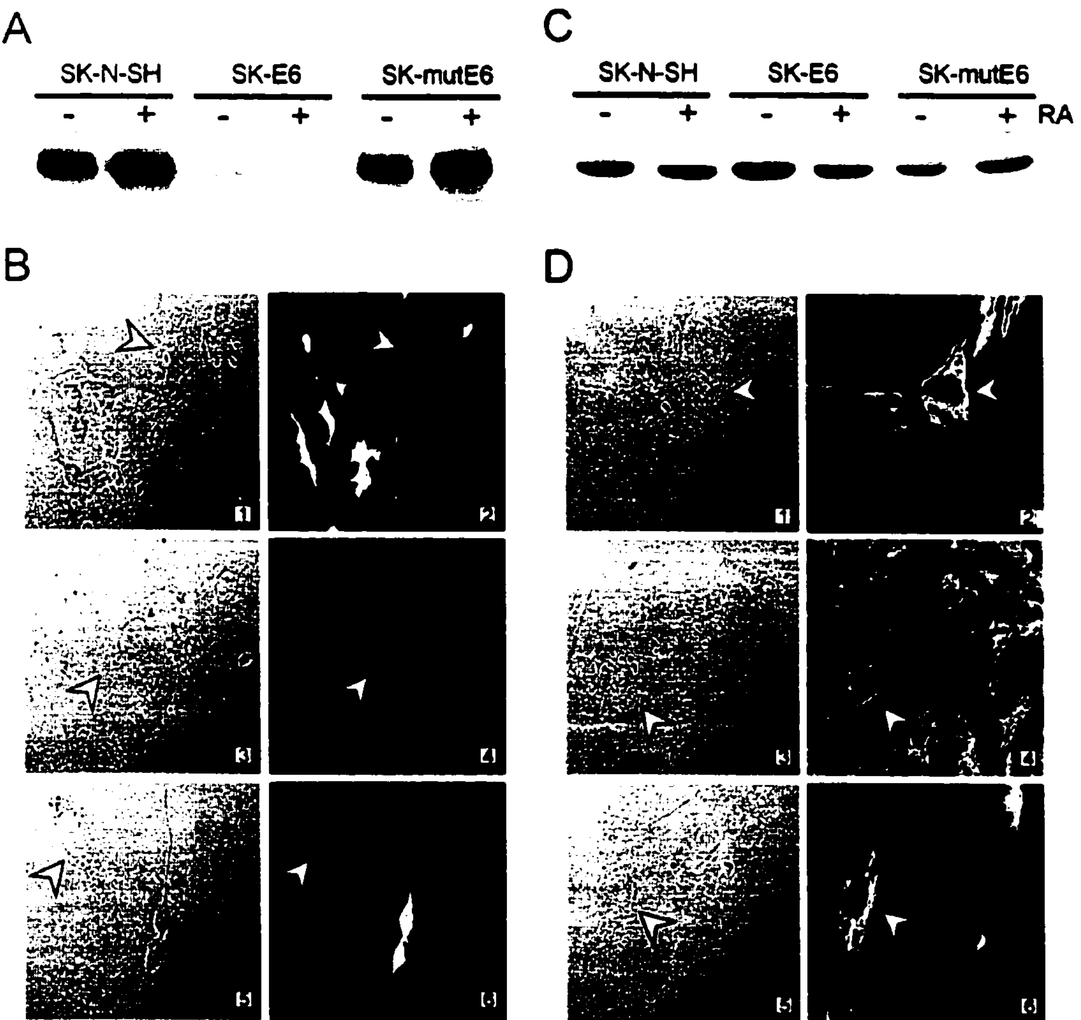


Figure 23. Response of SK-N-SH cells to RA treatment. SK-N-SH cells were treated with 5 μM RA for 1 week and the morphological response determined by microscopy. **Panel A** and **B** represent phase contrast micrographs of SK-N-SH before and after RA treatment respectively. The arrow in B points towards neurite extensions while the white arrow heads in both panels point to the S-type cells present in both untreated and treated populations.



A
SK-N-SH
SK-E6
SK-mutE6
- + - + - +
B
C
SK-N-SH
SK-E6
SK-mutE6
- + - + - + RA
D

Figure 24. Characterization of response of SK-N-SH, SK-E6 and SK-mutE6 cells to RA treatment. After 1 week of 5μM RA treatment, cells were harvested and protein extracts used to determine neurofilament and vimentin expression by Western blot analysis. Untreated cells were harvested after 4 days due to confluency of the plates. For immunofluorescent staining, cells were plated onto coverslips and 48 hours later 5μM RA was added. Coverslips were harvested 1 week later and indirect immunofluorescence was performed to determine the level of neurofilament and vimentin expression. **Panel A**. Neurofilament protein expression in cells before and after treatment with RA. **Panel B** shows the expression of neurofilament in the 3 different cell lines by immunofluorescent staining. **1** and **2** are reflective light and immunofluorescent confocal images of RA treated SK-N-SH cells respectively. **3** and **4** are confocal images of SK-E6 cells while **5** and **6** are confocal images of SK-mutE6 cells after RA treatment. Note the expression of neurofilament protein in the neurite extensions after RA treatment, not observed in Figure 4 B 1 and 2. **Panel C**. Expression of vimentin was determined by Western analysis in the 3 cell lines before and after RA treatment. **Panel D**. Expression of vimentin was also determined by indirect immunofluorescence in the 3 cell types. **1** and **2** are reflective light and immunofluorescent images of SK-N-SH cells after treatment with RA respectively. **3** and **4** are images of SK-E6 cells while **5** and **6** are images of SK-mutE6 cells after treatment with RA. White arrowheads in panels B and D point to S-type cells within the cell population. All images were taken using a 40X objective lens and are at the same brightness and contrast settings within each panel.

and SK-mutE6 cultures again suggesting that the S-type cells are resistant to the antiproliferative effects of RA (Figure 24 C and D). We conclude from these experiments that SK-E6 cells are resistant to the antiproliferative effects of RA treatment as are the S-type cells of the parent SK-N-SH population.

E6 transfected SK-N-SH cells lack anchorage independent growth

Previous studies have shown that S-type cells isolated from the mixed neuroblastoma cell population are less tumorigenic based on growth properties in soft agar assays and tumor formation in nude mice (La Quaglia & Manchester, 1996). To further test if the E6 transfected SK-N-SH cells also displayed similar characteristics as the S-type cells clonally expanded from mixed neuroblastoma populations we compared their colony forming ability in 0.6% soft agar to the parent and the mutant E6 transfected SK-N-SH cells. Consistent with previous reports of the S-type cells, the E6 transfected cells were also unable to form colonies in soft agar assays when compared with the parent and mutant E6 transfected SK-N-SH cells, both of which are predominantly N-type (Table 3). These results indicate that the outcome of the reduction of p53 in the E6 transfected cells affects characteristics other than morphology including the ability to exhibit anchorage independent growth.

Table 3. Anchorage independent growth properties of SK-N-SH, SK-E6 and SK-mutE6 cells assessed by colony formation in soft agar.

Cell line	Percent Colony Formation in 0.6% soft agar
SK-N-SH	73.7 ± 5.6
SK-E6	4.6 ± 3
SK-mutE6	71.8 ± 4.6

Discussion

Our results indicate that p53 plays an important role in the transdifferentiation process of neuroblastoma cells. SK-N-SH cells have elevated levels of wild-type p53 protein (Davidoff et al., 1992). Inactivation of this p53 by HPV-16 E6 mediated degradation led to a striking change in the phenotype of these cells. SK-E6 cells adopted a more flattened fibroblast-like morphology and showed increased vimentin and decreased neurofilament expression characteristic of transdifferentiation from an N- to S-type lineage

Such S-type cells have been shown to spontaneously occur in N-type populations and it has been suggested that both cell types share a common heritage (Biedler et al., 1988; Ross et al.. 1983). Although these spontaneously occurring S-type cells and those that are induced by HPV-E6 transfection appear phenotypically identical, we have determined that the S-type resulting from the E6 transfection show marked down regulation of p53 while the S-type cells derived from spontaneous transformation of SK-N-SH cells actually show an increase in the levels of p53 relative to the N-type cells. Thus, it appears that the S-type morphology can occur regardless of the levels of p53 protein present. Such a phenomenon would be predicted if other events, in addition to p53 expression might be involved. Nevertheless, the SK-E6 cells have one critical difference that distinguishes them from the spontaneously derived S-type cells; they lack the capacity to transdifferentiate from S- to N-type cells. This is supported by the observation that the phenotype is stable in that no N-type cells have appeared in the SK-

E6 cell population over several passages. This strongly suggests that the N-type morphology requires the presence of p53 protein.

Previously it was reported that a reduction in the level of p53 protein occurs after RA treatment of neuroblastoma cells (Sidell & Koeffler, 1988; Tieu et al., 1999). However, in our hands neither the parent SK-N-SH nor the SK-mutE6 cells exhibited a change in p53 levels after RA treatment. This may be due to the presence of S-type cells in the population that are resistant to the antiproliferative effects of RA and that have elevated levels of p53, which compensates for the low levels of p53 in the N-type cells which are initially predominant in the cultures. Induction of differentiation by RA in the parent SK-N-SH cells resulted in neuronal differentiation of the N-type cells while the S-type cells formed a basal layer of flattened fibroblast-like cells that did not possess the morphological characteristics of neuronal differentiation. Also, we did not observe an increase in the expression of neurite-like projections or neurofilament protein in E6-transfected S-type cells similar to S-type cells present in the parent SK-N-SH population. The fact that spontaneous S-type cells showed elevated p53 levels in comparison to N-type cells while E6-derived S-type cultures showed reduced p53 levels suggests that the level of p53 expression does not play a role in the RA resistance exhibited by cells of the S phenotype. Thus, our results with the E6 transfected SK-N-SH cells did not support the results observed by Schlett and Madarasz, who demonstrated neuronal differentiation by RA in neuroepithelial cell lines generated from mice lacking functional p53 (Schlett & Madarasz, 1997). The difference in outcome between our experiments and those reported by Schlett and Madarasz may be due in part to the fact that their cells were derived from

cerebral vesicles of mouse embryos lacking functional p53 whereas, the SK-N-SH cells are neuroblastoma cells derived from a bone marrow metastasis and presumably have additional genetic alterations.

When tested for their ability to form colonies in soft agar assays, the E6 transfected cells were unable to grow in an anchorage independent manner. Anchorage independent growth of cells has been used classically as a marker for cell tumorigenicity along with the ability to form tumors in nude mice. As such, our results suggest that reduction of wild-type p53 protein expression leads to a less tumorigenic cell type. In light of p53's role as a tumor suppressor protein, these findings are contradictory to what might be expected in cells in which wild type p53 levels are reduced. One possible explanation for these results is suggested by what is known about the oncogenic mechanism of mutant p53 (Zambetti & Levine, 1993; Zambetti et al., 1992b). The role of mutant p53 as an oncogene is partly due to its dominant negative behavior and subsequent inactivation of wild-type p53. However, mutant p53 has also been shown to behave as an oncogene in cells lacking the expression of wild-type p53, indicating oncogenic effects of mutant p53 independent of its dominant negative interactions. In this regard, over-expressed mutant p53 protein that is cytoplasmically sequestered can cause cells lacking wild-type p53 to become tumorigenic in nude mice (Dittmer et al., 1993). Given these observations, it is tempting to speculate that the wild-type cytoplasmically sequestered p53 protein observed at a high incidence in neuroblastoma cells could be playing a role similar to mutant p53 by enhancing tumorigenic potential. Our lab will investigate this possibility in more detail in future experiments.

CHAPTER 5

Discussion and Perspectives

The progression of a normal cell to a cancerous one involves the inactivation of multiple different genes suggesting that the tumorigenesis process is one that involves multiple steps. Systematic analysis of genetic changes involved during the evolution of colon cancer cells has confirmed that this process involves the inactivation of several different genes involved in several different pathways (Vogelstein et al., 1988). In addition, the incidence of cancer as a function of age also suggests that multiple genetic changes are required for tumorigenesis to occur. Cancer cells differ from normal cells in several different important characteristics such as the loss of differentiation, increased invasiveness and decreased drug sensitivity, suggesting that a process of cellular evolution and not just uncontrolled cell proliferation is important in the development of tumors. During multi-stage tumorigenesis, inactivation of p53 by mutation or other means is believed to be a common and requisite step in this process (Sherr, 1996).

The significant role p53 plays in tumor development is represented by the high incidence of tumors in which the p53 gene is mutated. Over 50% of all human tumors have mutations in the gene that codes for the tumor suppressor protein p53, while in some tumor types such as colon tumors, over 80% of the tumors have mutations in p53 (Baker et al., 1989; Hollstein et al., 1991; Nigro et al., 1989; Vogelstein et al., 1988). Interestingly, other tumor types such as neuroblastomas exist in which the incidence of p53 mutation is very low (Davidoff et al., 1992; Moll et al., 1995). However, in these

latter tumors, it is believed that other mechanisms of p53 inactivation exist. In these tumors, and in some breast and colon tumors in which the p53 is wild-type, the p53 has been found to be physically sequestered in the cytoplasmic compartment of the cell (Davidoff et al., 1992; Moll et al., 1995; Moll et al., 1992). The third mechanism by which p53 inactivation occurs is by increased degradation of the protein by either the cellular oncogene mdm2 or by viral oncogenes such as the human Papilloma Virus-16/18 E6 protein (Huibregtse & Beaudenon, 1996; Mietz et al., 1992; Momand et al., 1998). Both oncogenes work by ubiquitinating p53 protein rapidly and thereby targeting it for proteasome mediated degradation. For the cellular protein mdm2 to act as an oncogene, it has to be over expressed in the cell, which is usually observed due to gene amplification. Potential other mechanisms of p53 inactivation exist which have not been identified as yet. All of these observations reveal that p53 inactivation is critical for tumor development and that inactivation of p53 can occur by at least the three distinct mechanisms described above: mutation, cytoplasmic sequestration and enhanced degradation.

The ALTR cell system

The ALTR system was developed to understand the steps involved in the regulation of p53, and through that, pathways other than those described above that can also lead to p53 functional inactivation. Since the ALTR cell system was selected under conditions that required inactivation of functional p53, the resulting genetic screen composed of the panel of ALTR cell lines is a powerful tool in understanding the

mechanisms by which p53 is regulated. We have already gathered a lot of important information about p53 regulation from the ALTR cell system. Postulated defects in some of the ALTR cell lines have been schematically represented in Figure 25.

Similar to tumors, we observed p53 to be inactivated by gene mutation in approximately 50% of the ALTR cells. Part of the rationale in selecting A1-5 cells to generate the ALTR system genetic screen was that A1-5 cells had multiple copies of the temperature-senstive p53 plasmid transfected into them. This would circumvent mutation of p53 as the mechanism of its inactivation and allow the inactivation of genes involved in the activation of p53 to preferentially occur, thus allowing for the growth of colonies at 32°C. Since we observed the existence of mutations in the p53 protein in some of our ALTR cell lines, our data suggest that only a single copy of the temperature-sensitive p53 plasmid is being expressed in these cells. Silencing of the other copies of the p53 plasmid may have occurred during the generation of the ALTR cell lines or our initial rationale was based on incorrect information and A1-5 cells have been transfected with only a single copy of the temperature-sensitive p53 plasmid which is actually being expressed.

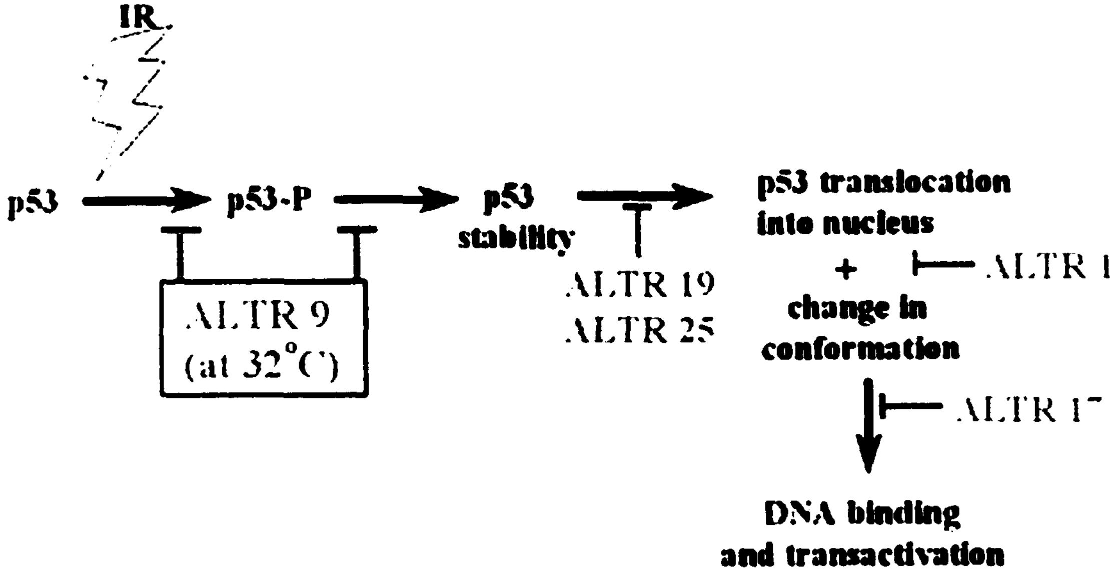


Figure 25. Postulated defects in some of the ALTR cell lines studied.

The mutations identified in all of the ALTR cells have been found located in the DNA binding region of p53, once again relating back to the observations in tumors in which the same is observed. More interesting though is the fact that since the p53 mutation rate in the ALTR cells coincides with what is observed in tumors, it also suggests that in tumors in which p53 is wild-type by sequencing, the p53 pathway is still inactivated through other mechanisms. Often times in a tumor, this inactivation may occur downstream of p53, however, in the 6 ALTR cell lines tested (ALTRs 1, 3, 9, 17, 19 and 25), of which 3 had mutations in p53, the other 3 cell lines (ALTR 1, 17 and 25) were found to have p53 that was incapable of binding DNA or transactivating a reporter construct under the control of a p53 response element suggesting that inactivation of p53 occurred upstream to DNA binding in these cells. These results were consistent with our rationale for developing this system, since we wanted to preferentially inactivate genes involved in the activation of p53 rather than downstream effector genes of p53 such as p21. It will be interesting to determine if inactivation of genes involved in the activation of p53 is also observed in other ALTR cell lines in which the p53 is wild-type. The results with the 6 cell lines tested however, do indicate that besides gene mutation, the steps involved upstream of p53 regulation are also important in inactivation leading to a nonfunctional p53 protein.

Subcellular localization of p53 in ALTR cells

One of the most striking observations in the ALTR cell system was the frequency of cytoplasmically localized p53 in the ALTR cells at 32°C. At 39°C, all the ALTR cell lines had p53 localized to the cytoplasm similar to A1-5 cells at this temperature. However, shifting temperature to 32°C, which causes the p53 in A1-5 cells to translocate into the nucleus, did not lead to a similar translocation of p53 into the nucleus in most of the ALTR cell lines. These results suggested that cytoplasmic sequestration of p53 was an effective and important mechanism of p53 inactivation in these cells. This has been observed previously in tumors in which the p53 is wild-type, such as neuroblastomas and breast and colon tumors. However, in the ALTR cells, the genotype of the p53 in these cells did not correlate with their subcellular localization; e.g., ALTR 9 and ALTR 17 cells have nuclear localized p53 but the former cell line has a mutation in the DNA binding domain of p53. In addition, ALTRs 1, 19 and 25 have cytoplasmically localized p53, but only the p53 in ALTR 19 cells is mutated in the DNA binding region. Previously, it was believed that the point mutation at residue 135 of the temperature sensitive p53 was important in determining the conformation and hence the localization of the protein. Thermodynamic instability of the protein at 39°C led to an inability of the protein to fold into the wild-type conformation and the drop in temperature to 32°C allowed appropriate folding in spite of the point mutation. Our results contradict this notion since the p53 in ALTR 1 and ALTR 25 cells has not acquired any additional mutations and is still maintained in a mutant conformation in the cytoplasm at 32°C. These findings suggested that some other modification to p53 or another protein

determined the localization pattern of p53 in these cells and not the genotype of the protein.

The increased incidence of cytoplasmic sequestration of p53 in the ALTR cells at 32°C does however, indicate that there are many steps involved in the regulation of nuclear localization of p53. If fewer steps were necessary for p53 translocation and maintenance in the nucleus, we would probably not have observed over 70% of ALTR cell lines in which the p53 was cytoplasmically localized at 32°C. In a normal cell, p53 shuttles into and out of the nucleus in the absence of a stress signal. The signaling mechanism that leads to its import are not clearly understood, but its export is mediated through p53's association with mdm2, which in turn associates with CRM1, a protein involved in the nuclear export machinery (Freedman & Levine, 1998; Henderson & Eleftheriou, 2000). Such nuclear export of p53, when exaggerated, can be an indirect mechanism that also leads to a p53 protein that appears to be cytoplasmically 'sequestered' (Stommel et al., 1999). The mechanisms involved in this hyperactive export of p53 still remain to be elucidated but may involve the leucine rich nuclear export signal present in the tetramerization domain of p53. It is possible that this mechanism of p53 inactivation is present in a few of the ALTR cell lines in which the protein appears to be cytoplasmically sequestered and remains to be investigated.

We were able to trap p53 in the nucleus of A1-5 cells at 39°C as well as ALTR 1 cells but not in ALTR 25 cells at 32°C by Leptomycin B, a specific inhibitor of CRM1 function (data not shown). In A1-5 cells at 39°C, the ability to trap p53 in the nucleus in the absence of a stress response indicates normal shuttling of p53. The fact that we

observe the same result in ALTR 1 cells however, suggests that the nuclear import machinery is intact in these cells, as also evidenced by their ability to translocate and remain in the nucleus by Ionizing radiation as well as UltraViolet light, suggesting that the defect in these cells may be due to an inability of the protein to be maintained in the nucleus in response to the temperature shift. Since we were unable to trap p53 in the nucleus in ALTR 25 cells after treatment to Leptomycin B as well as exposure to IR or UV, the defect in these cells appears to be due to an inability of the p53 in these cells to be translocated into the nucleus. These results, taken together, suggest that we have two distinct classes of ALTR cell lines in which the p53 is cytoplasmically sequestered. In one class, the p53 is capable of translocating into the nucleus in response to stresses other than temperature shift while in the second class, the p53 is not able to translocate into the nucleus in response to any types of stress signals tested so far.

Conformation of p53 in ALTR cells correlates with subcellular localization

The conformation of p53 was also found to be important in its regulation. We observed a strong correlation between the conformation of p53 and its subcellular localization. Mutations in p53 did not correlate with the protein's tertiary structure since we found ALTR cells such as ALTR 9, in which p53 was nuclear localized and in a wild-type conformation but also had acquired an additional mutation in its DNA binding region. The p53 protein in A1-5 cells also represents a similar situation, in that it is in the mutant conformation when in the cytoplasm, but when induced to go into the nucleus by either temperature shift or exposure to Ionizing radiation, there is an increase in p53's wild-type conformation. The close relationship between subcellular localization and

conformation of p53 initially suggested to us that a single post-translational modification was responsible for both nuclear translocation of p53 and its change in conformation to wild-type. However, our results with the ALTR 1 cells and their p53 response to IR suggested that the two events were, in fact, independent of each other. In these cells, we observed p53 translocation into the nucleus immediately following IR, but we did not observe a corresponding change in the conformation of the protein to wild-type. These results suggest that translocation of p53 into the nucleus can occur without a change in the protein conformation. Also, since we have not observed wild-type conformation of p53 in a single ALTR cell line in which the p53 is cytoplasmically localized, we believe that translocation into the nucleus must occur before the protein can undergo a change in its conformation to wild-type. The presence of p53 in the nucleus does not cause it to, by default, assume a wild-type conformation either, based on the results obtained in ALTR 1 cells. These results then, also suggest that two independent post-translational modifications must occur to p53 for the resultant events of nuclear translocation and change in conformation to wild-type to occur. These modifications could include but are not restricted to phosphorylation, dephosphorylation, and acetylation of p53, which could then lead to changes in protein-protein interactions and the conformation of p53 itself. Dissecting the pathways that are responsible for p53's translocation into the nucleus and its change in conformation will be the scope of future studies in our laboratory, and the panel of ALTR cells, particularly, ALTRs 1, 17 and 25 will be very valuable in determining the modifications involved in these steps of p53 regulation.

Degradation of p53 protein by calpain

When p53 is activated by a stress signal, one of the first steps involved in its activation is its increase in stability. The half-life of human p53 protein dramatically changes from 15-30 min in a normal cell to 3-8 hours in response to stress signals. The rapid turnover of p53 in a normal, unstressed cellular environment is thought to be a multiple step process: p53 shuttles into the nucleus where it associates with mdm2, another cellular protein, which acts as a ubiquitin ligase, the p53 protein then, in association with mdm2 must be exported out of the nucleus, and while in the cytoplasm gets ubiquitinated and subjected to proteasome mediated degradation. In fact, over-expression of mdm2 is a well-defined mechanism of p53 inactivation, since it will not allow stabilization of p53 even under situations of stress. It has been known for a while that p53 could be targeted for degradation by calpain in vitro (Kubbutat & Vousden, 1997; Pariat et al., 1997). This degradation is independent of the subcellular location of p53, as determined by the presence of ubiquitous calpains present in the cell, and the degradation of p53 in vitro by calpain present in nuclear and cytoplasmic fractions of the cell. It was of interest to find calpain mediated degradation as the mechanism of p53 inactivation in ALTR 9 cells.

Interestingly, calpain mediated degradation of p53 could be regulated by post-translational modifications such as phosphorylation at Serine 18 was of even greater interest since it suggests that calpain may also play a role, in addition to p53's association with mdm2, in maintaining the stability of p53 in vivo. The phosphorylation event of p53 at Ser18 has also been shown to be important in p53's ability to associate with

mdm2. The region spanning the mouse equivalent of amino acids 12-20 has been shown to not only be the site of p53's association with mdm2, but also the site at which calpain cleaves p53. This, by association, suggests that if p53 is not already associated with mdm2, it will be cleaved by calpain, unless phosphorylated at Serine 18. This also suggests that there are redundant pathways in place in the cell needed to ensure p53's efficient degradation, unless phosphorylated at the Serine 18 residue, which is indicative of cellular stress. Such stringent control of p53 activity is important in the cell to guarantee inappropriate p53 activation, which would otherwise lead to cell cycle arrest or apoptosis if not tightly regulated.

Our results suggest that the p53 in ALTR 9 cells was inactivated by calpain mediated degradation. These findings are novel in that no previous reports have shown that p53 could be inactivated by calpain cleavage, even though they did show p53 to be a target for calpain. Whether the p53 in ALTR 9 cells is targeted for degradation due to enhanced calpain activity or due to other reasons is not known at this time. Calpain activity has been shown to be upregulated in certain tumors and may be targeting a number of proteins in the ALTR 9 cells in a non-specific manner and will be addressed in the future. It will be of interest to determine if calpain mediated inactivation of p53 is observed in tumors as well. Of particular interest is the fact that the p53 in ALTR 9 cells has acquired a mutation in addition to the existing substitution of Valine to Alanine at residue 135. Pariat et al found some p53 mutant proteins to be more sensitive than wild-type p53 in their susceptibility to calpain cleavage, suggesting that the mutation acquired in ALTR 9 cells may have also, in turn, made this p53 a better target for calpain cleavage.

Since the p53 in ALTR 9 cells could induce p21 function, it was still capable of binding DNA and transactivation, in spite of the additional mutation. This is of particular relevance if calpain mediated cleavage is found to be a mechanism of p53 inactivation in tumors, since it would suggest that resistance to calpain mediated degradation could stabilize the p53 in these tumors and therefore lead to a functional p53 protein. Also, in ALTR 9 cells, the p53 could be stabilized by IR and p21 induction could be observed. Hence, in tumors in which p53 stability is compromised by calpain, IR would be an effective treatment strategy since, based on our results with the ALTR 9 cells, it could also potentially lead to functional reactivation of the p53 in these tumors since in our system it was possible to reactivate a mutant p53 protein and induce p21 protein expression. Of the various types of mutant p53 that exist in tumors, some have been shown to be active for DNA binding and transactivation under certain conditions suggesting reactivation of such mutants as a potential therapeutic target in the treatment of cancers.

Physical sequestration of p53 in the cytoplasm

Previously, it has been reported that p53 can be effectively inactivated by its exclusion from the nucleus (Kubbutat & Vousden, 1997; Pariat et al., 1997). This is an important mechanism of p53 inactivation in light of p53's function as a transcription factor (Shaulsky et al., 1991b). Other studies have also shown that in tumors in which wild-type p53 is cytoplasmically sequestered, the stability of the protein is increased through mechanisms not clearly understood (Zaika et al., 1999a). These reports do suggest, however, that exclusion of the p53 from the nucleus also causes the protein

stability to be prolonged. This is consistent with the mechanism described earlier involving p53 degradation by mdm2, which requires nuclear export of the p53-mdm2 complex. Nuclear import of p53 is thought to be regulated by the association of its nuclear localization sequence (NLS1) present in the carboxy-terminus of the protein, with importin alpha (Kim et al., 2000; Liang & Clarke, 1999; Shaulsky et al., 1990b). A recent study has shown that if importin alpha function is compromised by truncation of the protein, cytoplasmic sequestration of p53 is observed as a result (Kim et al., 2000). It is possible therefore, that in cells in which wild-type p53 is cytoplasmically sequestered, its translocation into the nucleus has been abrogated due to inactivation of importin alpha. This could be the mechanism of cytoplasmic sequestration and hence p53 inactivation in some of the ALTR cell lines, such as ALTR 25, in which p53 is incapable of translocating into the nucleus in response to stress signals tested, such as temperature shift, IR or UV light exposure.

Another mechanism by which p53 may appear cytoplasmically sequestered is by enhanced export of the p53 out of the nucleus (Stommel et al., 1999). This study used the CRM1 inhibitor LeptomycinB to demonstrate that p53 could be trapped in the nucleus in neuroblastoma derived cells in which the p53 appeared to be cytoplasmically sequestered. Since CRM1 is a nuclear export protein involved in the export of several different proteins out of the nucleus including mdm2 which is responsible for shuttling p53 out of the nucleus, it was believed that blocking its activity demonstrated that in cell lines with 'cytoplasmically sequestered' p53, the p53 was shuttling through the nucleus but that its export was proceeding at a faster rate than import thus giving the appearance of

'cytoplasmic sequestration'. An alternative explanation for these results could be due to the fact that Leptomycin B affects the export of several different proteins in the cell, it is possible that this causes undue stress to the cell leading to the activation of p53 similar to other stress responses and leads to the translocation of p53 into the nucleus at which point it gets trapped in the nucleus as well. Our results with Leptomycin B in A1-5 and ALTR cell lines suggest this scenario as a possibility as well, since trapping of p53 in the nucleus by Leptomycin B is only observed in those ALTR cell lines in which the p53 translocates into the nucleus in response to IR and UV exposure.

Cytoplasmically sequestered p53 plays a role in neuroblastoma cell morphology

When we eliminated p53 from a neuroblastoma derived tumor cell line, SK-N-SH expressing wild-type p53 that is cytoplasmically sequestered, we observed a dramatic change in the morphology of these cells. Neuroblastoma cells are found, in culture, as a heterogeneous population of N- and S-type cells, representative of neuronal and Schwannian or substrate-adherent type cells respectively (Ross et al., 1988; Ross et al., 1983). Elimination of p53 by HPV16-E6 caused a predominantly N-type population of cells to convert into an exclusively S-type population. This change in morphology was confirmed with the use of markers specific for each cell lineage.

We determined the level of p53 protein in the N- and S-type cells of the two cell types in the parent SK-N-SH population and found the levels of p53 expressed in the two cell types to be different. The S-type cells in the parent SK-N-SH population expressed more p53 protein than that observed in the N-type cells of the population. These results

were unexpected since they were in contrast to our experiments in which manipulation and reduction of p53 protein led to the S-type cell morphology. However, the differences in the levels of p53 in the N- v/s the S-type cells suggests that even though a reduction in the level of p53 is not necessary to see the morphologic conversion of these cells, it is sufficient for the conversion to take place. Isaacs et al described the p53 in neuroblastoma cells of the N-type to be present in a punctate form in the cytoplasm, while in the S-type cells, a diffuse staining pattern was observed for p53 (Isaacs et al., 1998). This suggests that the p53 in the N-type cells is modified differently from the p53 in S-type cells. However, they also showed the p53 in the N-type cells to be incapable of nuclear translocation in response to genotoxic stress, in contrast to our experiments which showed p53 in both N- and S- cell types to be capable of translocating into the nucleus in response to IR. Subtle differences between the cell lines used in our experiments could be responsible for the differences observed. Nonetheless, our results demonstrating a change in the morphology of the cells to the S-type also suggest that the p53 in the N-type cells is modified differently from that in the S-type cells. It is possible that the p53 in the N-type cells forms protein-protein interactions that are different from those observed in the S-type cells, and that these interactions are important in maintaining the N-type morphology. Elimination of the p53 in the N-type cells could then, by default, lead to the S-type cell morphology.

The change in morphology to the S-type cell was also significant since the S-type cell is less tumorigenic than the N-type cell in the mixed population (Biedler et al., 1988). That degradation of wild-type p53 could cause the conversion of cells to a less

tumorigenic cell type is in direct contrast to the normal function of p53 as a tumor suppressor protein. In light of the enhanced stability of this wild-type p53 protein that is cytoplasmically sequestered, it is possible that the wild-type p53 behaves phenotypically similar to mutant p53, which also displays the similar characteristic of being very stable in the cell. The modification in N-type cells may therefore only affect the protein's stability, which in turn affects the type of protein-protein interactions that the p53 displays in the cytoplasmic compartment of the cell, which eventually leads to the protein assuming a role in maintaining the N-type morphology of the cell and behaving as an 'oncogene'.

This characteristic of p53 behaving as an oncogene has been described in cells expressing mutant p53. In the absence of endogenous p53, introduction of mutant p53 led to an enhanced tumorigenic phenotype in otherwise non-tumorigenic cells (Shaulsky et al., 1991c). These data suggest that p53 can exhibit 'gain of function' behavior and behave as an oncogene. Our results suggest that wild-type p53, when over expressed, can also display such 'gain of function' oncogenic behavior by maintaining the more tumorigenic N-type cell. It would be particularly important to determine if this type of behavior is exhibited by p53 in all cell types in which the protein is stable and cytoplasmically sequestered since a number of tumors exist, particularly breast and neuroblastoma derived tumors, in which p53 has been described to be cytoplasmically sequestered and over expressed due to enhanced stability. It would be of relevance in the treatment of such tumors if p53 could be targeted for degradation by gene therapy or

small molecule treatment, which could lead to a less aggressive tumor type that is easier to treat.

These data open up multiple avenues of investigation in understanding the regulation of p53 function and consequently inactivation. The ALTR model system will be valuable in understanding the multiple and redundant steps involved in p53 regulation that lead to such stringent control of its activity. The information obtained from the ALTR cell system can then be confirmed in tumors in which p53 is inactivated, thereby leading to a better understanding of how p53 can be reactivated in tumors. In addition, the ALTR system will also provide information about other pathways that are regulated by the same proteins that regulate p53, and will broaden our overall understanding of p53-dependent and independent cell cycle regulation. Such investigations are currently in progress in our lab.

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