DNA BINDING AND SELECTIVE GENE INDUCTION BY DIFFERENT FORMS OF THE P53 PROTEIN

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FOROOZAN A. MAYELZADEH

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Foroozan A. Mayelzadeh entitled: DNA BINDING AND SELECTIVE GENE INDUCTION BY DIFFERENT FORMS OF THE P53 PROTEIN.

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

Jesse Martinez 4/14/2006
Tim Bowden 4/14/2006
Mark Nelson 4/14/2006
Bernard Futscher 4/14/2006
Qin Chen 4/14/2006

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

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director: Jesse Martinez  Date: 4/14/2006
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Signed: Foroozan Mayelzadeh
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DEDICATION

Hey! You whom I've discovered so late
I speak to you
just the way the grass speaks to the prairie
the cloud to the storm
the rain to the sea
the bird to spring
the tree to the forest
because I've found your roots
because your voice is familiar with mine

In the bright silence
I've wept with you
for all the living
and in the dark cemetery
I've sung with you
the most beautiful songs
since the dead of this year
were the most amorous of the living.

-A. Shamloo

To my Beloved Parents, Aghdas and Reza Mayelzadeh

&

To the ever lasting memory of my darling brother, Ramin Mayelzadeh.
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ABSTRACT

When cells are challenged by genotoxic stress, the tumor suppressor protein p53 promotes adaptive responses, including cell cycle arrest, DNA repair, or apoptosis. How these distinct fates are specified through an action of p53 protein is not known. It has been suggested that p53 levels, its post-translational modifications or the cell’s genetic background aid p53 in deciding cells’ fate. As a transcription factor, additional evidence indicates that differences in the DNA-binding ability of p53 protein to its response elements are also important determinants of whether p53 will activate growth arrest genes or apoptotic genes. Here we utilized a set of mutant cell lines which, unlike the parental A1-5 cell line which expresses a mouse tsp53 and becomes growth arrested at 32°C, are capable of growth at this same incubation temperature. We found that the tsp53 in the two mutant cell lines, ALTR-17 and ALTR-24, could induce expression of p21, the principle growth arrest gene induced by p53, although to a lesser extent than in parental cells. Interestingly, evaluation of the conformation of tsp53 using conformation-specific antibodies showed that the protein, which we showed could induce expression of p21 reporter construct, existed in different forms which were found to bind DNA using ChIP assays. We also showed, using microarray technology, that the different forms of p53 are capable of inducing/repressing various sets p53-inducible genes. We conclude that the tsp53 may stably exist in various forms capable of binding DNA and decide cell fate through a p53 transcription-dependent pathway.
CHAPTER 1

INTRODUCTION

I. Introduction to Tumorigenesis and tumor formation

All cancers arise from genetic alterations making cancer a genetic disease. Cancer usually arises in a single cell where genes that control the replication of the cell become damaged allowing it to reproduce uncontrollably. The cell's progress from normal to malignant seems to follow a series of steps; each controlled by a different gene or set of genes hence the multi-step process of tumorigenesis (Figure 1-1) involving activation of oncogenes, alterations of genes involved in DNA repair and genomic stability and also inhibition or inactivation of tumor suppressor genes. Thus, most human tumors carry alterations in multiple genes affecting cell proliferation, cell survival, and cell’s genomic stability.

In 1990, Fearon and Vogelstein proposed a model of successive genetic changes leading to colorectal cancer. They suggested that 5 to 7 mutations accumulate and lead to a progression from normal epithelium to metastatic carcinoma through hyperplastic epithelium–early adenoma–intermediate adenoma–late adenoma–and carcinoma. The genes in which mutations occur at steps in this process include APC on chromosome 5, KRAS on chromosome 12, DCC on chromosome 18, and TP53 on 17p. The model proposes that these mutations cooperate, act cumulatively and finally confer a growth advantage to the cell. P53 functions as the guardian of the integrity of the genome and is extensively studied (Levine, 1997, Jayaraman et al, 1997, Prives and Hall, 1999). Loss of p53 function causes disruption of several checks and balances in the cell, which paves
the way to genomic instability. Mutations in the p53 occur widely in human cancers. Indeed the p53 protein has been found to be mutated in more than 50% of all known human cancers.
Figure 1-1. Genetic changes associated with colorectal Tumorigenesis. APC mutations initiate the neoplastic process, and tumor progression results from mutations in the other genes indicated. MMR deficiency speeds up this process. K-RAS is an oncogene that requires only one genetic event for its activation. The other specific genes indicated are tumor suppressor genes that require two genetic events (one in each allele) for their inactivation. Chromosome 18q21 may contain several different tumor suppressor genes involved in colorectal neoplasia, with DCC gene proposed as a candidate. P53 mutation is one of the last mutations which drive the tumor cells from late adenoma stage into early carcinoma stage. A variety of other genetic alterations have each been described in a small fraction of advanced colorectal cancers. These may be responsible for the heterogeneity of biologic and clinical properties observed among different cases. (Kingzler et al., 1996)
Hereditary Cancers due to mutation in p53 protein

About 5-10% of cancers are due to an inherited predisposition in which all cells of the individual will contain the same mutation. Mutations in p53 can be inherited through the germline and are known to be the cause of a hereditary cancer syndrome known as Li-Fraumeni syndrome. Many of these p53 mutations lead to substitution of one amino acid for another in the part of p53 protein that binds to DNA. Other types of mutations include deletions within the gene again rendering the protein non-functional.

Nearly 250 independent germline TP53 mutations have been reported and that most were associated with Li-Fraumeni syndrome or Li-Fraumeni-like syndrome (Valery, 2003). Patients with Li-Fraumeni develop early-onset breast cancer and soft tissue tumors or sarcomas. These patients are also at risk for other early-onset cancers such as leukemia and tumors of the adrenal glands, bones, brain, lung, pancreas, and skin. These cancers are often diagnosed in childhood. The risk of developing any of the additional tumors associated with Li-Fraumeni syndrome is 50 percent by age 30 and 90 percent by age 70. An unusual feature of this syndrome is that about 50 percent of the people who develop cancer in one tissue also develop a second or new primary cancer.
II. The Discovery of p53

The p53 protein was discovered through serologic and virologic studies of tumor cells. DeLeo et al. (De Leo et al. 1979) showed that mice exhibited a humoral response towards a p53 kilodalton protein in methylcholanthrene-induced tumor cell lines. Next it was shown that the p53 protein was more prevalent in cells transformed by the Simian Virus 40 (SV40) than in normal cells and that p53 could bind to the Large T antigen from SV40. (Chang et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). P53 was originally classified as an oncogene, since initial research had revealed that transfecting cells with p53 caused cells to undergo transformation. But in 1989, Bert Vogelstein, Arnold Levine and others showed that p53 is actually a tumor suppressor, and that it is altered in the majority of colon cancers. It was discovered that researchers had been studying missense mutants of the TP53 gene instead of the wild-type gene. Ironically, the missense mutations found in the original TP53 cDNA clones provided a key tool to understanding the pathological activity of this protein. P53 has the ability to form tetramers allowing it to behave in a dominant-negative fashion. It was also discovered that certain missense p53 mutants 'gain oncogenic activity'. Research also revealed that the viral proteins block the DNA-binding surface of p53, thus inhibiting its role as a transcription factor. Ten years later, this link categorized p53 as a tumor suppressor protein and transcription factor. Since its discovery there has been so much interest that Science hailed the p53 protein as the "Molecule of the Year" in 1993.
III. Functional role of p53

P53 plays a role in senescence, differentiation and antiangiogenesis. However, the best characterized functions of the p53 protein are initiation of cell cycle arrest, apoptosis in response to genotoxic damage and DNA repair (Lane et al. 2002). P53 regulates these pathways primarily as a sequence-specific transcription factor. Although transcription independent activities of p53 for induction of apoptosis have been documented (Bennett et al., 1998; Mihara et al., 2003), it remains unknown whether this represents a true in vivo event.

A. P53 and the cell cycle checkpoints

Control of cell cycle progression is one of the most important aspects of all biological processes and by definition uncontrolled cell proliferation leads to tumorigenesis. Cell division progresses through an oscillatory pattern of cyclin-dependent kinase (Cdk) activities. The CDKs are themselves regulated by the synthesis and degradation of their regulatory subunits, the cyclins. Destruction of cyclins through ubiquitin-proteasome system inactivates Cdk activities and mediates uni-directional transitions from one cell cycle stage to the next stage.

The cell cycle is an ordered set of events (Figure 1-2). The $G_1$-phase stands for “GAP-1” and is required for cell growth and preparation of DNA synthesis. The $S$-phase stands for “Synthesis” and replicates the genome. The $G_2$-phase is “GAP-2” and needed for cell growth and preparation for mitosis. The finally $M$-phase stands for “Mitosis” in which cells segregate duplicated chromosomes. There are 3 main checkpoints which control the cell cycle in eukaryotes. The first checkpoint is located at the end of the cell
cycle's G1 phase, just before entry into S phase, which makes the key decision of whether the cell should divide, delay division, or enter a resting stage. The G1 phase checkpoint, also known as the restriction point, is the main point of action of p53. Cells that progress through this point are committed to enter S phase, where DNA synthesis and replication will occur. If a cell is not ready, or external conditions are not appropriate for the S phase, then the cell may enter G0 phase, a quiescent stage. The second checkpoint is located at the end of G2 phase, triggering the start of the M phase. P53 also plays a role in maintaining cells in G2 when exposed to genotoxic stress. If this checkpoint is passed, the cell initiates the many molecular processes that signal the beginning of mitosis. The third checkpoint is located during metaphase, also known as the mitotic spindle checkpoint, triggering the exit from mitosis and cytokinesis and the beginning of G1.
Is Machinery for Replication intact?  
Is cell ready to replicate?

Cells ready for Cytokinesis?

Is the DNA mutation-free and fully replicated?

Figure 1-2. Schematic model showing the cell cycle and its checkpoints. During G1 cell growth begins and the replication machinery is checked. During S-phase chromosomes divide and duplicate and the cell continues to grow. In G2 phase, cell continue to grow and the duplicated chromosomes are checked for mutations. During M phase the cell undergoes cytokinesis and divides into two daughter cells.
Role of p53 in G1 & G2 Arrest

In addition to the cyclins, another key regulator of the cell cycle is the p53 protein. P53 regulates the cell cycle at all three checkpoints. It regulates both G1 and G2 phases of the cell cycle (Kastan et al. 1992, Bunz et al. 1998) and is also involved in regulating the mitotic spindle checkpoint preventing endoreduplication of 4N cells (Meek, 2000). The implicating role of p53 in multiple cell cycle checkpoints is a tale-telling sign to the crucial role of p53 in suppression of amplification of genetic alterations which can lead to cancer. Therefore, it is only reasonable to find that p53 is mutated in more than 50% and deregulated in 100% of human cancers.

P21\textsuperscript{WAF1} is the main player of the G1 phase arrest within the cell cycle (El-Deiry et al. 1993). It is a member of the p27 and p57 KIP family of universal cell cycle inhibitors (Gorospe et al. 1999). P21 can bind to proliferating cell nuclear antigen (PCNA) and inhibit progression of DNA replication (Boulaire et al. 2000, Rousseau et al. 1999). P21, a p53 downstream target gene, also inhibits complex formation between various cyclin/cdks proteins; i.e. Cyclin E-Cdk2 and CyclinB-Cdk1 (Flatt et al. 2000) [Figure 1-3]. As a result, pRb is not phosphorylated, and it inactivates E2F-1 through recruitment of histone deacetylase (HDAC1) [Brehm et al. 1998]. These data are further confirmed by the findings of Deng et al. (1995) who had reported that mice lacking p21\textsuperscript{WAF1} undergo normal development, but are defective in G1 checkpoint control.
Figure 1-3. A simplified schematic model describing p53-dependent G1 arrest. Upon activation, p53 moves into the nucleus and transcriptionally activates the p21 gene. Next, p21 protein inhibits Cdk2/CycE complex formation, leading to G1 phase arrest.
Another downstream target of p53, 14-3-3 protein has been implicated in response to genotoxic stress leading to G2-phase cell cycle arrest (Hermeking et al. 1997). P53 and its target genes, p21 and 14-3-3 proteins, inhibit the cyclin B/Cdk1 activity. CycB-Cdk1 complex formation is essential for cell’s transition into mitosis. The 14-3-3–σ protein is not a cdk inhibitor itself, and its mode of action is different from that of p21wAF1 protein. Peng et al. (1997) were able to show that 14-3-3–σ sequesters the phosphorylated form of Cdc25C phosphotase, thereby preventing dephosphorylation and activation of the cyclin B/Cdc2 complex. Also, Chan et al. (1999) presented a second role for 14-3-3–σ G2 arrest. They reported that the 14-3-3 protein sequesters Cdc-2 in the cytoplasm, thereby inhibiting it from moving into the nucleus during G2-phase. Although the role of p53 in activating G2 arrest is vague, its ability to induce the expression of p21 and 14-3-3–σ is essential (Chan et al. 1999) [Figure 1-4].
Figure 1-4. A simplified schematic model describing p53-dependent G2 arrest. Upon activation p53 moves into the nucleus and induces expression of p21 and 14-3-3 genes. P21 inhibits the cdk1/cycB complex formation which leads to G2 phase arrest. 14-3-3 protein sequesters Cdc25C in the cytoplasm leading to inhibition of activation of the Cdk/cyc complex and thereby causes G2 phase arrest.
P53 also plays a key role in regulating chromosomal segregation which is the third and final cell cycle checkpoint, a.k.a. mitotic spindle checkpoint (Tarapore and Fukasawa, 2000). It was reported by Sablina et al. (1999) that although p53 does not control the spindle assembly, it mediates G1-like arrest in response to disruption of microtubule system. Induction of G1-like arrest inhibits reentry into of the cell into the S-phase, thus preventing endoreduplication. Just like in G1-phase this checkpoint depends on p21’s ability to inhibit cyclin E-Cdk2 which has been shown to be the complex that drives initiation of centrosome duplication (Lacey et al. 1999) [Figure 1-5].
Figure 1-5. A simplified schematic model describing p53-dependent Mitotic Spindle Checkpoint. Under normal conditions, in response to disruption of microtubule system, p53 moves into the nucleus and induces gene expression of p21 which in turn leads to inhibition of Cdk/Cyc complex formation and a G1-like arrest. However, deregulation of this pathway will lead to endoreduplication.
**B. Role of p53 in the induction of Apoptosis**

Apoptosis, a.k.a. programmed cell death, is an orderly process by which a cell commits suicide. The phenotypes of a cell undergoing apoptosis include: DNA condensation, cell fragmentation into membrane-bonded apoptotic bodies, and finally ingestion by phagocytes of the immune system (Lodish et al., 2000). P53 plays an important role in the induction of apoptosis in response to genotoxic damage. It has been demonstrated that p53 induces apoptosis through multiple pathways (Bates and Vousden, 1999). It is important to keep in mind that a full apoptotic response requires both transcription and transcription-independent functions. Hence, there are two major apoptotic p53-dependent pathways known as: the “Intrinsic” Pathway and the “Extrinsic” Pathway (Figure 1-6)

i. **P53-dependent intrinsic apoptotic Pathway**

The Intrinsic apoptotic pathway is a transcription-dependent pathway. Following DNA damage, p53 translocates into the nucleus and binds DNA, transactivating a multitude of pro apoptotic genes such as Bax, Puma, Noxa, etc. Those proteins in turn, trigger the release of cytochrome c into the cytoplasm. Cytochrome c is a key regulator of this pathway because once it is released from the mitochondria the cell is irreversibly committed to die. Cytochrome c then associates with Apaf-1, caspase-9, and ATP to form an “Apoptosome”. The apoptosome then activates caspase-3, which in turns activates the caspase cascade and the degradation phase of apoptosis.
ii. **P53-dependent extrinsic apoptotic pathway**

Following exposure to stress, p53 can also activate the extrinsic apoptotic pathway (Bouvard et al. 2000). Extrinsic apoptotic pathway depends on the induction of p53 downstream target genes such as the transmembranes protein, Fas (Muller et al., 1998). The cell-surface receptor Fas (CD95/Apo-1) is a key component of this pathway (Nagata and Golstein, 1995). The pathway is initiated by binding of death activator protein FasL to the transmembrane death receptor. This binding allows interaction with the cytoplasmic adaptor protein FADD, and procaspase-8 that activates membrane-proximal activator caspases 8. The activation of caspase 8 leads to caspase activation which leads to apoptosis (Ethell & Buhler, 2003).

A transactivation-independent, pro-apoptotic activity of p53 has been described in several experimental systems (Caelles et al 1994, Haupt et al.1995, Chen et al. 1996, Wagner et al, 1994, Moll et al.). One mechanism describing this phenomenon was postulated by Marchenko et al. (2000) to be a direct import of p53 into the mitochondrial matrix to act on ROS generation. Interestingly it was found that the activation of cytosolic p53 can induce mitochondrial cytochrome c release in cyoplasts and in a cell-free system through a mechanism requiring its intact N-terminus (Shuler et al. 2001). Hence, it is possible that transcription-independent functions of p53 co-operate with its transcription-dependent functions in the induction of apoptosis.
Intrinsic and Extrinsic Death Pathway

Figure 1-6. Schematic model describing the Intrinsic and Extrinsic Apoptosis Pathways (Johnstone et al. 2002). Intrinsic and extrinsic pathways of apoptosis. The extrinsic pathway is triggered by death receptor engagement, which initiates a signaling cascade mediated by caspase-8 activation. Caspase-8 both feeds directly into caspase-3 activation and stimulates the release of cytochrome c by the mitochondria. Caspase-3 activation leads to the degradation of cellular proteins necessary to maintain cell survival and integrity. The intrinsic pathway occurs when various apoptotic stimuli trigger the release of cytochrome c from the mitochondria.
C. P53’s role in DNA Repair

The p53 protein plays an important role in DNA repair via several distinct pathways. One such pathway by which p53 exhorts its effect on the removal of potentially mutagenic errors after DNA damage is named the nucleotide excision repair (NER) pathway. NER is an evolutionarily conserved DNA repair pathway with the ability to remove a wide range of DNA damaging adducts induced by environmental as well as carcinogenic agents (De Laat et al. 1999). Following exposure to DNA damage induced by UV light, p53 induces GADD45 protein, which in turn binds to the damaged DNA and affects lesion accessibility (Therrien et al. 1999). The most prevalent adduct removed by NER pathway is the UV-induced cyclobutane pyrimidine dimers (Hanawalt, 2002) and the pyrimidine–pyrimidone photoproducts (LeClerc et al. 2002). The p53-dependent induction of ribonucleotide reductase gene which is involved in DNA repair following IR, UV and drug induced DNA damage, is another testament to p53’s involvement in various repair pathways (Nakano et al. 2000. Tanaka et al. 2000).
Figure 1-7. Schematic of function of p53 (Levine, 1997). P53 is activated by DNA damage in a response pathway involving ATM/ATR. Post translational modifications occur to the protein that lead to gene activation and alteration in cell cycle, DNA repair, and apoptosis.
IV. P53 Target Genes

The tumor suppressor protein p53 plays an essential role in the cell and is normally present in all cell types. P53 is probably the most studied of the transcription activators. It is interesting to note that many forms of DNA damaging agents including ionizing radiation, UV light, anti-cancer drugs such as topoisomerase inhibitors, inhibitors of transcription, DNA cross linking agents, and environmental agents have all been shown to alter p53 activity. And that is not all; non-genotoxic stresses such as hypoxia, microtubule disruption, oncogene activation, and replicative senescence are also known to modify and activate p53. The p53 protein is at the center of a large network of proteins involved in cellular damage detection and control. It is the sensor of the integrity of the DNA and the cell’s wellbeing.

P53 functions as a transcription factor (El-Deiry et al 1992). It binds as a tetramer to specific genomic sites that have a symmetrical arrangement of two 10 bp palindromes, separated by 0-13 bp: PuPuPu(CA/TA/TG)PyPyPy (0-13N) PuPuPu(CA/TA/TG)PyPyPy. Cho et al. (1994) demonstrated through the three dimensional structure of p53 protein, that the core domain of p53 is involved in DNA binding and that p53 which is mutated in these positions fail to bind the target DNA. It has also been shown that the amino acid residues of p53 that make contact with the DNA exhibit the highest mutation rate in cancer and the most mutated of them have been coined as “hot spot” mutations. These results have defined the DNA sequence element (a.k.a. Response Element) with which p53 interacts in order to activate transcription of its downstream target genes.
Recent developments in the human genome sequencing efforts have led to identification of p53 binding sites on a genomic scale resulting in global identification of p53 target genes. Analysis of p53 binding sites throughout the human genome suggests the existence of many p53 regulated genes (Tokino and Nakamura, 2000). It was predicted that the expression of about 200-400 genes might be controlled by p53 transactivation (El Diery et al. 1992). This will certainly help us understand cellular networks which are controlled by p53.

P21\textsuperscript{waf} was the first identified target of p53 which is involved with growth suppression effect of p53. P21 is also known for its role in inducing senescence (Noda et al. 1994) and most importantly it is also identified as a universal inhibitor of CDK which blocks the cell cycle at G1/S transition and inhibits cell growth (Xiong et al. 1983, Harper et al. 1993). P53’s role as an inducer of apoptosis had been discovered early on (Yonish-Rouach et al. 1991), however the mechanism of p53 mediated apoptosis was not understood. It wasn’t until 1995 when Miyashita et al. and Owen-Schaub et al. published their finding of the first identified mediators of p53 induced apoptosis: Bax and Fas/APO1. Based on this data it was clear that the transcription activation function of p53 fell into two main categories where various yet specific genes were induced by p53. First category involved cell cycle regulation leading to growth arrest and the prototype transactivated gene for this category was p21. The second category involved programmed cell death and involved genes responsible for apoptosis such as Bax. Today there are more categories that need to be added to this list, as p53 is also the main activator of genes involved in senescence, DNA repair, angiogenesis, cell motility and
also cell differentiation (Almog and Rotter, 1998, Bates and Vousden, 1999, Colman et al.
The choice between transcription activation of either of these pathways is the remaining
question of p53 function. This choice is strongly linked to the understanding of how a
cell decides whether to enter cell cycle arrest or to undergo apoptosis upon p53 induction.

Three models have been proposed to explain how p53 determines a cell’s fate
(Lane 2001):

“Protein Level” Model: This model predicts that the downstream effects of p53
are protein level dependent. For instance, as demonstrated by Chen et al. (1996)
tetracycline-induced regulation of p53 showed that at low concentrations the p53 protein
induced growth arrest while at higher concentrations p53 triggered apoptosis.

“Cellular Background” Model: This model proposes that cellular background
determines the p53-mediated effects within a cell. This model is supported by
observation made by Smart et al. (1999) that activation of p53 by Leptomycin B (nuclear
export inhibitor) in different cell lines resulted in senescence in normal fibroblasts and
apoptosis in neuroblastoma cells.

“Selective gene activation” Model: This model hypothesizes that the various
modifications of p53 determine its affinity for promoters in the p53 target genes. For
instance as shown by Olive et al. (2004), knock-in mutations that affect key amino acids
within the DNA-binding domain of the p53 protein prevented the activation of p53’s
downstream target genes triggering apoptosis, but did not affect p53’s ability to induce
the genes involved in cell cycle arrest.
Additional evidence indicates that differences in the DNA-binding sequences within the promoter regions of the p53-responsive genes are also important determinants of whether p53 will activate growth arrest genes or apoptotic genes (Qian, et al. 2002). Although in vitro data supports all three models, the Protein Level model seems to be a crude and overly simplistic model for explaining p53’s role in deciding cell fate. However, the Cellular Background and the Selective Gene Activation models are highly plausible and together can shed some light on the factors which influence p53 in pushing cells into one pathway vs. another.

As mentioned previously, the wide range of p53’s biological effects can in part be explained by its activation of expression of a number of target genes. Here is a partial listing of p53 transactivated genes as they are categorized by their function within the cell:

1. **Apoptosis genes:**

   - *Induction of apoptosis:* Atm, Bax, Bbc3, Casp2, Dapk1, Foxo3, Myc, Perp, Plagl1, Prkca, Pten, Tnf, Trp63.
   - *Anti-apoptosis:* Bcl2, Birc5, Dapk1, Hdac1, Rela.
   - *Regulation of apoptosis:* Apaf1, Bak1, Bid, Brca1, Btg2, Casp9, Cradd, Fadd, Mcl1, Pycard.
   - *Other apoptosis genes:* Amid, Bag1, Bnip3, Daxx, E2f1, Faf1, Nfkb1, Ppp1r13b, Siah1a, Sirt1, Tnfsf6, Tp53, Tradd, Traf4, Traf6, Trp53bp2, Trp73.
2. **Cell cycle genes:**

- **Cell cycle arrest:** Cdkn1a, Cdkn2a, Chek1, Gadd45a, Gtse1, Msh2, Pmp22, Sesn1, Sesn3.
- **Negative regulation of cell cycle:** Atm, Brca1, Pten, Pycard, Rb1, Tp53, Trp63, Trp73, Tsc2, Wt1.
- **Regulation of cell cycle:** Aatf, Brca2, Ccne2, Cdk4, E2f1, E2f3, Esr1, Frap1, Jun, Kras2, Sfn.
- **Other cell cycle genes:** Aurkb, Ccng1, Ccng2, Ccnh, Cdc25a, Cdc25c, Cdc2a, Cdk7, Gak, Mdm2, Parc, Plk, Ppm1d, Pttg1, Rad53, Trp53bp2.

3. **Cell growth, proliferation and differentiation genes:**

- **Cytokines:** Cx3cl1, Il6, Segh3a1, Tnf, Tnfsf6.
- **Cell growth:** Bbc3, Cyr61, Esr1, Sch1, Wrn.
- **Regulation of Cell proliferation:** Bap1, Brca1, Cdk4, Cdkn1a, Fanca, Mdm4, Myc, Sch1, Tnf, Tp53, Traf6, Tshr, Wig1.
- **Cell differentiation:** Btg2, Hif1a, Myod1, Ndr1, Nfl1, Stat1, Stat5a, Wt1.
- **Others:** Jun, Kras2, Mdm2, Numb, Pml, Pttg1, Tsc1, Tsc2.

4. **Cell motility and adhesion genes:**

- **Cell adhesion molecules:** Aatf, Cspg2, Cx3cl1, Cyr61, Ddr1.
- **Extracellular matrix proteins:** Cspg2, Cx3cl1, Cyr61, Il6, Tnfsf6.
- **Others:** Pten, Serpinb5.

5. **DNA repair genes:**

- Apex1, Atm, Atr, Brca1, Brca2, Ercc1, Fance, Lig4, Msh2, Prkde, Pttg1, Rev3l, Xrcc4, Xrcc5.
V. P53 Regulation

The growth suppressive properties of the p53 tumor suppressor protein are activated upon DNA damage. The activation of p53 is reflected in increased p53 levels which are, at least in part, the result of an extended half-life of the protein. Although this suggests that stabilization of p53 is an intrinsic feature of p53 activation, the mechanisms involved in p53 degradation and stabilization are thought to be more complex. The importance of p53 in tumor development has been confirmed by p53 knockout mice that show a remarkable predisposition to lymphoma and other types of cancers (Donehower et al., 1992). The p53 protein is subject to tight regulation at multiple levels. This is achieved by a variety of positive and negative regulators, often creating feedback loops.

Three major modes of p53 regulation include:

- protein stability
- protein activity
- subcellular localization

Recent finding regarding molecular mechanisms governing these regulatory processes are helping us understand the regulation of p53 in more depth.

A. The regulation of p53 stability and the role of Mdm-2 protein.

Mdm-2 has been identified as the major negative regulator of the p53 protein (Momand et al. 2000, Freedman et al, 1998). In 1996 Haupt et al. proposed a “Masking Model” of p53 by Mdm-2 protein, where it binds to and renders the transactivation domain of p53 unavailable. It was later confirmed by Momand et al. (2000) that Mdm2 binds the N-terminus of p53 occupying residues 18-28 within the transactivation domain of p53. This is the region of the protein where it also interacts with transcription co-
factors such as TBP and TAFs (Momand et al., 2000) and also p53’s coactivator, p300 (Wadgaonkar and Collins, 1999). Another important region required for p53 degradation by Mdm2 is within residues 92–112 (Gu et al. 2000). On the other hand, the polyproline region (residues 62–91) of p53 may provide protection for p53 from Mdm2. p53 lacking this region is excessively sensitive to inhibition and destabilization by Mdm2 (Berger et al. 2001). Another important piece of data is that oligomerization of p53 appears to be essential for the degradation of p53 by Mdm2 (Maki, 1999). P53 transactivates the Mdm-2 gene, and in turn Mdm-2, through a negative feedback loop, binds to and tags p53 for destruction (Momand et al., 2000). It was discovered that p53’s degradation by Mdm-2 is through an ubiquitin-proteasome pathway (Haupt et al. 1997). Over-expression of Mdm2 in many cancers is often sufficient to inactivate p53 without further mutation (Momand et al. 2000). The importance of Mdm2 in this negative regulation is demonstrated by the lethal effect during early stages of embryonic development of mdm2 knockout mice. The early mortality of these mice is stopped by inactivation of p53 (Jones et al. 1995, Montes de Oca Luna et al. 1995). Moreover, the importance of Mdm-2 in keeping p53 in check was also emphasized by the in vivo study conducted by De Rozieres et al. (2000) where they showed that loss of Mdm2 is enough to induce p53-mediated apoptosis.
Figure 1-8. Cartoon of p53 showing major functional domains and positions of modifications within the amino and carboxyl termini. (Ryan et al. 2001). The human p53 protein comprises of several domains. The N-terminus(1-44) contains the transactivation domain, which is responsible for activating downstream target genes. A proline-rich region (58-101) mediates p53 response to DNA damage through apoptosis. The DNA Binding Domain (102-292) is a core domain which consists of a variety of structural motifs. The oligomerization domain (325-356) consists of a β-strand, which interacts with another p53 monomer to form a dimer, followed by an α-helix which mediates the dimerization of two p53 dimers to form a tetramer.
B. Regulation of protein activity
   
i. Structure and Conformation

At the biochemical level, one feature that is critical for normal function of a cell 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). Hainaut et al. demonstrated that some metal chelators could perturb the wild-type conformation of p53 to a mutant conformation, which also led to inhibition of its ability to bind DNA in electrophoretic mobility shift assays (Hainaut & Milner, 1993). Published 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 and Watson, 1990). For example, genotypically wild type p53 found in a mutant conformation promotes cell growth and only behaves as a tumor suppressor when present in a wild type conformation.

Previous published data indicates that p53 seems to be inactive for sequence-specific DNA binding, whereas various posttranslational modifications in the p53 C-terminal domain, binding of the monoclonal antibody PAb421, which recognizes an epitope within the p53 C-terminus, or deletion of the 30 C-
terminal amino acids, strongly enhance sequence-specific DNA binding under certain *in vitro* conditions (Hupp et al. 1994). This data implies that the C-terminus negatively regulates sequence-specific DNA binding of p53 (Hupp et al. 1992). Hupp et al. (1992) explained the inhibiting effects of the C terminus by what they called the “conformation” model, which postulates that the p53 protein exists in two distinct conformations termed “latent” (for DNA binding inactive p53) and “activated” (for DNA binding active p53). According to this model, the C-terminal domain in “latent” p53 directly interacts with the core domain and inhibits sequence-specific DNA binding of p53 (Hupp et al. 1992). A conformational switch that converts latent p53 into an “activated” form relieves this allosteric inhibition.

**ii. Posttranslational Modifications**

The p53 protein was reported by Appella et al. (1999) to be posttranslationally modified on at many sites (Figure11). A review of p53 posttranslational-modification literature reveals that phosphorylation in response to DNA damage is the most prevalent modification at most sites on the p53 protein. The N-terminus of the p53 is phosphorylated on numerous Serines and Threonines; i.e. S6, S9 (Fuchs et al. 1995), S15 (She et al. 2000), S37 (Tibbetts et al. 1999), S46 (Oda et al. 2000), S20 (Chehab et al. 2000), S33 (Kishi et al. 2001), Thr-18 (Sakaguchi et al 2000). However, it seems that the carboxy terminus of p53 may be more ornamented with various posttranslational modifications ranging from phosphorylation [S315 and S392], to acetylation [K320, K373,
K382] (Ito et al. 2001) and finally sumolyation [K386] (Gostissa et al. 1999, Rodriguez et al. 1999). Interestingly individual sites seem to respond differently to specific stress signals. For instance, it was reported that Serines 15, 9 and 6 are phosphorylated for a short period of time following exposure to ionizing radiation, however when exposed to UV light a less rapid yet prolonged phosphorylation of these sites occurs (Shieh et al. 1997, Siliciano et al. 1997, Sakaguchi et al. 2000).

Posttranslational modifications and the modifiers regulate p53’s activity through many different mechanisms. For instance, Chk2 phosphorylation of p53 at S20, renders p53 more stable, because it hampers Mdm2’s ability to bind p53 (Chehab et al. 2000). While K320 acetylation, by p53 co-activators p300/CBP, enhance p53’s sequence specific transactivation along with its stability within the cell. (Figure 1-9)
Figure 1-9. DNA damage-induced posttranslational modifications of p53. The bar represents the 393 amino-acid p53 polypeptide; regions associated with transactivation (TA), sequence-specific DNA binding (DBD), nuclear localization (NLS), tetramerization (TET), and DNA mediated negative regulation of specific DNA binding (REG) are indicated (not to scale). The positions of known phosphorylations and acetylations are represented by ovals and squares, respectively; the number of the amino acids modified also is given, and possible modifying enzymes are listed. The response to ionizing radiation is believed to initiate from DNA strand breaks that are sensed directly or indirectly by ATM, which phosphorylates p53 directly and activates other kinases (e.g. Chk1 and Chk2) that phosphorylated p53 and MDM2. Upon exposure to UV light, ATR, p38MAPK and the CK2/FACT complex are activated and phosphorylate a distinct but overlapping set of residues. P53 and MDM2 are modified by conjugation to SUMO-1 through an E1/Ubc9-mediated mechanism. Acetylation of p53 C-terminal residues is mediated via a cascade initiated by N-terminal phosphorylations that modulate acetyltransferase binding to p53. Similarly, phosphorylation of Ser6 and Ser15 may facilitate phosphorylation of Ser9 and Thr18 by CK1. (Appella and Anderson, 2001).
C. Regulation of p53’s subcellular localization.

Placing p53 in the appropriate intracellular location is another important regulatory mechanism of p53’s activity. In 1990, Shaulsky et al. showed that the p53 protein shuttles between the cytoplasm and the nucleus in a cell cycle-dependent fashion. The accumulation of p53 in the nucleus is critical for its tumor suppressive activity and naturally its cytoplasmic sequestration provides the means by which cancerous cells proliferate in the presence of a transcriptionally active p53 as has been detected in tumors such as neuroblastomas, breast and colon cancer (Moll et al. 1996, Momand et al. 1997). In 2000 Lu et al. showed that Mdm2 is responsible for the nuclear exclusion of p53 in certain cancers. Also, it was shown that the adenoviral E6 protein, E1B protein and the HBx protein of Human Papilloma Virus keep p53 in the cytoplasm (Thomas et al. 1999, Wienzek et al. 2000).

Another cause of p53 misdistribution is defects in its import/export machinery. p53 is imported into the nucleus following its association with importin alpha and beta via its Nuclear Localization Signal sequence (NLS). P53 has three NLS sequences (Figure 1-10), with NLSI residing between residues 316 to 325 (Kim et al, 2000), NLSII residing between residues 369 to 375 and NLSIII residing between residues 379 to 384 (Liang and Clarke, 1999). NLSI is most important for p53 translocation into the nucleus and mediates the interaction between p53 and importin-alpha (Kim et al. 2000). It was also reported by Liang and Clarke (1999) that residues 326 to 355 which reside immediately to downstream of NLSI might be sites of binding of p53 cytoplasmic anchor proteins and hence they also play an important role for the cytoplasmic sequestration of
p53 by masking NLSI. Some of the currently known cytoplasmic binding proteins of p53 include: tubulin, hsc70, hsc84, F-actin and vimentin (Klotzsche et al. 1998). Also Parc has been suggested as a p53 cytoplasmic anchor by Nikolaev and Gu (2003). Aside from NLS associated defects, p53 could become sequestered within the cytoplasm due to a mutation within any of the proteins in the importin complex, for example, Kim et al. (2000) reported that truncated form of importin-alpha identified in breast cancer cell inhibits nuclear import of p53. Another important regulatory means of p53 protein degradation is its export out of the nucleus (Roth et al 1998, Freedman and Levine, 1998). As P53 protein posses a Nuclear Import Signal which guides the protein into the nucleus, it also posses a Nuclear Export Signal (NES) residing on residues 340 to 351 (Stommel et al. 1999) which guide it out of the nucleus and into the cytoplasm to be degraded by proteasomes. It was suggested by Boyd et al. (2000) and Geyer et al. (2000) that p53 must be ubiquitinated by Mdm2 prior to its nuclear export. It is believed that ubiquitination of p53 exposes its NES hence allowing p53 to interact with nuclear exporting protein, CRM1, and facilitate p53 export.
Figure 1-10. Cartoon of the location of the NLS and NES on the p53 protein. Three putative nuclear localization signals (NLS) have been identified in the C-terminus, through sequence similarity and mutagenesis. The most N-terminal NLS (NLSI), which consists of 3 consecutive Lysine residues to a basic core, is the most active and conserved domain. NLSII and NLSIII are located within residues 370-376 and 380-386 respectively. Two putative nuclear export signals (NES) have been identified. N-terminus NES is located within residues 11-24. The leucine-rich C-terminal NES, found within the oligomerization domain, is highly conserved and it has been suggested that oligomerization can result in masking of the NES, resulting in p53 nuclear retention. (Berkson et al. 2005).
VI. P53 Deregulation in Tumors

Frequency of mutations within p53 varies depending on the type of cancer, however it was reported by Beroud and Soussi (1998) that more than 50% of human cancers posses a mutation on the p53 gene (Figure 1-11). It has been reported that more than 90% of p53 mutations are missense mutations which lead to a single amino acid change (Prives, 1994). P53’s activity can be deregulated via these alterations in its gene, depending on the location of the alteration.

A. Mutations may occur in regulatory or promoter regions. A mutation in the promoter region can result in a decrease or absence of p53 expression in the cell.

B. Mutations may also occur in the protein coding region of the gene can impact the activity of the protein in two general ways:

- Decrease in the activity of p53 as a transcription factor. Which in turn effects the expression of the downstream target genes of p53 including p21 (a protein involved cell cycle regulation), Bax (a protein involved in the induction of apoptosis), and thrombospondin-1(an angiogenesis inhibitor).
- Change in p53 protein conformation that
  - Makes it more susceptible to degradation by MDM-2. Low levels of p53 in the cell will not be able to perform its functions as tumor suppressors.
Inhibit its DNA binding ability as it changes the p53 core domain structure.

Mutations in p53 have been shown to be predominantly within its DNA binding domain (Figure 1-12). However Levine et al. (1991) had pointed out that there exists a bias in the site of mutation and the tissue type where for example missense mutation of amino acid 175 is more prevalent in lung and colon cancers than any other cancer. These correlations between the site of mutation and the type of cancer suggest that they may be a result of the specificity of a specific mutagen to which the tissue is most often exposed.
Figure 1-11. 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.
Figure 1-12. Schematic showing the frequency of mutation of the amino acid residues in p53. The numbered regions represent the five regions that are conserved across species. These regions are target of 90% of p53 mutations found in human cancers as a single mutation within this domain can cause a major conformational change (Harris, 1993).
VII. Introduction to the ALTR Cells

Our overall goal for this project was to understand the mechanism of how p53 plays a role in deciding cell fate. Although there have been great advances in the field of p53, it is still unclear how P53 is involved in deciding cell fate. Of the models proposed to explain how this occurs, data supporting the “Cellular Background” and “Selective Gene Activation” models are strongest. Hence our objective was to find a model system that could investigate both models.

A genetic approach was used to address the complexity of p53’s role in deciding cell fate. Previously, A1-5 cells had been used to generate the genetic screen. A1-5, a rat fibroblast cell line, had been transformed by the introduction of activated Ha-ras and mouse temperature sensitive p53 (Martinez et al, 1991). The temperature sensitive p53 protein has an amino acid substitution from Valine to Alanine on residue 135. Hence, at 39°C the p53 protein assumes a mutant conformation and is sequestered in the cytoplasm. However, at the permissive temperature of 32°C, p53 moves into the nucleus and assumes a wild type conformation. Upon its translocation into the nucleus p53 binds DNA in a sequence specific manner and transactivates growth arrest genes such as p21 (Figure 1-13).

Normally wild type p53 expressing cells maintain a low level of levels of the protein when not under stress. This characteristic of cells makes studying the p53 protein and its mechanism of function a difficult endeavor. A unique feature of the A1-5 cells, making them a precious model system for our studies, is the high levels of p53 they
maintain at all times. Hence, the mechanism of p53 transactivation can be easily studied using biochemical techniques.
Model System

Rat embryonic fibroblast cell line  \[ \text{A1-5 cells express temperature-sensitive p53} \]

32°C  \[ \text{wild-type p53} \]

p21 induced growth arrest

39°C  \[ \text{mutant p53} \]

Figure 1-13. Schematic representation of the localization and function of temperature sensitive p53. (courtesy of Supria Gaitonde) Temperature sensitive p53 val135 in the A1-5 cells assumes the wild type conformation and is capable of translocating into the nucleus at 32°C and behaves as a Transcription Factor leading to transactivation of downstream target genes such as p21 leading to cell cycle arrest. However at 37°C the protein is in a mutant conformation and unable to move into the cytoplasm.
A genetic screen was developed to identify mutants in which p53 was functionally inactivated at various stages along its route. Based on previous unpublished data, it was known that A1-5 cells expressed multiple copies of the plasmid expressing temperature sensitive p53 protein, making inactivation by mutation in the p53 expressing plasmid unlikely. Inactivating the protein through deregulation of some aspect responsible for its activation was expected.

The genetic screen was developed by mutagenizing A1-5 cells with a low concentration of EMS at 39°C. The EMS treated cells were then transferred and incubated at 32°C (Gaitonde et al. 2000) [Figure 1-14]. As shown by the model, the p53 in A1-5 cells moves into the nucleus at 32°C and binds DNA and transactivates 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. Using this procedure, 40 colonies were isolated, from which 20 have been successfully maintained and to some extent characterized. These cells were designated ALTR for A1-5 Low Temperature Resistant cells (Table1).

Among the isolated ALTR cell lines, 16 cells have cytoplasmically sequestered p53, some of which contain a mutation and some which have a wild-type p53. However, the remaining four cell lines, ALTRs 9, 17, 18 and 24 all seem to express copious levels of p53 with an intact nuclear localization mechanism, which allows p53 to appropriately move into and out of the nucleus. However, after sequencing the p53’s expressed in
Mutagenesis of A1-5 cells. Selection of ALTR cell lines

Figure 1-14. Schematic representation of the development of the ALTR cell system (Courtesy of Supria Gaitonde). A1-5 Low Temperature Resistant cell lines were developed upon exposure of A1-5 parental cells to low dose EMS. At 32°C the p53 in A1-5 cells moves into the nucleus and binds DNA and induces p21 mediated growth arrest. Mutant cells containing a mutation within p53 or its downstream pathway can grow at 32°C and form colonies. Using this procedure, 40 colonies were isolated, from which 20 have been successfully maintained and to some extent characterized.
these cell lines it was discovered that ALTR-9 and ALTR-18 express a mutant p53 protein, whereas ALTR-17 and ALTR-24 express a p53 which is identical in sequence to the p53 expressed in the parental A1-5 cells. Surprisingly both cell lines, expressing wild type and nuclearly localized p53, exhibited a growth arrest resistant phenotype at low temperatures. This feature makes the cell lines not only quite interesting but also ideal for studying p53’s transactivation mechanism once it has translocated into the nucleus.
<table>
<thead>
<tr>
<th>P53 Mutational Status</th>
<th>Nuclear @ 32 °C</th>
<th>Cytoplasmic @ 32 °C</th>
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</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>ALTR-17</td>
<td>ALTR-1</td>
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<tr>
<td></td>
<td>ALTR-24</td>
<td>ALTR-7</td>
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<td>ALTR-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALTR-25</td>
</tr>
<tr>
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<td>ALTR-3&lt;sup&gt;Arg153&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>*ALTR-18</td>
<td>ALTR-4&lt;sup&gt;Pro174&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>ALTR-26&lt;sup&gt;Arg263&lt;/sup&gt;</td>
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</tbody>
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Table1. Summary of p53 subcellular localization and Mutational status in ALTR cell lines. The missense mutation of each mutant cell line is listed as superscript. * indicates that the mutations in these cells have not be verified.
VIII. Statement of objectives:
Owing to the fact that p53’s structure has been shown to effect its activation and its ability to function as a transcription factor, which is known to be the most important function of the p53 tumor suppressor protein, it is important to determine how p53’s structure correlates with its function in-vivo. **Hypothesis:** P53’s transcription factor activity is directly regulated by its conformation and form leading to activation or suppression of downstream target genes and finally to induction of cell cycle arrest or apoptosis. To test the above hypothesis my research focused on three areas.

**Specific Aim I:** To determine if various structural forms of p53 exist in cells and whether the various forms of the p53 protein can bind DNA in-vivo using the genetically altered rat fibroblast cell lines, “ALTR” cells, and conformation-specific antibodies.

**Specific Aim II:** To resolve the ability of various forms of p53 to induce or repress downstream target genes using Microarray technology.

**Specific Aim III:** To resolve the relationship between p53 structure, function and biological outcome.
CHAPTER 2

MATERIALS AND METHODS

Cell culture and antibodies

A1-5 cells are rat fibroblast cell lines transfected with multiple copies of the temperature-sensitive murine p53val135 gene (Martinez et al., 1991). A1-5 and ALTR cell lines were maintained in DMEM medium, containing 10% fetal bovine serum, 100 u/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Gaithersburg, MD, USA) with 5% CO2. A1-5 cells were incubated at 37°C unless otherwise noted, and ALTR cell lines at 32°C. Antibodies specific for p53 were PAb 421, 240, 246 and 242 (kindly provided by Dr Arnold Levine).

Immunoprecipitation

Cells grown on 10cm² plates to 90% confluency at the appropriate temperature were used to immunoprecipitate p53 using conformation-specific antibodies. Briefly, cells were labeled with 200 mCi ³⁵S-Methionine (New England Nuclear, Boston, MA, USA) per plate in DMEM containing 10% FBS for 1h after starving for 1h with DMEM (Methionine-free) containing 10% fetal bovine serum. Cells were lysed on ice for 10 min in 1 ml Lysis buffer (50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 5% NP-40, 1 mM PMSF, and 1 mM aprotinin, leupeptin and pepstatin A) (Sigma Chemical Co.). The supernatant was retained and 30 ml 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 scintillation counter equal amounts of the cell lysates were incubated with 100 ml of antibody and 30 ml ProA-SEPHADEX beads and the tubes rotated at 4°C
overnight. The beads were washed three times with 1 ml SNNT buffer (50 mM 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 24 h before developing. Quantitation of $^{35}$S-labeled p53 protein was done using densitometry analysis using Scion Image software.

**Indirect immunofluorescence and image processing**

Cells were plated onto coverslips at an approximate concentration of 2 X 105 cells per 60 mm2 plate. The plates were then incubated at the appropriate temperature for 20h. Coverslips with attached cells were rinsed three times in cold PBS (containing 3 mM KCl, 1 mM KH2PO4, 0.2 mM MgCl2, 137 mM NaCl, and 8 mM Na2HPO4, pH 7.5), and cells fixed to the coverslips using methanol:acetone (1 : 1) for 5 min, at 20°C. For A1-5 and ALTR cells, purified PAb 242 antibody was applied and the coverslips were incubated at room temperature for 1 h after which, they were rinsed three times with cold PBS. Goat anti-mouse antibody conjugated to FITC (Sigma Chemical Co.) at a 1 : 1000 dilution was then applied for an incubation time of 30 min, 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.
**Chromatin Immunoprecipitation Assays**

Chromatin immunoprecipitations using the p53 antibodies was performed. Cells were rinsed in HBSS with 0.1% EDTA and treated with 1% formaldehyde for 10 minutes at 37°C to form DNA-protein cross-links. The cells were rinsed in ice-cold HBSS with 0.1% EDTA containing protease inhibitors (1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml pepstatin A), scraped, and collected by centrifugation at 4°C. Cells were then resuspended in PIPES buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40) containing protease inhibitors and incubated for 10 minutes on ice. Cells were then collected by centrifugation and resuspended in a sodium dodecyl sulfate lysis buffer containing protease inhibitors and incubated on ice for 10 minutes. The DNA-protein complexes were sonicated to lengths between 200 and 1000 bp as determined by gel electrophoresis. Samples were centrifuged at 14,000 rpm at 4°C to spin out cell debris, and then the supernatant was diluted 10-fold with ChIP dilution buffer containing protease inhibitors. One tenth of the sample was set aside for input control, and the remaining sample was then precleared with Protein A Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). Following preclearing, the samples were split into thirds, with two of the three samples treated with anti-P53 (PAb 242, PAb 421), whereas the third sample was left as minus antibody (-Ab) control. All samples were rotated overnight at 4°C. The chromatin-antibody complexes were collected using Protein A Sepharose and then sequentially washed with the manufacturer’s low-salt, high-salt, and LiCl buffers, then twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA). The chromatin-antibody complexes were eluted and the DNA-protein cross-links were reversed with 400 mM
NaCl at 65°C for 4 hours for all samples, including the input DNA control. All samples were treated with proteinase K, and the P53±enriched fractions of genomic DNA were recovered by phenol/chloroform extractions and ethanol precipitations, which were later quantitated using a BioPhotometer (Eppendorf Scientific, Westbury, NY). Quantitative real-time PCR was used to analyze ChIP DNA, using the ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). PCR amplification of the P53’s RE-1 of P21 promoter and GAPDH were done utilizing Syber Green (primer sequences are available upon request). Amplifications were done as outlined in Applied Biosystems, SYBR Green PCR MasterMix protocols for P21 and GAPDH, respectively. GAPDH was amplified using the printed universal conditions for 40 cycles, whereas RE-1 of P21 was amplified using the following conditions; 95°C for 1 minutes followed by 40 cycles of 94°C for 30 s, and 57°C for 45 s, and 68°C for 15s. For each experiment, the threshold bar was set within the linear range of the PCR amplification. The resulting Ct and Rn files were exported to Microsoft Excel for data and graphical analysis. Ct is the number of PCR cycles necessary to reach fluorescence intensity (an indirect measure of PCR product) within the linear range of PCR amplification. Quantification was determined by applying the comparative Ct method, as described in the ABI 7000 sequence detection user guide and others.

**Quantitative real time RT-PCR for RE-1 of P21**

A1-5 parental cells grown at either 37°C or 32°C (20h incubation), and ALTR-17 and ALTR-24 were grown at 32°C. After the cells were 70% confluent, RNA was isolated followed by real-time RT–PCR. For real-time RT-PCR analysis of p53’s RE-1 of P21
promoter and GAPDH gene expression, a reverse transcription step was performed using I-script cDNA synthesis kit (Biorad), 125 ng of total RNA in a 50uL reaction. The reverse transcription reaction was incubated at 25C for 10 min followed by 48ºC for 30 min., 95ºC for 5 min. and a chill at 4ºC. Each 25 uL PCR reaction consisted of 4uL of cDNA added to 12.5 uL of SYBR Green PCR MasterMix (ABI), 1uL of gene-specific primer mix (reverse and forward primer sequences are available upon request), and 7.25 uL of PCR water. The PCR conditions were 95ºC for 10 min., followed by 40 cycles of 95ºC for 15 sec. alternating with 60C for 1 min. p53’s RE-1 of P21 promoter and GAPDH-specific PCR was performed and the data collected using the ABI Prism 7000 real-time sequence detection system (Applied Biosystems). Dissociation curve was obtained at the end of each reaction to verify the presence of a single product with the appropriate melting point temperature for each product. The presence of a single product from each reaction and its size were also checked on a 2% agarose gel stained with Ethidium Bromide. Differences in p21 expression in our parental and ALTR cell lines were determined using the equation 2deltaCt, where the p21 Ct value for each sample is subtracted from its corresponding GAPDH Ct value for normalization.

Transient Transfection Assay

Transient Transfection was conducted by cationic lipid-mediated DNA Transfection using Lipofectamine. Briefly, 3.0X10^5 cells were seeded In 35 mm dish and the cells cultured until they reached confluency of 80%. The cells were washed with Serum/antibiotic-free DMEM medium prior to Transfection. Aliquotes of 2ug of p21-luc plasmid and 50ng of renilla luciferase plasmid or 3 ug of MDM2-luc plasmid and
50ng of renilla luciferase plasmid were purified with Qiagen tips and 2ul of lipofectamine reagent diluted in 100 ul of serum/antibiotic-free DMEM medium were mixed gently and incubated at room temperature for 60 min. After adding 800 ul of serum/antibiotic-free DMEM medium the lipid-DNA complex was placed onto the cells. Transfection was performed at 37°C in an incubator under 5% CO₂ for 1h. After Transfection the lipid-DNA complex was replaced by fresh DMEM including 10% FBS and antibiotics. The Cells were then cultured for 1.5 hours before harvest. For luciferase reporter assays cells were washed three times with PBS and lysed on the dish with 200 ul lysis buffer (Promega, Heidelberg, Germany) for 15 min at room temperature. The cell lysate was cleared by centrifugation (14 000Ug for 5 min). Twenty ul of the lysate were mixed with 100 ul luciferase assay reagent and assayed in a luminomtere according to the Promega instruction manual. Unless indicated each assay was performed in triplicate and repeated four times. Data are presented as mean values with the respective standard deviation.

**Adenovirus transfection**

Mammalian cell lines plated in 60 mm culture dishes (in DMEM containing 10% fetal calf serum) were infected, at appropriate temperatures, for duration of 48h one day after plating. The cultures used for this experiment had attained a confluence of 50% prior to transfection. Purified Adenovirus-p21 was added directly to the culture medium to give a final concentration of 5.0 x 10⁹ pfu/ml. Cells were also infected with an Empty vector as a positive control. Previously, the susceptibility of different cell lines to adenovirus-mediated gene transduction was determined by visualization of GFP in cells infected with adenovirus expressing GFP. Next, the infected cells were pulse labeled with 10 μCi/ml
H\textsuperscript{3}-thymidine (Perkin Elmer, USA) for 35 minutes at 37°C. Next cells were washed 4 times with ice cold PBS. Next, 500 µL of Lysis buffer (50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 5% NP-40, 1 mM PMSF, and 1 mM aprotinin, leupeptin and pepstatin A) (Sigma Chemical Co.) was added and cells were pipetted vigorously. Next, 500 µL of TCA was added to each sample and samples were left on ice for 10 minutes. Next the samples were added to Manifold and washed first with 1ml of 70% ethonal and then with 1ml of acetone. Next, disintegrations per minute were estimated by liquid scintillation counting.

**Preparation of nuclear extracts**

A1-5 cells and their growing conditions have been in detail elsewhere (Martinez et al., 1991). To prepare nuclear extracts, twenty 10 cm plates of A1-5 cells were grown to 95% confluency at 37°C and then shifted to 32°C for 3.5 h. Cells were scraped from the plates and nuclear extracts prepared as described by Dignam et al. (1983). Protein concentration of the extracts, determined using the Biorad protein assay, was typically about ten micrograms per microliter. Three different extracts were used in these experiments.

**Electrophoretic mobility shift assays**

To test for DNA binding activity, 10 nanograms of labeled oligonucleotide, 1 mg acetylated bovine serum albumin (GIBCO/BRL, Gaithersburg, MD), 2 mg poly d(I-C) (Boehringer Mannheim, Indianapolis, IN), and 25 mg of proteins from the nuclear extracts were combined in a 20 ml reaction mixture that contained final concentrations of 72 mM KCl, 13% glycerol, 10.6 mM HEPES (pH 7.9), 0.1 mM EDTA and 0.8 mM
MgCl2. Reactions were incubated at 15°C for 1 h, and then applied to a 1x tris-borate, 5% polyacrylamide gel containing 2.5% glycerol. Gels were equilibrated at 4°C and pre-electrophoresed at 200 V for at least 1 h prior to applying samples to prevent thermal denaturation of p53val135. After electrophoresis, gels were dried and then autoradiographed on Kodak X-Omat® film at 7808°C with an intensifying screens.

**Oligonucleotides**

The following oligonucleotides were used in these studies:

p21, \[5\text{'}-\text{GATCGAACATGTCCCAACATGTTG-3\text{'}, \]
\[5\text{'}-\text{CTAGCAACATGTTGGACATGTTC-3\text{'}, \]

Double stranded probes were made by annealing equal molar quantities of the single stranded oligonucleotides and labeling with α-32P-dATP using Klenow polymerase.

**Oligo GEArray® Rat p53 Signaling Pathway Microarray**

Oligo GEArray® Rat p53 Signaling Pathway Microarray kit was obtained from SuperArray Inc. (Bethesda, MD, USA). A1-5 and ALTR cells were plated at a density of 1.5 x 10⁴/mL on 10-cm dishes (Falcon) were cultured until 70% confluence. The A1-5 cells were divided into two groups: incubated at 37°C and incubated at 32°C (for 20hrs). A1-5 at 37°C served as the control group. Next, total RNA was isolated with the use of a RNeasy Midi Kit (Qiagen Inc., Valencia, CA, USA), and 3g RNA was used as a template to generate Biotin-16-dUTP-labeled cDNA probes according to the manufacturer's instructions. The cDNA probes were denatured and hybridized at 60°C with the SuperArray membrane, which was washed and exposed with the use of a chemiluminescent substrate. To analyze the SuperArray membrane, we scanned the membranes via a CCD camera and imported it into Adobe Photoshop as a TIFF file. The
image file was inverted, and the spots were digitized with the use of GEarray analyzer
program (SuperArray Corp.), and normalized by subtraction of the background as the
average intensity value of 2 blank spots. The averages of 2 GAPDH spots were used as
positive controls and set as baseline values with which the signal intensity of other spots
was compared.
CHAPTER 3

VARIOUS FORMS OF P53 CAN BIND DNA

Abstract

P53 is a tumor suppressor gene that plays a crucial role in suppressing tumorigenesis by inducing either cell cycle arrest or apoptosis in cells with DNA damage. In more than 50% of tumors p53 is inactivated by gene mutations. However, there have also been reports of tumor cells in which p53 remains wild type and is present in elevated concentrations. Here we utilized a set of mutant cell lines which, unlike the parental A1-5 cell line which expresses a mouse tsp53 and becomes growth arrested at 32°C, are capable of growth at this same incubation temperature. We found that the tsp53 in the two cell lines, ALTR-17 and ALTR-24, was identical to the parental A1-5s and concentrated in the nucleus at 32°C. Examination of both lines revealed that p21 was induced at 32°C, although to a lesser extent than in parental cells and that the p21 genes were not mutated. Interestingly, evaluation of the conformation of tsp53 using conformation-specific antibodies showed that the protein existed in different forms, which were found to bind DNA using ChIP assays, and which we showed could induce expression of a p21 reporter construct. We conclude that the tsp53 may exist in various forms capable of binding DNA.
I. Introduction


Wild-type p53 is structurally flexible (Milner, 1995, Hainaut et al, 1995) and during cell division it reversibly assumes distinct conformations (Milner et al, 1990, 1991). Hupp et al. (1992) showed that p53’s conformation also regulates its DNA binding activity. Under normal conditions, p53 is present in cells in a latent form which is unable to bind DNA. However, as the allostERIC model (Hupp et al., 1995) predicts a switch between the latent and active conformations of the p53 through repositioning of the C-terminal domain of the protein can activate DNA binding activity of p53’s core domain. Interestingly, different conformations of p53 correlate with different cellular growth characteristics, with the wild-type conformation correlating with growth suppression and mutant conformation promoting cell proliferation in genotypically wild-type p53 expressing cells (Milner et al, 1995). Both mutated and wild-type

Mutations that inactivate p53 are frequent and occur in more than 50% of all human tumors suggesting that inactivation of p53 is a key step in tumorigenesis (Bartek, 1990). That p53 is a conformationally labile protein whose structure is easily disrupted is strengthened by the relationship between mutations and structure of the protein. The crystal structure of the p53 core domain bound to consensus DNA (Cho et al, 1994) revealed that the DNA-binding ability of p53 can be altered through two different mechanisms. First is a class of mutants referred to as DNA contact mutants, which causes loss of sequence-specific DNA binding without necessarily having a discernible effect on the secondary structure. Many such single residue changes occur in the DNA binding region and render the mutant product unable to bind to DNA and promote transactivation (Benchimol, 2001). Interestingly, the majority of the other mutants are classified as “structural” because they alter the structural elements which function in the positioning of residues that interact directly with DNA and disrupting these is predicted to inactivate p53’s transactivation activity which is the main biochemical activity responsible for the tumor suppressor function of p53 (Chao et al, 2000 and Jiminez et al, 2000). However, in some cancers the p53 protein contains no mutations yet is

The high incidence of p53 mutations in the DNA binding region of p53 and the analyses indicating that different mutations lead to different phenotypes suggest that mutant p53 may contribute to a more aggressive tumor. Hence the various p53 forms can be classified according to their conformation and behavior. In the present study, we tested these predictions by adopting a genetic approach, designed to select for alterations in the pathways that lead to p53 activation. Cells expressing the temperature sensitive p53val135 (tsp53) gene, were mutagenized and cells that were resistant to the growth inhibitory properties of wild-type p53 induced by incubating at the non-permissive temperature of 32ºC were selected and characterized (ALTR cells). We selected and further characterized two of these lines in which the p53 was localized to the nucleus at 32ºC. In an effort to show a relationship between the biological activity of p53 with its conformation and form, ALTR cells were examined for potential changes in conformation and for retention of p53 function and DNA-binding activity. Our analysis suggests that p53 can exist in several alternative forms that are capable of binding DNA. Our data suggests that the form of wild-type p53 and its conformation are closely linked to transcriptional events, which may play an important role in determining cell fate.
II. Results

*P53 in the ALTR cells is nuclearly localized at 32°C.*

Generation of the ALTR cells has been described previously. Briefly A1-5 cells, which express a tsp53, become growth arrested at 32°C in a p53 dependent manner (Martinez et al., 1991, Gaitonde et al, 2000). ALTR cells were generated by mutagenizing A1-5 cells and then selecting for cells that could grow at 32°C. Two cell lines, ALTR-17 and ALTR-24, were selected for further characterization. To confirm that the ALTR cells proliferate at 32°C we conducted growth curves (Figure 3-1). We show that at 32°C the growth rate is slow but that the cells do proliferate when compared to A1-5 cells incubated at same temperature, which are completely arrested. Interestingly the growth rate of ALTR-24s was slightly greater than that of ALTR-17s. We next examined the subcellular localization of tsp53 using indirect immunofluorescence (Figure 3-2). When studying the subcellular localization of tsp53 in these cells, the photomicrographs show that the tsp53 in both ALTR-17 and ALTR-24 cells translocates from the cytoplasm at 37°C and is concentrated in the nucleus at 32°C, similar to what we observe in parental A1-5 cells, however, A1-5 cells become growth arrested and the ALTR cell lines do not. This suggested that the tsp53 in the ALTR cells is non functional as a growth suppressor.
Figure 3-1. Growth curve of the Parental A1-5 and the ALTR cells following temperature shift. Cells were grown in 10% FCS DMEM for 192 hours and harvested every 24 hours and the number of cells counted. The curve indicates the number of cells observed per cell line for each temperature.
Figure 3-2. 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 h and indirect immunofluorescence using PAb 242 to determine the subcellular location of p53. (A, B) represent confocal images of A1-5 cells maintained at 37°C and 32°C respectively. Confocal images of ALTR-17 cells (C and D) and ALTR-24 cells (E and F) maintained at 37°C and 32°C respectively, are also represented. Photographs were taken using a 60x objective lens.
Non-growth arrest phenotype of the ALTR cells is not due to lack of p21WAF-1 expression.

It was paradoxical that the tsp53 could induce growth arrest in A1-5 cells when localized to the nucleus but not in the ALTR cells. One simple explanation is that the tsp53 in the ALTR cells was mutated and so we sequenced the p53 transcript in its entirety in both cell lines and found that it was identical to that found in the parental cell line (data not shown).

Another possibility was that the p21WAF-1 protein was either not expressed or could be defective. P21 mediates cell cycle arrest by binding to and inhibiting cyclin-cyclin-dependent kinase complexes; kinase activity of these complexes is essential for the coordinated transitions between cell cycle phases (Deng et al, 1995; Gu et al, 1993; Harper et al, 1993; Xiang et al, 1993). To test this hypothesis, we measured levels of p21WAF-1 protein via western blot (Figure 3-3a). Following temperature shift from 37°C to 32°C, p21 protein’s level is increased in the parental A1-5 leading to cell cycle arrest. Unexpectedly, we observed that levels of p21WAF-1 increased in the ALTR-17 and ALTR-24 cell lines although expression is reduced relative to the parental A1-5s at 32°C. To confirm our western blot results, we utilized Quantitative Real Time PCR to assess the levels of p21WAF-1 mRNA and compared them with GAPDH which was used as a control (Figure 3-3b). In accordance with our previous data, we observed that p21WAF-1 is induced in the both cell lines at 32°C, with a higher induction in ALTR-17s. Furthermore, when the p21WAF-1 gene was sequenced we found it to be wild type in both cases (data not shown).
These observations suggested two possibilities, either that the cell cycle inhibitory pathway downstream of p21WAF-1 was nonfunctional or that the level of p21WAF-1 induced by the tsp53 in the ALTR cells was insufficient to bring about growth arrest. To investigate this all three cell lines were infected with a p21WAF-1 expressing adenovirus (kindly provided by Dr. Vaziri) and then the infected cells pulse labeled with H\(^3\)-thymidine (Figure 3-4). As can be seen the p21WAF-1 adenovirus caused a marked reduction in H\(^3\)-thymidine incorporation in the A1-5 cells (37°C) as well as in both ALTR cells when compared with the untreated controls and cells infected with the empty vector. This indicated that the p21 pathway downstream of the p21WAF-1 protein was in tact. Our results also implied that, although the tsp53 in the ALTR cells was capable of inducing gene expression, its transactivation function was compromised.
Figure 3-3. Presence of p21WAF-1 in the ALTR cells. (A) Western blot analysis was performed to determine the expression of p21 protein and B-actin in the total cell extracts obtained from parental A1-5 and ALTR cells at 37°C and 32°C. Cells were plated on 60-mm² dishes and harvested at 80% confluency, and 130 mg of protein was used for Western blot analysis. B-actin was detected as a loading control. (B) Total RNA was isolated and following a Reverse Transcription step, Real Time PCR was performed and levels of p21 transcript was measured in A1-5 at 37°C (negative control) and 32°C (positive control) and ALTR-17 (32°C) and ALTR-24 (32°C). The data was normalized against GAPDH control and experiment repeated in triplicate.
Figure 3-4. Adenovirus-P21 induction by indigenous p53 in A1-5 and ALTR cells. A1-5 cells incubated at 37°C and 32°C were used as negative and positive control, respectively. A1-5 cells, ALTR-17 (32°C) and ALTR-24 cells (32°C) were transfected with Ad-p21 and Empty Vector control (vec) or not transfected (NT). Next all cells were pulse labeled with H\(^3\)-thymidine and the incorporation was measured with scintillation counter.
Conformation of p53 in ALTR cells differs from A1-5 parental cells and from each other.

The secondary structure of the p53 protein is an important regulator of its functioning and has been shown to control its ability to bind DNA in a stable manner (Hupp et al, 1992, McLure and Lee, 1999; Wolkowicz et al., 1998). Because the tsp53 in A1-5 cells is known to undergo changes in conformation and because the tsp53 in the ALTR cells appeared to be capable of inducing p53 target gene expression we examined the conformation of the tsp53 in each of the cell lines by immunoprecipitation using a series of antibodies (Pab240, Pab246, Pab421, and Pab242) which recognize conformational epitopes on the p53 protein (Figure 3-5). Examination of parental A1-5 cells at 37°C and 32°C showed that, as expected, the quantity of PAb 246+ protein increases markedly at the lower incubation temperature (Figure 3-5a). Interestingly, there is a similar increase in the quantity of PAb242+ protein and a concomitant decrease in the quantity of Pab421+ protein in the A1-5 cells (Figure 2-5c,d). Hence, the tsp53 protein exhibits distinctly different epitope availability at the two incubation temperatures. Analysis of the tsp53 protein in the ALTR lines reveals that tsp53 in these cells exhibits a different phenotype. Immunoprecipitation with each of the four antibodies was markedly reduced in the ALTR-17 cells suggesting that all of the epitopes were occluded relative to those of the tsp53 protein in A1-5 cells at 32°C (Figure 3-5). Interestingly, the protein in ALTR-24 cells showed a 246/240 ratio that was similar to that seen in the parental cells with the quantity of Pab246+ protein increased relative to the Pab240+ protein (Figure 3-5). Surprisingly, the quantity of Pab421+ protein was increased relative to the quantity of
Pab242+ protein, which is the exact opposite of that seen in A1-5 cells (Figure 3-5c,d). Collectively these results indicate that, even though the primary sequence of the tsp53 in the ALTR cells remains the same as that in the A1-5 cells, the secondary structure differs from that seen in the parental cells which suggest that p53 exist stably in different conformations.
Figure 3-5A. 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 35S-methionine labeling, cells were harvested, and after quantitation using a scintillation counter, 4 x 10^6 c.p.m of cell lysate immunoprecipitated using either PAb 246 (wild-type), PAb 240 (mutant), PAb 421 (C-terminus) or PAb 242 (N-terminus). The immunoprecipitated protein was resolved by SDS-PAGE analysis on 10% polyacrylamide gels.
Figure 3-5B. 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 35S-methionine labeling, cells were harvested, and after quantitation using a scintillation counter, 4 x 10⁶ c.p.m of cell lysate immunoprecipitated using either PAb 246 (wild-type) (5a), PAb 240 (mutant) (5b), PAb 421 (C-terminus) (5c) or PAb 242 (N-terminus) (5d). The immunoprecipitated protein was resolved by SDS-PAGE analysis on 10% polyacrylamide gels. Densitometry analysis was performed to determine the quantity of p53 immunoprecipitated by each antibody.
TSp53 differentially induces expression of P21 and MDM2 in the A1-5 and ALTR cells.

It has been suggested that distinct forms of the p53 protein may bind differentially to the promoters of specific p53 target genes (Gu et al, 1997; Liu et al, 1997; Wang et al, 1995). This in conjunction with our results suggesting that the tsp53 in ALTR cells existed in different forms prompted us to test whether different p53 target genes were selectively activated by the tsp53 in the ALTR cells. Binding of p53 to the consensus element can drive the transcription of reporter genes (Funk et al., 1992), and the p53 DNA binding element has been shown to be present in promotor regions of several genes including p21waf1/cip1 (El-Deiry et al., 1993; Harper et al., 1993) and the mdm2 gene (Barak et al., 1993). We previously demonstrated that the p21WAF-1 and MDM2 response elements could be categorized into distinct classes based on their ability to bind distinct sets of tsp53 containing complexes in A1-5 cells (Martinez et al.,1997). Hence, the p21WAF-1 and Mdm2 p53 response elements were chosen for comparison.

Transient-transfection assays using luciferase reporter constructs containing either the p21WAF-1 (WWP-Luc kindly provided by B. Vogelstein) or the Mdm2 p53 response element (MDM2-Luc kindly provided by Moshe Oren) were used to evaluate the transactivation properties of the tsp53 in the cell lines (Figure 3-6). As expected the most robust activation of both reporters occurs in A1-5 cells incubated at 32°C. Although the level of activity of the two reporters is markedly reduced in both ALTR cell lines relative to A1-5 cells at 32°C, it is interesting to note that the reduction was greater for the Mdm2 reporter (Figure 3-6b) than it was for the p21 promoter (Figure 3-6a). Moreover, the tsp53 in ALTR-17 cells appeared to be more capable of activating the p21 promoter
(~65% of A1-5s at 32°C) than the tsp53 in ALTR-24 cells (~50% of A1-5s at 32°C) (Figure 3-6a) and it is consistent with the greater induction of p21 protein in ALTR-17s (Figure 3-3a) and the reduced proliferation rate of ALTR-17 cells relative to ALTR-24 cells. These results suggest that the transactivation properties of the tsp53 proteins in the two ALTR lines are different.
Figure 3-6. Kinetics of p21WAF1/CIP1 and Mdm2 reporter activity in A1-5 and ALTR cells at the 32°C. A1-5, ALTR-17 and ALTR-24 cells were transfected in six-well dishes with either the WAF1-luc (6A) or Mdm2-luc reporters (6B) and Renilla-Luc as internal control. After transfection, the cells were transferred to 37°C, and luciferase activities were measured after 1.5 hr. Luciferase activities at any particular time point were corrected for variations in transfection efficiency using the internal control. Similar results were obtained in three separate experiments.
**DNA-bound p53 can exist in different forms.**

Our previous experiments suggested that the tsp53 in the ALTR-17 and 24 cells existed in aberrant conformations that were still able to induce low levels of p53 target genes expression. To explore this further we used Chromatin Immunoprecipitation (ChIP) assay to determine whether the forms of tsp53 expressed in the ALTR cells could bind the p53 response elements of the endogenous p21WAF-1 gene in a sequence specific manner. DNA-protein complexes were immunoprecipitated from the three cell lines with PAb 421, PAb 242, PAb246, and PAb240. Primers which amplified p53 response element number one in the p21 promoter (El Deiry, 1993) were then used in quantitative real time PCR to amplify the immunoprecipitated DNA (Figure 3-7). As expected, little or no tsp53 was bound to DNA in A1-5 cells incubated at 37°C. However, in A1-5 cells incubated at 32°C there was a strong signal with both Pab242 and PAb246 indicating that tsp53 was bound to the p21 promoter in these cells and that it had a wild-type conformation and that the extreme N-terminus of the proteins was open (Figure 3-7a,d). Unexpectedly, we found that the Pab421 antibody did not detect tsp53 binding in A1-5 cells at 32°C even though this antibody has strong affinity for tsp53 (Figure 3-7c) and although there is strong induction of p21 under these conditions (Figure 3-3). The only antibody that generated a signal, although weak, in the ALTR-17 cell line was PAb 246 suggesting that some of the protein in this cell line was present on the DNA in the wild type form (Figure 3-7a). The absence of signal with the remaining antibodies, suggested that none of the other epitopes detected by our antibodies were available. However in
ALTR-24 cells, strong signals were generated with PAb421 and PAb246 indicating that tsp53 was bound to DNA (Figure 3-7c,a), but that the epitopes available for immunoprecipitation were different from the tsp53 in the parental A1-5s and the ALTR-17 cells. Collectively, our results suggest that DNA-bound p53 can exist in a number of alternative forms.
Figure 3-7. P53 Response Element number one (RE-1) of rat p21 promoter demonstrates occupancy by various forms of p53. Chromatin Immunoprecipitation assay was performed on ALTR-17 and ALTR-24 cell lines both incubated at 32°C. A1-5 cells at 37°C and 32°C were used as negative and positive control, respectively. Various forms of p53 were immunoprecipitated using PAb 246 (wild-type) (5a), PAb 240 (mutant) (5b), PAb 421 (C-terminus) (5c) or PAb 242 (N-terminus) (5d) and each experiment was repeated in triplicate.
III. Discussion

In this study we utilized two mutant cell lines, ALTR-17 and ALTR-24, that are resistant to the growth inhibitory effects of tsp53 to examine the relationship between structure and function and found evidence that tsp53 can exist stably in several different forms that are capable of affecting gene expression. In vitro and in vivo studies indicate that tsp53 folds into a wild-type conformation at 32°C and binds DNA and induces cell cycle arrest or apoptosis (Milner and Medcalf, 1990). However, in the ALTR cells although the tsp53 was nuclearly localized at 32°C cell proliferation was not inhibited. This lack of growth arrest could not be explained by a defective p21 pathway since the p21WAF-1 gene in both the ALTR lines was sequenced and found to be wild-type and expression of p21WAF-1 using a p21-adenovirus did inhibit DNA replication. Curiously, we found that there was significant activation of a p21WAF-1 reporter construct in both the ALTR lines indicating that the tsp53 in both lines retained some activity. However, in both cases endogenous p21 expression was reduced and the extent of activation of a p21 reporter was less than what was seen in parental A1-5 cells. This suggested that the function of tsp53 in the ALTR lines was compromised. However, it seems likely that the level of p21 expression that is achieved in the ALTR cells is insufficient to bring about complete growth arrest. Nevertheless, the tsp53 proteins retain some transactivation capacity and the tsp53 in the ALTR-17 cells appears to have a greater capacity for induction of the p21WAF-1 reporter than does the protein in ALTR-24 cells. This is consistent with the slower growth rate of the ALTR-17 cells.
Analysis of the tsp53 protein in the ALTR cell lines suggested that the conformation of these proteins was distinctly different from that observed for the protein in A1-5 cells. A trivial explanation for this aberrant secondary structure would be that the tsp53 had sustained additional mutations that affected its conformation. There is extensive evidence in the literature indicating that some classes of mutations can result in an improperly folded structure that inactivates p53’s DNA binding and transactivation activities (Hamelin et al, 1997). However, we found no additional mutations when we sequenced the tsp53 transcript in its entirety eliminating this as a possible explanation. On the other hand when we examined the tsp53 proteins with a series of antibodies we found that the protein exhibited distinctly different epitope availabilities. The PAb246 and PAb240 antibodies are well known as conformationally specific antibodies (Milner et al, 1987, 1990, Yewdell et al, 1986, Gannon et al, 1990) and the PAb242 and PAb421 antibodies detect epitopes that are at the N- and C-termini; respectively, we determined that the tsp53 in the two ALTR lines was distinctly different by using these conformation specific antibodies. Immunoprecipitation was markedly reduced for all of the proteins in ALTR-17 cells indicating that the epitopes were unavailable, however, the 246/240 immunoprecipitation profile for the tsp53 in ALTR-24 cells was similar to that seen in A1-5 cells. One possibility is that the tsp53 in the ALTR-17 cells is conformationally mutant. However, in this case we would expect that the protein would be immunoprecipitated by Pab240. A second possibility is that the tsp53 in ALTR-17 cells is extensively bound with other cellular proteins which obscure the epitopes for these
antibodies. In support of this it should be noted that neither the Pab421 nor the Pab242 epitopes are considered sensitive to conformation.

In spite of their altered secondary structure the tsp53 proteins are still capable of affecting p53-target gene expression. ChIP analysis demonstrated that the tsp53 in ALTR-24 cells was capable of binding DNA as a strong signal was obtained with both PAb246 and PAb421. Surprisingly, although the tsp53 in A1-5 cells at 32°C was also bound to DNA it did not ChIP with PAb421 but rather with PAb242. This indicates that the epitope availability on the two proteins is different and suggests either that the two proteins are in a different conformation or that they are interacting with other cellular proteins that occlude specific epitopes. We were only able to demonstrate a weak protein-DNA binding between tsp53 and the endogenous p21 promoter in the ALTR-17 cells via PAb 246. This result is inconsistent with p21 promoter activation and p53-dependent p21 expression in these cells. A simple explanation for the inability of tsp53 in the ALTR-17 cells to generate a signal in the ChIP assays might be that the protein in ALTR-17 cells exists in a novel conformation that enables it to bind the promoter of some p53-inducible genes but which exhibits different secondary structure that can not be detected with our set of antibodies. Hence, it may be that the tsp53 in ALTR-17 cells is dysfunctional but may still be capable of binding DNA.

P53 is a structurally labile transcription factor whose structure may be modified in conjunction with the cell’s response to genotoxic stress (McLure et al, 1999, Meplan et al, 1999, Appella & Anderson, 2001). Our results support the notion that p53 can exist in several alternative forms. However, it is unclear whether the difference in epitope
availability that we observe is due to genuine differences in secondary structure or whether it is due to the tsp53 proteins being bound to different cellular proteins in various cell lines. It is possible that the ALTR cells have a defect in either the folding machinery for p53 or in the complex formation pathway which leads to p53’s response-element recognition and DNA binding which is responsible for triggering its transactivation/repression ability.

It has been suggested that p53’s ability to induce different biological outcomes when activated may be due to selective activation of p53 target genes by the protein (Deb et al., 1992, 1994, 2002; Cadwell and Zambetti, 2001). Our results indicate that the form of p53 in the nucleus can be a potent mechanism for inactivating p53’s normal function. It is tempting to speculate that the protein’s structure behaves in a lock and key model, different conformations of the protein interact with specific complexes and each unique complex transactivates/represses certain response elements.

It is evident that knowledge of the p53 status of a tumor, with respect to its form, may be required to more accurately predict the disease course. We expect our ALTR cells to provide us with a deeper understanding of how p53 is regulated and its role in several signal transduction pathways.
CHAPTER 4

FUNCTIONAL CAPABILITIES OF P21, AS A CDK/CYC INHIBITOR, IN THE ALTR CELLS

I. Introduction

The cell cycle machinery is composed of regulatory cyclin subunits complexed to cyclin-dependent kinase (cdk) subunits (Morgan, 1995). Cyclin E/cdk2 is active in mid G1 to S phase; cyclin A/cdk2 is active from S phase to M phase, whereas cyclin B/cdk1 is active at the G2-M transition (Sherr, 1993). Inhibitory proteins, which associate with and inactivate the kinases, further regulate cyclin/cdk's (Sherr et al. 1995). INK4 family proteins: p15, p16, p18, and p19, inhibit CDK4/CDK6, and Cip/Kip family proteins, which consist of p21, p27, and p57, inhibit all of the CDKs (Connell-Crowley et al. 1998, Kato, 1999, Hengstschlager et al. 1999). p21’s cDNA was cloned independently in three ways: Cip1, a cyclin-dependent kinase 2 (CDK2)-binding protein (Harper et al, 1993); Waf1, p53-inducible protein (El-Deiry et al. 1993); and Sdi1, a senescent-inducible protein (Noda et al. 1994). The p21 gene is induced through p53-dependent and independent pathways in cells after various stresses. P53-dependent p21 induction occurs by IR or UV irradiation, which causes DNA damage (El-Deiry et al. 1993). Nutrition starvation, contact inhibition, terminal differentiation, or aging of cells triggers p53-independent p21 induction that is brought about by transcription factors, including STAT family protein (Chin et al. 1996), C/EBPα (Timchenko et al. 1996), MyoD (Halevy et al. 1995, Parker et al. 1995), and vitamin D3 receptor (Liu et al. 1996). In any case, p21 induces cell cycle arrest by inhibiting CDK activity necessary for Rb inactivation.
Because of the pivotal role of cyclin E/cdk2 in cell cycle progression, molecules that inhibit their activities should have antineoplastic properties. Here we examined the function of p21 in ALTR cells to elucidate its role in the regulation of the cell cycle of ALTR cells and thus to understand how p21 fails to induce G1 arrest in these cells.

II. Results

*RT-PCR amplification and sequencing showed no mutation in p21 gene.*

Our previous data revealed that growth arrest is attenuated in our temperature sensitive p53 expressing ALTR-17 and ALTR-24 cell lines when incubated at 32°C. This finding suggested that perhaps p21 is not functional in these cells. Therefore, we performed reverse transcription-PCR analysis and sequenced the coding region of the p21 cDNA from both cell lines and the parental A1-5 cells. The p21 cDNA sequences in all cell lines examined were identical to the wild-type sequence (Figure 4b-1).
Figure 4-1  p21 sequence alignment. RT-PCR amplification and sequencing showed no mutation in p21 gene of neither A1-5 parental cell lines nor ALTR-17 nor ALTR-24 Cell lines when compared to the published p21 rat sequence.
Co-immunoprecipitation assays reveal p21 is able to bind CDK/cyclin complex in the ALTR cells.

It is well established that when functional p21 binds to cyclin E-cdk2 complexes it inhibits the kinase activity of cdk2 (Harper et al. 1993, Harper et al. 1995, Xiong et al. 1993). Because the p21 coding sequence of the ALTR cells contained no mutations, we hypothesized that the inability of p21 to suppress cdk2 activity in ALTR cells is because p21 does not bind to active cdk2 in these cells. Therefore, we performed a series of sequential IPs with antibodies, first against p21 and then against cdk2, to determine whether p21 binds to cdk2-cyclin E complexes. In extracts of parental A1-5 cells incubated at 37°C, very little cdk2/cycE co-precipitated with p21 (Figure 4-2, lanes 1 and 2) suggesting that p21 could not bind to this complex. However, most of the p21 in A1-5 parental cell line at 32°C, ALTR-17 and ALTR-24 cells (32°C) was immunoprecipitated in complex with cdk2 and cyclin E (Figure 4-2). The sequential IP studies using anti-cdk2 antibodies resulted in co-precipitation of cdk2 in the ALTR cell extracts (Fig. 4-2). The presence of these complexes with p21 supports the hypothesis that cdk2 is able to bind to p21. These observations combined with our p21 adenovirus transfection assay results suggested that the p53 pathway downstream of p21 is intact, and points to p53 itself as the main culprit in the non functional p53 growth arrest pathway in the ALTR cells.
Figure 4-2. Co-immunoprecipitation assay revealed association of p21Waf1/Cip1 with cyclin-CDK complexes at 32°C. Immunoprecipitates of Cdk-2 or p21 following temperature shift of each cell line to either 32°C were subjected to SDS-PAGE and blotted for p21 or cdk2, respectively. They show that although ALTR cells express lower amounts of both p21 and active cdk2 (lower band) both associate with each other but to a lesser extent than seen in A1-5 cells at 32°C. P21 was detected using a monoclonal anti-p21 antibody (Santa Cruz Laboratories). Cdk-2 was detected using monoclonal anti-cdk2 antibody (Santa Cruz Laboratories).
**Gel shift assay showed DNA binding capability of p53 in ALTR-24 but not ALTR-17 cell lines.**

We next investigated the ability of nuclearly localized p53 of the ALTR cells to sequence-specifically bind DNA. Nuclear extracts were prepared from parental A1-5 cells grown at 37°C (negative control) and 32°C (positive control) and also both ALTR cells grown at 32°C. The extracts were tested, using the electrophoretic mobility shift assay (EMSA), for binding with the p21 oligonucleotide. No DNA binding was observed in A1-5 parental cells at 37°C (Figure 4-3). This was expected as we had previously shown that at this temperature p53 is sequestered in the cytoplasm and is in the mutant conformation. However, p53 DNA binding was observed in A1-5 cells at 32°C. Based on our previous data, where p53 was seen to move into the nucleus and assume a wild type conformation, this observation was also expected. In accordance with our previous ChIP assay results, the EMSA assay also revealed that p53 is capable of binding DNA in the ALTR-24 cell line but not in the ALTR-17 cells. This confirms again that there must exist differences between p53 DNA binding complex in the ALTR-17 cells and the complex present in the ALTR-24s.
Figure 4-3. Characterization of p53<sup>val135</sup> DNA binding activity using Gel shift assay. Radiolabeled p21 oligonucleotide was incubated either without extract (probe), nuclear extract prepared from A1-5 cells (37°C & 32°C) or nuclear extract from ALTR-17 or ALTR-24 cells (32°C).
III. Discussion

The analysis of the p21 gene status allowed us to exclude the presence of any mutations that may account for the non-induction of G1 arrest, indicating that p21 is a potentially functional CDK inhibitor in these tumors. This was further verified by the immunoprecipitation assays which indicated that p21 was able to interact with Cdk2/ CycE complexes. Hence the lack of growth arrest phenotype in the ALTR-17 and ALTR-24 cell lines was not due to inability of p21 to associate with and inhibit these kinase complexes. One possible explanation of the lack of growth arrest characteristic of these cells is that p21 induction levels in ALTR cells are insufficient to inhibit CDK activity. Recent data indicate the existence of a dose dependent effect with regard to the ability of p21 to inhibit cyclin-CDK complexes. Zhang et al. (1994) showed that at least two molecules of p21 are required to completely abolish the growth-promoting role of the CDK complex. Hence, it is possible that even if produced by the over-expressed p53 in the ALTR cells, the quantity of p21 produced in these cells is under the level required to promote G1 arrest. Further more, the p21 protein has been shown to have a short half-life and ubiquitin-dependent and -independent modes of degradation. Zhu et al. (2005) indicated that Cdk2 activity may also target p21 for degradation. They suggested that in a situation where p21 levels are high inactive Cdk2 can stabilize p21, whereas when p21 levels are low, active Cdk2/CycE may promote p21 degradation.

Our previous studies showed that p21/WAF1 expression was p53-dependent in the ALTR cells. In the present study, we found that there was negative correlation between p53 and p21/WAF1 these cell lines. We hypothesize that due to p53’s inability
to properly bind DNA in the ALTR cells, p53-dependent p21/WAF1 activation had been disrupted and resulted in decreased p21 protein in these cells. Without p53 transactivation p21/WAF1 remained at low level and no longer functioned as a mediator in growth suppression in the ALTR cells.
CHAPTER 5

MICROARRAY ANALYSIS STUDIES OF ALTR CELLS

I. Introduction

The ability of p53 to induce apoptosis or cause cell cycle arrest through transactivation of its target genes is critical for its function as a tumor suppressor (El-Deiry et al. 1992). Jimenez et al. (2000) showed that mice possessing p53 mutated at the transcription activation domain generated by gene targeting were prone to tumor formation similar to p53 deficient mice demonstrating the importance of the transcription domain in p53 function. The main outcome of p53 activity is cell cycle arrest and apoptosis and various cell lines respond differently to the activation or introduction of p53 (Levine, 1997). It is not clear what controls the cells' choice between growth arrest and apoptosis in response to p53, but a detailed understanding of this decision may provide a better insight into cancer therapy. Recently, the use of DNA microarrays allowed for the global analysis of gene expression that characterizes the state of the cell in response to changing stimuli and to p53 (Polyak et al., 1997; Maxwell and Davis, 2000; Yu et al., 1999; Zhao et al., 2000; Kannan et al., 2000; 2001). In our study we used the A1-5 cells and their derivatives containing temperature-sensitive p53 (ts-p53<sup>val135</sup>) which acquires wild-type conformation and activity upon changing the temperature from 37°C to 32°C (Martinez et al. 1990). It has been shown previously that different cell lines expressing this ts-p53 respond differently at 32°C. For example the human lung cancer cell line H1299 stably expressing this ts-p53 exhibits growth arrest and not apoptosis,
whereas the mouse myeloid leukemic cell line LTR6 stably expressing this p53 exhibits marked apoptosis at 32°C (Levy et al., 1993; Yonish-Rouach et al., 1993).

In this study, using oligonucleotide microarrays, we analyzed p53 regulated genes in the ALTR cells which unlike their parental cell line have lost the ability to undergo growth arrest. ALTR system was analyzed previously showing that upon shifting the temperature to 32°C, p53 rapidly moves into the nucleus and p53 in the ALTR 24 cells is capable of binding DNA however, in ALTR-17 cell line we were not able to show DNA binding by p53 protein. We suggest that the differential forms of p53 which exist in the ALTR cells may be responsible for the various phenotypes displayed by the ALTR cells.

II. Results

*P53 signaling pathway Microarray indicates differences in gene expression between parental A1-5 and ALTR cells.*

It has been suggested that p53’s ability to induce different biological outcomes when activated (apoptosis vs. growth arrest) may be due to selective activation of p53 target genes by different modifications or forms of the protein (Chin et al., 1992; Deb et al., 1992, 1994, 2002; Cadwell and Zambetti, 2001). Also previous studies of gene expression of tsp53-expressing cell lines using northern blots, found many genes that were activated (like MDM2, p21) or suppressed (like c-myc) after incubation at 32°C (Levy et al., 1993). This notion, in conjunction with our previous data, prompted us to examine and compare p53 target gene expression in the ALTR cell lines. We utilized the Oligo GEArray® Rat p53 Signaling Pathway Microarray kit from SuperArray Inc. (Bethesda, MD, USA) which is designed to profile the expression of 113 key genes
known to be induced by p53. As a control, we used A1-5 cell line incubated either at
37°C (negative control) or at 32°C (positive control). RNA from all four cell lines was
collected and a biotinylated probe prepared from each of the lines which were incubated
at 32°C and additionally from A1-5 cells which were incubated at 37°C. Next, the
microarray membranes were washed with their image was captured using a CCD camera
(Figure 5-1).
Figure 5-1. Digital capture of the membrane arrays following hybridization with biotinylated mRNA extracts for the parental A1-5 (37°C & 32°C) and the ALTR cells (32°C). A. A1-5 (37°C), B. A1-5 (32°C), C. ALTR-17 (32°C), D. ALTR-24 (32°C).
Before analyzing the data, we normalized the data by subtracting genes whose expression was induced or repressed in A1-5 cells at 37°C from the ALTR array data to remove background changes in gene expression. Next, using the provided online analysis software, scatter plots were created. Scatter plots of control A1-5 cells at 37°C and 32°C demonstrate the difference in gene expression between the two temperatures (Figures 5-2 & 5-3). Since the superarray membrane is p53 pathway-specific the number of genes screened and analyzed is limited and we have considered any change of 1.5 folds or more to be significant. Most of the genes were within the 1.5fold range of expression. However, ALTR cell lines (32°C) showed progressive modulation in gene expression when compared to A1-5’s at 37°C and 32°C. When we compared A1-5s incubated at 37°C to the ones incubated at 32°C (Figure 5-2& 5-3 A) and also to the ALTR cells incubated at 32°C (Figure 5-2B&C) there was an obvious increase in scattering of these selected group of genes which was much more pronounced in the parental cells than either ALTR cell line, where many of these genes show an up or down regulation of expression by more than the 1.5fold range.
Figure 5-2. Gene Expression Clusters. A. Comparison of genes induced and repressed by more than 1.5folds between parental A1-5 cells at 32°C vs. 37°C. B. Comparison of genes induced and repressed by more than 1.5folds between parental ALTR-17 cells at 37°C vs. A1-5 cells at 37°C. C. Comparison of genes induced and repressed by more than 1.5folds between parental A1-5 cells at 37°C vs. A1-5 at 37°C.
Figure 5-3. Gene Expression Clusters. A. Comparison of genes induced and repressed by more than 1.5folds between parental A1-5 cells at 32°C vs. 37°C. B. Comparison of genes induced and repressed by more than 1.5folds between parental ALTR-17 cells at 32°C vs. A1-5 cells at 32°C. C. Comparison of genes induced and repressed by more than 1.5folds between parental ALTR-24 cells at 32°C vs. A1-5 at 32°C.
To further analyze the profile of gene expression and to distinguish genes activated by p53 from those that are changed in the control cell line we used cluster analysis. In processing the data for cluster analysis, we scaled up to 100 all the expression values that were less than 100 (and therefore may be due to noise), we then calculated the correlation of the genes. The correlation coefficient was used to express similarity. The formula for the correlation coefficient between two-dimensional profiles is:

\[
\rho_{X,Y} = \frac{\sum_{i=1}^{n} (X_i - \mu_X)(Y_i - \mu_Y)}{\sqrt{\sum_{i=1}^{n} (X_i - \mu_X)^2 \cdot \sum_{i=1}^{n} (Y_i - \mu_Y)^2}}
\]

The resulting data were subjected to complete linkage cluster analysis. Genes that were more similar were joined at lower heights in the dendrogram and those that were less similar were joined at higher heights. The Average linkage method was used to join the clusters to form a tree. ‘Average’ tends to be equally good with data that are in long chains or in clumps.

Figure 3c-4 depicts the results of the cluster analysis that contains four lanes. The first lane is A1-5 at 32°C used as positive control, the second lane represents A1-5 at 37°C used as negative control, the third lane is ALTR-17 (32°C) and the fourth lane is ALTR-24 (32°C). The cluster analysis revealed three general clusters (A-C shown in Figure 5-3). Cluster C represents mostly the house keeping genes. Cluster B represents generally genes which are mostly repressed in the parental cell line at 32°C where p53 is
in the nucleus and active. Finally Cluster A represents the genes which are mostly upregulated in the parental cell line at 32°C.
Figure 5-4. Clustering results using super-array clustering program for the 113 genes that were activated by p53 following temperature shift. The dendrogram of the genes. Each cluster is represented by a box colored according to the per cent of primary target genes. The genes are ordered according to the dendrogram on the left. The color represents Maximum induction (red) or minimum induction (green).

To compile a list of genes consistently modulated by p53, we also analyzed the
data using K-mean clustering. In K-means clustering, the goal is to break objects into
groups that have a low variance within clusters and a large variance across clusters. The
genes in the same cluster have more similar expression patterns than with the genes in
other clusters. Using the Euclidean distance method a K-means relationship for the genes
was created and diagramed. The number of genes implicated in each cluster is the header
information. Individual arrays are listed on the x-axis and data set values (post-
Normalization and Background correction) are placed on the y-axis (Figure 5-4). This
analysis provided a more detailed clustering of the genes consisting of 6 clusters. Clusters
5 and 6 represent the house keeping genes. Clusters 3 and 4 are mostly containing genes
involved in apoptosis. Clusters 1 and 2 consist mostly of DNA repair genes and cell
cycle regulation genes.

To better distinguish the differences in gene induction/repression by tsp53 in the
ALTR cells, we next compiled a list of genes consistently modulated by p53. We
selected only those genes with greater than 1.5fold induction or repression in comparison
to A1-5 cells at 32°C. This resulted in 17 upregulated genes (Table 3) and 21 down
regulated genes (Table 2) in both ALTR cell lines. The upregulated genes described in
Table 3 are represented in clusters A and the down regulated genes described in Table 2
are represented in cluster B of the clustergram previously presented (Figure 5-3).

Next the genes regulating p53 were grouped according to their functional
relationship to p53 protein and divided into the functional clusters that are implicated in
apoptosis, cell cycle, cell growth and proliferation, differentiation and DNA repair. (See
www.superarray.com for details). As can be seen in Figure 5-5, following temperature
shift from 37°C to 32°C, the nuclearly translocated p53 in both A1-5 cells and ALTR cells causes both induction and repression of various genes. The figure represents genes induced or repressed by more than 1.5 fold. Interestingly when we compared the genes induced or repressed by p53 in the ALTR cells compared to the parental cell line a change in profile was observed. In parental A1-5 cells, incubated at 32°C, ten genes were up-regulated and 6 genes down regulated. As motioned previously, we grouped the upregulated and down regulated genes into categories based on each gene’s function; i.e. DNA repair, cell cycle regulation, Cell adhesion and motility, cell growth and differentiation and apoptosis (Tables 2 & 3). However, through a closer look at the genes activated and repressed in the ALTR cells we can see that there is clearly a difference in gene induction/repression pattern in those cells. Induced/repressed genes in the ALTR-24 cell line are listed in figure 5-7b. The figure displays four upregulated and eight down regulated genes which differ from the genes expressed in the parental cell line at the permissive temperature. On the other hand, ALTR-17’s upregulated and down regulated genes are listed in figure 5-7c, where in comparison to the parental cell line at 32°C only three genes seem to be uniquely upregulated in this cell line and surprisingly nine genes are uniquely down regulated.

Interestingly each ALTR cell line also exhibits its own unique combination of induced or repressed genes. Tables 2 and 3, respectively, list both common and unique genes induced or repressed in the ALTR cell lines after comparing them to the genes induced or repressed in the parental A1-5 cells at 32°C. Further study of the genes listed in these tables reveals that ~66% of genes upregulated in the ALTR-17 cell line, in
a p53-dependent manner, are involved in the apoptotic response. The other 44% of upregulated genes are involved in cell cycle regulation. Similarly, ALTR-24 cell line exhibits the same ratio of 66% apoptotic to 44% cell cycle regulating genes, however, the genes induced were different. On the other hand, when examining the repressed genes in ALTR-17 cells, we noticed that they were mostly involved in normal cell proliferation (e.g. Serpin5b). However, genes mostly repressed in ALTR-24 cells were involved in DNA repair. Perhaps it is the specific combinations of genes induced which ultimately determines the phenotype of the cell.
Figure 5.5. K Mean Clusters. Clustering results using super-array clustering program for the 113 genes that were activated by p53 following temperature shift. K mean clustering takes advantage of differential gene expression between cell lines.
Figure 5-6. Detailed K-mean Gene Clusters. Cluster A represents genes which have an expression level is within the range of 0-200. Cluster B represents genes whose expression level is within the range of 0-400.
Figure 5-6. Detailed K-mean Gene Clusters. Cluster C represents genes which have an expression level is within the rage of 200-600. Cluster D represents genes whose expression level is within the range of 300-500.
Figure 5-6. Detailed K-mean Gene Clusters. Cluster E represents genes which have an expression level within the range of 400-600. Cluster F represents genes whose expression level is within the range of 500-800. These two clusters mostly represent the housekeeping genes.
Figure 5-7. Comparison study of genes induced in the various cell lines. A. Bar graph representing genes induced or repressed (by more than 1.5-folds) in the A1-5 cells incubated at 32°C when compared to same cells incubated at 37°C. B. Bar graph representing genes induced or repressed (by more than 1.5-folds) in the ALTR-24 cells incubated at 32°C when compared to A1-5 parental cells incubated at 32°C. C. Bar graph representing genes induced or repressed (by more than 1.5-folds) in the ALTR-17 cells incubated at 32°C when compared to the parental A1-5 cells incubated at 32°C.
Table 2. Microarray analysis of gene down-regulated in parental A1-5 and ALTR cells at 32°C. Total RNA was isolated from 70% confluent plates. RNA was analyzed with a Oligo GEArray® Rat p53 Signaling Pathway kit. The relative expression of genes was normalized to average signal of GAPDH expression. Blank spots was used as a negative control for background subtraction. Genes with more than 1.5-fold change in signal intensity were selected.
Table 3. Microarray analysis of gene upregulated in parental A1-5 and ALTR cells at 32°C. Total RNA was isolated from 70% confluent plates. RNA was analyzed with a Oligo GEArray® Rat p53 Signaling Pathway kit. The relative expression of genes was normalized to average signal of GAPDH expression. Blank spots was used as a negative control for background subtraction. Genes with more than 1.5-fold change in signal intensity were selected.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Function</th>
<th>Genes in ALTR Cells Vs. A1-5 (32C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc</td>
<td>Myelocytomatosis viral oncogene homolog</td>
<td>Both</td>
</tr>
<tr>
<td>Serpinb5</td>
<td>Serine (or cysteine) protease inhibitor</td>
<td></td>
</tr>
<tr>
<td>Ccne2</td>
<td>Similar to cyclin E2</td>
<td></td>
</tr>
<tr>
<td>Fadd</td>
<td>Fas (TNFRSF6)-associated via death domain</td>
<td>ALTR 17</td>
</tr>
<tr>
<td>Plk1</td>
<td>Polo-like kinase 1</td>
<td></td>
</tr>
<tr>
<td>I16</td>
<td>Interleukin 6</td>
<td></td>
</tr>
<tr>
<td>Xrcc4</td>
<td>Similar to XRCC4, DNA repair</td>
<td></td>
</tr>
<tr>
<td>Msh2</td>
<td>MutS homolog 2</td>
<td></td>
</tr>
<tr>
<td>Rela</td>
<td>V-rel reticuloendotheliosis viral oncogene homolog A</td>
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</tr>
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<td>Stat5a</td>
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<td></td>
</tr>
<tr>
<td>Wig1</td>
<td>Wild-type p53-induced gene 1</td>
<td></td>
</tr>
</tbody>
</table>
III. Discussion

Since p53 in all our cell lines has been found by sequencing to be wild-type (Gaitonde et al, 2000), it leads us to conclude that the form of p53 in these cell lines must be different and the change in form is significant enough to alter p53’s DNA binding and transactivation capability. Although there is a strong correlation between wild-type conformation of nuclear p53 and its DNA binding and transactivation ability, our microarray comparative study of the parental A1-5 cells and the ALTR cells indicated that it is the final form of p53 which dictates cell fate. The statistical procedures that we used to identify genes that were significantly differentially regulated in the ALTR cells compared with A1-5 controls resulted in the identification of several genes that exhibited fold changes of more than 1.5, indicating their strong transactivation. In deciding a cell's fate, the differential transactivation of a gene by more than 1.5 fold may have a significant impact when added to the cumulative effects of changes in the expression of several genes in the cell. In this initial analysis, we studied expression of 113 p53-regulated genes listed in SuperArray website. For the genes differentially regulated at 32°C, a detailed cluster analysis of the gene tree and correlograms provided graphic insight into possible coordinated gene function within a gene category, and combined with the analysis of fold expression differences, provided insight into possible differential p53 transactivation ability. In the nucleus of the ALTR 17 cells almost equal populations of mutant and wild-type conformations of p53 were observed. Although our previous ChIP assay results failed to show DNA binding ability of p53 in this cell line, the array data clearly shows induction and/or repression of several p53 target genes. This
suggests that p53 in ALTR 17 cells assumes a form unrecognizable by the conventional conformation specific antibodies such as PAb242 or 421. In spite of this, as shown by the microarray data, the form that p53 assumes in this cell line allows it to bind to certain response-elements and cause repression or induction of certain p53 target genes.

Interestingly the genes induced via this form of p53 are mostly involved in regulation and induction of cell proliferation and growth and the genes repressed via this form of p53 were mostly involved in induction of cell cycle arrest and apoptosis. In spite of the many differences between ALTR 17 and ALTR 24 cell lines; ie.P53’s conformation/form, its DNA binding ability and the p53-transactivated/transrepressed genes, in ALTR 24 cells like ALTR17 cells, genes transactivated were mostly involved in cell cycle regulation and apoptosis and genes inhibited were ones involved in induction of cell proliferation and DNA repair. Perhaps it is the specific combinations of genes induced which ultimately determines the phenotype of the cell.

These results indicate that these cells have a defect in either the folding machinery for p53 or in the complex formation pathway which leads to p53’s response-element recognition and DNA binding which is responsible for triggering its transactivation/repression ability. Our results indicate that the form of p53 in the nucleus can be a potent mechanism for inactivating p53’s normal function and that the transactivation process is closely linked to the protein's conformation. Perhaps the protein’s conformation behaves in a lock and key model, different conformations of the protein interact with specific complexes and each unique complex transacivates/represses certain response elements.
The two ALTR cells used during this study will be important reagents for examining the mechanisms involved in regulating p53 transactivation/repression ability. ALTR 17 and ALTR 24 cells will aid in dissecting the two closely linked processes of p53 transactivation which involve its conformation in the nucleus and its association with other proteins involved in complex formation for DNA binding. Changes in conformation of p53 may be mediated by post-translational mechanisms or by association with other proteins which allow p53 to fold appropriately and hence bind its response-elements differentially, either of which may be mutated in the ALTR17 or 24 cell lines. We are currently characterizing the defects in the ALTR17 and ALTR24 cells that lead to the inactivation of p53 in an attempt to understand its regulation. We expect our ALTR cells to provide us with a deeper understanding of how p53 is regulated and its role in several signal transduction pathways.
CHAPTER 6

CONCLUSIONS AND DISCUSSION

The significance of p53’s role as a tumor suppressor is represented by the high incidence of tumors in which p53 is mutated. It has been reported that in 50% of human cancers p53 is mutated (Baker et al. 1989, Hollestein et al. 1991, Nigro et al. 1989, Vogelstein et al. 1988). In the other 50% of cancers, where p53 is non-mutant, it has been reported that its function is deregulated through various mechanisms.

The experiments presented here demonstrate for the first time that (i) p53 can exist stably in various conformations; (ii) Wildtype temperature sensitive p53 can bind DNA in various forms in vivo; (iii) Different forms of p53 can activate a subset of genes transactivated by the wildtype p53.

Mutating the parental A1-5 cells generated ALTR cell lines. A1-5 cells have been transfected with multiple copies of the temperature sensitive p53 plasmid. Our studies were focused on two ALTR cell lines: ALTR-17 and ALTR-24. Both cell lines contain a temperature sensitive p53 identical to the parental A1-5 cells capable of normally translocating into the nucleus at 32°C. Surprisingly, unlike the parental cell line, neither of the ALTR cell line is able to growth arrest at 32°C. Since it is well known that p53’s main function is brought about by its DNA binding capability, this phenotype makes these two ALTR cell lines an ideal system to study regulation of p53 transactivation.

One of the most striking observations in ALTR-17 and ALTR-24 cell lines was their inability to undergo cell cycle arrest regardless of detectable levels of p21 at 32°C. However, transfecting the cells with p21-adenovirus, which augments the levels of p21
protein in the cell, did stop cell proliferation and protein synthesis in these cells. These results suggested that p21’s Cdk-inhibitory effect in the ALTR cells was concentration dependent and low level expression of p21 was an effective and important mechanism of p53-dependent growth arrest pathway inactivation in these cells. This has been observed previously in some tumors such as primary human sarcomas. Hence, our data suggest the existence in ALTR cells of a mechanism of functional inactivation of the p53 pathway occurring upstream of the CDK inhibitor p21, pointing to p53 itself as being the main perpetrator in the inactivated growth inhibitory pathway in these cells.

As mentioned previously, p53 can be structurally divided into three main regions: N-terminal, core and C-terminal domains. While its C-terminal domain is responsible for p53’s tetramerization, it is the core domain that accounts for DNA binding (Cho et al. 1994). In general, p53 core domain mutations either affect amino acid residues directly involved in DNA binding, being classified as contact mutants, or they affect residues involved in maintenance of the native fold, being classified as structural mutants. Both types of mutations lead to inactivation of p53’s wild-type function as a tumor suppressor.

Interestingly p53 in the ALTR cell lines is identical to the temperature sensitive p53^{val135} in the parental A1-5 cells without any further mutations. However, when the conformation of the protein was tested using conformation specific antibodies such as: PAb 246, which detects the wild type conformation, PAb 240, which detects the mutant conformation, PAb 421, which posses an epitope at the C-terminus of the protein, or PAb 242, with an epitope at the N-terminus, we were able to show that p53 in each ALTR cell line exhibits its own unique structural form different from the p53 present in the parental
A1-5 cells at 32°C. Most interestingly, in tumors p53’s conformation is a target for disruption leading to deregulation of its activation. The non-mutated wild-type p53 protein is often found within cells in a latent state and when activated in response to various intracellular and extracellular signals it assumes an “active” conformation, translocates into the nucleus and induces or represses its respective downstream target genes. However in some tumors, p53 is mutated in such a way which renders it unable to assume an “active” conformation and hence unable to induce cell cycle arrest or apoptosis. The DNA binding domain of wild-type p53 is revealed by specific tertiary folding of the polypeptide, this being destroyed leads to protein inactivation (Cho et al. 1994).

Since our results suggest that p53 exists stably in an alternative conformation in each ALTR cell line, we were intrigued to look into the consequences of such differences on DNA binding ability of p53. Studying the mechanism of DNA binding activity of the p53 protein is vital to understanding its function as a tumor suppressor. Evidence for the biological relevance of a wildtype p53 with an alternative conformation lies in the observation that several mutants with misfolded conformations play an important role in tumor development, as well exhibiting dominant negative effects, i.e. they can drive the active, wild-type protein into an inactive, mutant conformation (Milner & Medcalf, 1991, Sun et al. 1993). Also, It has been reported that certain mutations induces a considerable distortion in the p53 protein conformation when compared to the wild-type protein, perturbing the structure of loops L2 and L3 as well as residues in helix 2, loop L1, strand S2 and strand S20, and rendering the protein unable to bind DNA. Even though majority
of p53 mutants, with an oncogenic capacity, contain a point mutation within the protein's DNA binding domain, some tumors exhibit wild type p53 proteins without the capacity to bind DNA and cause gene transactivation. Hence, characterization of the DNA binding activity of the wild-type protein could provide insight into what other factors may be responsible in converting this tumor suppressor into an oncogene.

Overall, our ChIP assays, testing DNA binding ability of p53 in vivo, was in support of our hypothesis and showed that p53 may bind DNA in various forms in vivo. At this point it is unclear to us what comprises the different forms of p53 in the ALTR cell lines. Two possibilities exist and both possibilities seems plausible and can be supported by published data

i. Differential association with various DNA binding complexes

ii. Specific conformational changes rendering p53 more suitable towards associating with certain promoters/repressors and not others.

-Posttranslational modifications have been associated with conformational change in p53

As a transcription factor p53 has been reported to associate with many different proteins including basal transcription factors such as: TAF6, TAF9, hTAF9, TBP, TFIID, and TFIIB. TAFs have been proposed to serve as “co-activators”, mediating signals from transcription factor to the basal machinery. Some of these co-activators, such as TBP and TFIID, can stimulate p53 binding to DNA with directly binding to the N-terminus of the p53 protein them selves and without interacting with the DNA (Chen et al. 1993). Thut et al. (1995) reported that direct interaction between p53 and components of TFIID,
TAFII40 and TAFII60, is required for p53-dependent transactivation of certain genes.
Interestingly, it has also been reported that some transcription factors bind specifically to
the C-terminus of p53, i.e., PC4, JMY, and promote p53 transactivation of genes such as
Bax, which is involved in the p53-dependent apoptotic pathway. Other proteins such as,
53BP2, which was identified in a yeast 2-hybrid screen as a p53-binding protein, can also
inhibit the binding of p53 to DNA via interaction with the DNA-binding surface of the
p53 core domain. Finally, another well-known p53 interacting protein, MDM2, is known
to form a complex with a transactivation domain of p53. Relating this data back to our
findings we can see that the molecular background in each mutant ALTR cell line could
permit the differences in the epitope availabilities we observed in our
immunoprecipitation and ChIP assay studies. It is possible that any one or several of
these co-activators are augmented or mutated in each cell line leading to differential
binding of these co-activators to the p53 protein and hence differential DNA binding
patterns of the resultant complex. Clinically this is very interesting because it means that
these interfaces, where these proteins interact with p53 to form a DNA binding complex,
may be amenable to inhibition or activation by small molecule compounds that can help
restore the function of the p53 pathway in a subset of cancers.

Many strategies for rational cancer therapy have been pursued in recent years.
Among the strategies for cancer therapy used today, there is the development of drugs or
peptides that re-establish and stabilize the active conformation of p53 protein (Foster et al.
1999, Nikolova et al. 1998). Hence, it is essential to understand the molecular basis that
drives p53 folding and stabilizes its native conformation in order to understand why some
alternative conformations of the wild-type protein, lead to loss of protein function, and hence to tumors. As mentioned earlier, p53 exists stably in various conformations in each of the ALTR cell lines without having incurred any additional mutations as the parental A1-5 cells. One reason for the change in conformation of p53 could be post-translational modifications of the protein. Indeed, many phosphorylated forms of p53 are found in cells, and by phosphorylation p53 can be released from a latent state, in which it is unable to bind DNA, into an active, DNA binding state. One example of p53 activation by phosphorylation is the DNA-dependent protein kinase (DNA-PK). DNA-PK is activated by DNA damage, and one of its substrates is p53. DNA-PK phosphorylates Ser15 within the critical N-terminal region of p53, which controls the interaction of p53 with the transcriptional apparatus and with the MDM-2 protein. Indeed, recently it was demonstrated, that DNA-PK is required for the p53 response to occur (Woo et al., 1998). Another such example is the ATM kinase, the product of the ATM gene, which phosphorylates Ser15 in vivo (Banin et al. and Canman et al., 1998). Interestingly ATM is also able to activate specific phosphatases which dephosphorylate p53 at serine 376 and in turn enable DNA binding of p53 and its transcriptional activation. Another set of proteins, which bind dephosphorylated p53 at the C-terminus and activate it for DNA binding.

Recruitment of co-activators and Histone acetylation to the p21 promoter requires acetylation of p53 by CBP and TRRAP (Berger et al. 2001). P53 lacking the ability to be acetylated is severely defective for transcriptional activation and G1 arrest, yet it can bind DNA. This is a very close description of the phenotype seen in ALTR-24 cell line, where
low levels of p21 are expressed yet p53 is shown to bind DNA. Hence substitution at acetylation site can perhaps change p53’s conformation in such a way that it can no longer bind the response element in p21 gene yet it can bind other response elements in the genome. From these data it can be inferred that modified p53 proteins either activate only a specific subset of the genes induced by wild type p53. Indeed, our microarray results suggest that experimental evidence suggests that some each unique form of p53 present in each ALTR cell line can enhance the expression of a subset of genes upregulated in the parental cell line, while repressing expression of another unique subset of genes.

In recent years, the search for molecules able to rescue non-functional, misfolded p53 has been one of the most pursued therapeutic strategies (Friedler et al. 2002). Hence, the identification of important conformations of the protein at the edge of correctly folded/misfolded pathways, such as the ones that we have observed in the ALTR cells, may be helpful for a better characterization of such states and hence for the design of reactivating drugs.

Based on our data, we have proposed a model (Figure1) of how p53’s form relates to its ability to bind and transactivate/repress genes and lead to either cell cycle arrest or cell proliferation. We propose that at 37°C, P53 in the parental cell lines is in a form in which only the epitope for the mutant conformation-specific antibody is available and all other epitope are either hidden due to conformational changes or occluded by other p53 interacting proteins. Upon temperature change, to 32°C, the p53 protein in these cells undergoes a conformational change, assuming the “activated” conformation and epitopes
Figure 6-1. Schematic model of various p53 forms and their ability to bind DNA and cause growth arrest. The model proposes that p53 may exist stably in various forms in the cell. At 37°C p53 assumes a mutant conformation, recognized by conformation specific antibody PAb240, and is unable to bind DNA. At 32°C p53 may assumes a wildtype conformation, recognized by conformation specific antibody PAb246 and also by either PAb242 (as in A1-5 cells) and this form of p53 is also capable of binding DNA in such way that leads to activation of downstream genes responsible for growth arrest. However, p53 may also assume a second conformation at 32°C, recognized by PAb246 and PAb421 as seen in ALTR-24 cells. This conformation is also able to bind DNA yet induces a subset of downstream genes which play a role in cell growth and proliferation. Finally, at 32°C, p53 may assume a third form unrecognized by conventional conformation specific antibodies. This form also seems to be able to bind DNA and induce downstream genes involved in cell growth and proliferation.
for both the wildtype-conformation-specific and the N-terminus PAb 242 antibodies become available, however the mutant and the C-terminal epitopes are either hidden or occluded under these conditions.

Our panel of antibodies was unable to recognize any of the epitopes on the ALTR-17 cell line at 32°C. Based on our microarray data we were able to show that p53 does induce and repress a unique subset of genes in this cell line, hence we conclude that the p53 in these cells assumes a conformation where the epitopes recognized by our antibodies are occluded or hidden. We were also unable to show DAN binding of this cell line in our EMSA assay. There are various explanations for this finding. One simple explanation could be that the EMSA assay was not preformed optimally and the protocol needs to be modified and the experiment repeated. Another possibility is that the Oligonucleotides, representing the consensus p53-binding site in the p21/waf1 gene promoter, used correspond to the response element towards which the ALTR-17s’ form of p53 exhibits a very low affinity. It has been published (El Deiry et al, 1993) that p21 contains three response elements for the p53 protein and that p53 binds strongly to only one the elements. Hence repeating the EMSA assay with the other response elements might yield different results.

Finally our model indicates, in accordance with our data, that in ALTR-24 cell line at 32°C, p53 binds DNA in a yet different form, which is recognized by both the wildtype and the C-terminal recognizing antibodies and ignored by the mutant and the N-terminal antibodies. This is the exact opposite of the form seen in the parental A1-5 cells at the same temperature. Microarray data indicated that the panel of genes induced by
this form of p53 is different from the ones induced by either the A1-5 or the ALTR-17 cell line, and our growth curves indicates that the induced genes allow the cell to bypass growth arrest.

Taken together, our results indicate that a wild-type, native p53 can be induced to take on a mutant-like conformation which exhibits biophysical behavior similar to a hot-spot tumorigenic protein. Hence, through a better understanding of the p53 forms in both ALTR-17 and ALTR-24 cells, we might be able to characterize structurally this other stable alternative conformation of the wild-type p53 protein, providing targets for the development of antagonists capable of blocking protein misfolding, potential drugs against tumoral diseases.
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