THE ROLE OF p53 AMINO TERMINAL DOMAIN IN REGULATING p53 CONFORMATION

Thesis submitted to Jawaharlal Nehru University

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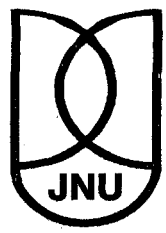
Doctor of Philosophy

In

Biotechnology

By

Amjad Ali



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Certificate

This is to certify that the work entitled **"The role of p53 amino terminal domain in regulating p53 conformation"** submitted to School of Biotechnology, Jawaharlal Nehru University, New Delhi, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Biotechnology, embodies original research work carried out **by Mr. Amjad Ali** in the School of Biotechnology, Jawaharlal Nehru University, New Delhi. He has worked under my guidance and supervision. This work is original and has not been submitted so far in part or full for any other degree or diploma of any other university.

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To beloved parents

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Amjad Ali

Abbreviations

%	Percentage
O/N	Overnight
°C	Degree Celsius
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
A_{405}	Absorbance at 405 nm
A_{595}	Absorbance at 595 nm
A_{600}	Absorbance at 600 nm
Amp	Ampicillin
APS	Ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate (BCIP)
bp	Base pair
BSA	Bovine serum albumin
Ci	Curie
cpm	Counts per minute
dl	Decilitre
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
gm	Grams
HEPES	N-2-hydroxyethylpiperzine-N-2 ethane sulphonic acid

IPTG	Isopropyl-β-D-thio-galactopyranoside
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
M	Molarity
mCi	Milli Curie
mg	Milligram
min/mins.	Minute/Minutes
ml	Millilitres
mM	Milli molar
NBT	Nitro blue tetrazolium chloride
ng	Nanogram
O.D.	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulphonyl fluoride
PNK	Polynucleotide kinase
pNPP	*para*-Nitrophenyl Phosphate
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Seconds
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA

TE	Tris EDTA
TEMED	N, N, N', N', tetramethyl ethylene diamine
Tris	Tris (hydroxymethyl) amino methane
U	Unit(s)
UV	Ultraviolet
μCi	Microcurie
μg	Microgram
μL	Microlitre
μM	Micromolar

Contents

Introduction

INTRODUCTION

p53: Structure and Function

The *p53* gene, first described in 1979, was the second tumor-suppressor gene to be identified. It was originally believed to be an oncogene, a cell-cycle accelerator but genetic and functional data obtained ten years after its discovery showed it to be a tumor suppressor (Vogelstein, 2000). The importance of p53 protein lies in the fact that the p53 protein does not function correctly in most human cancers. In about half of these tumors, p53 is inactivated directly as a result of mutations in the *p53* gene. While in many others, it is inactivated indirectly through binding to viral proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53 (Vogelstein, 2000).

In laboratory animals, the absence of p53 permits the occurrence of numerous genetic alterations, including gene amplification and abnormal chromosome numbers, and leads to the development of a variety of tumors in young adults (Donehower, 1992; Fukasawa, 1997; Yin, 1992). These findings underscore the pivotal nature of p53 in preventing tumour development and are consistent with the well established occurrence of loss of p53 function in the development of a wide range of human tumours (Hollstein, 1996). The importance of p53 in the cancer lies in the fact that p53 was the first commercially available gene therapy treatment to be approved (Pearson, 2004).

The human *p53* gene is localized to the short arm of chromosome 17 (17p13) and spans about 20 kb of DNA. It is composed of 11 exons, the first of which is non coding and is localized 8-10 kb away from coding exons, 2 to 11 (Benchimol, 1985; Isobe, 1986; Miller, 1986; Oren, 1985).The human p53 gene encodes a 393 amino acid protein that can be divided into four major functional domains (Fig. A). The amino terminal domain of p53 consists of an intrinsically disordered transactivation domain (TAD) and a proline-rich region. It is followed by the central, folded DNA-binding core domain that is responsible for sequence specific DNA binding. The core domain is connected to the tetramerization that regulates the oligomerization state of p53 domain via a flexible linker. At its carboxy terminus p53 contains the negative regulatory

2domain. This domain is natively unfolded and is rich in basic amino acids (mainly lysines) and binds DNA nonspecifically (Joerger, 2008). Cross species comparison of amino acid sequences of the p53 protein showed the existence of five highly conserved regions within the amino acid residues 13-23, 117-142, 171-181, 234-250 and 270-286 (Soussi, 1990; Soussi, 1996). These regions, termed domains I-V, were expected to be crucial for the p53 functions.

Although, p53 is a nuclear protein, but it shuttles between the cytoplasmic and nuclear compartments in a cell cycle dependent fashion (Liang, 2001). For the nucleo-cytoplasmic shuttling of p53 in the cell, it contains nuclear localization signal (NLS) and nuclear export signal (NES). Three NLS are reported in the p53 protein, out of which one is strong and spans between residues 316 -322. A highly conserved leucine-rich NES has been identified in the tetramerization domain of p53 (326-354) (Liang, 2001), a NES is also identified in the transactivation domain (amino acid residues 11-27) (Zhang, 2001). The p53 protein is a transcription factor that enhances the rate of transcription of genes that contain p53 binding site (sites). As transcription factor, p53 regulates the expression of genes involved in a variety of cellular functions, including cell-cycle arrest, DNA repair, and apoptosis (Vogelstein, 2000). The p53 protein was also shown to repress the transcription of a variety of genes including c-MYC, Cyclin B, VEGF, RAD51, and hTERT (Laptenko, 2006). In addition p53 also works in the transcription independent manner in the apoptotic pathway (Chipuk, 2004; Moll, 2005; Leu, 2004).

In normally growing cells, p53 protein levels are very low. In response to different cellular stresses, such as DNA damage, oxidative stress, inappropriate cell proliferation driven by oncogene activation, telomere erosion, nutrient deprivation, radiations and hypoxia, and so on, p53 is stabilized by covalent and noncovalent modifications, succeeding specific protein–protein interactions that in turn, rapidly increase p53 protein levels and induce the expression of genes involved in cell cycle arrest to allow repair processes or failing that, induce the expression of genes promoting apoptosis (Laptenko, 2006). Genotoxic damage caused by chemotherapy or radiotherapy induces p53 overexpression to control the rate of proliferating damaged cells, thus triggering the mismatch repair or apoptotic pathway. The p53 protein also has its darker side, as slight constitutive hyperactivation of p53 results in an alarming premature-ageing phenotype in

mice (Tyner, 2002; Maier, 2004). In humans, a polymorphism in p53 that results in a slight reduction in its activity is associated with an enhanced cancer risk, but also with increased longevity (van Heemst, 2005). How p53 might promote ageing is not yet clear, although a contribution of p53 to cellular senescence and the limitation of the proliferative capacity of stem cells has been proposed (Sharpless, 2004).

In a nutshell, p53 is primarily a sequence-specific transcriptional activator. It binds to cognate p53 responsive elements within the genome and activates the transcription of genes residing in the vicinity of these binding sites. Although p53 can also function in a transcription-independent manner (Fuster, 2007), the best understood functions of p53 have been attributed to its DNA binding and transcription activity.

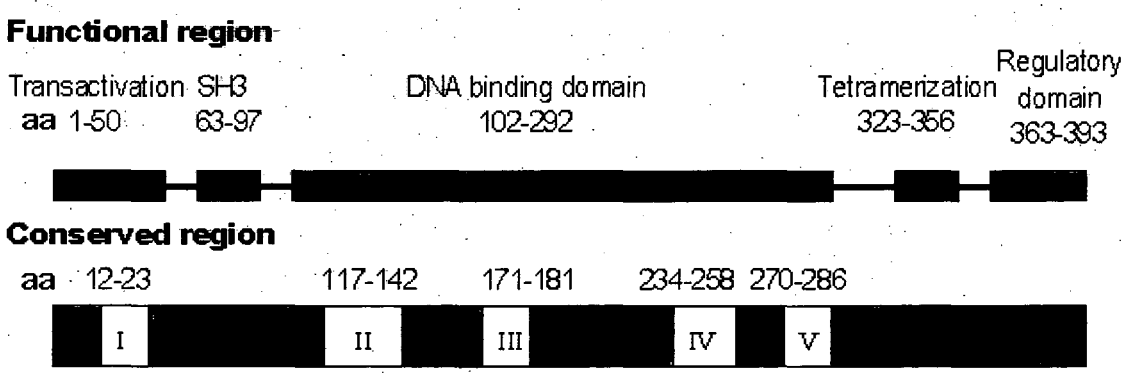


Fig. A. Structural and functional regions of the p53 protein Functional regions and corresponding amino acid residues are shown on top. The bottom represents the evolutionary highly conserved domains I-V. (May, 1999).

Amino terminal domain

The amino-terminal domain of p53 encompassing residues 1-93(Wells, 2008) of the protein is remarkably acidic and its primary structure is similar to other activation domains. It consists of two ill defined and contiguous transcriptional activation subdomains (amino acids 1–42 and 43–63) (Candau, 1997; Zhu, 1999) and an adjacent proline-rich domain (amino acids 62–91) containing five copies of the sequence "PXXP" (Cain, 2000) as well as a repressor domain

between the proline rich domain and the DNA binding domain (residues 100-116) (Curtin, 2005).

Structure of the amino terminal domain

Unlike the DNA binding and tetramerization domain which are well structured the amino terminal domain of p53 is natively unfolded (Bell, 2002; Dawson, 2003), although a residual secondary structure is observed in regions of functionally important hydrophobic residues (Lee, 2000). However recently using paramagnetic relaxation enhancement (PRE) it has been shown that the p53TAD contains some global organization to the transient secondary structures and this global organization is important for the function of p53TAD (Vise, 2007). The unbound full-length p53 transactivation domain is populated by an amphipathic helix and two nascent turns. The helix is formed by residues Thr18 -Leu26 (Thr-Phe-Ser-Asp-Leu-Trp-Lys-Leu-Leu), whereas the two turns are formed by residues Met 40 -Met 44 and Asp 48 -Trp 53, respectively (Lee, 2000). Amino acid residues 15–29 adopt α-helical conformation upon binding to a hydrophobic cleft in the N-terminal domain of MDM2 and MDM4 (Kussie, 1996; Popowicz, 2007). In addition *Chi* et al (2005) reported two mini-motifs (turn I, residues 40–45 and turn II, 49–54) which were shown to bind MDM2.

The regions of the nascent helical structure that are present in the native state of p53 become rigid and fully folded when the transactivation domain binds with the partner proteins. A characteristic is the presence of molecular recognition features, short segments of about 20 residues that undergo disorder-to-order transition upon binding to their protein or nucleic acid binding partners (Mohan, 2006; Vacic, 2007). Regions within TAD2 fold into amphipathic α-helices upon binding to replication protein A (Bochkareva, 2005) and to the Tfb1 subunit of yeast TFIIH (Di Lello, 2006). The amino terminal domain is also reported to take part in the formation of p53 tetramer. The N-terminus (residues 1-63) of one p53 monomer binds with the C-terminus (residues 323–393) of another p53 molecule to form a dimer (Okorokov, 2006).

The proline-rich region that links the TAD to the DNA-binding domain in human p53 contains five PXXP motifs (Walker, 1996) that mediate numerous protein-protein interactions in

signal transduction through binding to Src homology 3 domains (Kay, 2000). The PXXP motifs are not conserved among mammalian p53s, but the prevalence of prolines in this region is maintained (Toledo, 2007), indicating a functional or structural requirement for a certain degree of rigidity. Small angle X ray (SAXS) have shown that the proline-rich region (residues 64–92) was stiffer and has a tendency to adopt a polyproline II (PPII) structure that projects the TAD away from the core domain (Wells, 2008, Fig. B). The mutational studies on mouse models have shown that the putative protein docking sites in this region are dispensable for tumor suppression, whereas the length of the region is crucial (Toledo, 2007).These findings support a modular role of this domain, potentially as a spacer between the different functional domains.

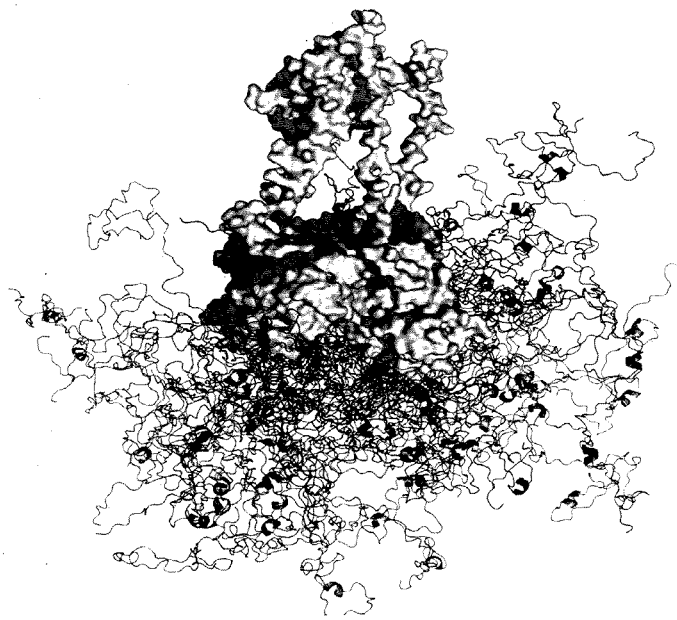


Fig. B. Modeling the N-terminal domain in the p53–DNA complex. p53CTetD (gray) and DNA (magenta) are shown in space fill mode. The flexible C-terminal domain is not shown. N-terminal domains forming the four difference monomers are shown in different colors for clarity. Twenty copies are shown for each monomer. (Wells, 2008).

Functions of the Transactivation Domain

The transactivation potential of transactivation domain mainly lies in the first transactivation domain, since the deletion of amino acid residues 20 to 42 completely abolishes the transactivation activity, which can be restored by substituting the transactivation domain of the VP16 protein at the same location (Pietenpol, 1994). Functional analysis of this region using

a library of point mutations at various residues revealed that a single point mutation was generally not able to completely abolish transactivational activity (Lin, 1994). However, simultaneous mutation of two hydrophobic residues Leu22 and Trp23 markedly impairs transactivation by p53 ((Lin, 1994). Mutation of residues Trp53 and Phe54 affect specifically the ability of p53 to regulate some of its pro-apoptotic target genes in some settings (Candau, 1997). These findings suggested that the overall structure of the transactivation domain rather than its sequence is important for the transactivational activity. These results also explained why mutations in the transactivation domain cannot be found in human cancers. Also unlike the DNA-binding region, which is highly sensitive to virtually any point mutation, the transactivation domain is sufficiently stable to be tolerant of a single mutational event (Soussi, 1996).

Further the amino terminus (residues Glu^{17} to Asn^{29}) is the region to which its negative regulator Mdm2 binds and targets p53 for both repression of its transcriptional regulation functions and also for proteasome-mediated degradation (Bond, 2005). CHIP that is responsible for the ubiquitination of wild type as well as mutant p53 and the chaperoning of p53 also binds with p53 amino terminus (Esser, 2005; Tripathi, 2007). The MDM2-binding region of the p53 TAD overlaps with parts of the binding site for the transcriptional coactivator p300, which is essential for the transcriptional function of p53. The p300 acetylates lysine residues in the C-terminal region of p53 (Goodman, 2000).

The p53 amino terminus is a site of post-translational modification of which phosphorylation is major change with 10 being observed within 100 amino acids of the N-terminus (Lavin, 2006) (Fig. C). These phosphorylations are brought about by a number of protein kinases that respond to different stress stimuli including ATM (mutated in ataxia-telangiectasia); ATR (A-T and Rad3-related), the checkpoint kinases, Chk1 and Chk2; Jun NH2-terminal kinase (JNK), p38 and others. The most frequently described phosphorylation is on Ser15 and occurs in response to different stress signals. This phosphorylation occurs rapidly in response to DNA double-strand breaks and is carried out by ATM (Lavin, 2006). ATM also mediates phosphorylation at Ser 6, 9, 15, 20, 46 and Thr 18 on p53 in response to ionizing radiation (Saito, 2002). Ser15 phosphorylation of p53 acts as a precursor for the subsequent

phosphorylation of Thr18 by another kinase such as Chk2 and may also have a bearing on Ser9 and Ser20 phosphorylations (Saito, 2003). Phosphorylations at Ser15, Thr18 and Ser20 stimulate the recruitment of other factors including p300, CBP and P/CAF that promote C-terminal acetylation that may prevent p53 from ubiquitination and subsequent degradation (Ito, 2001).

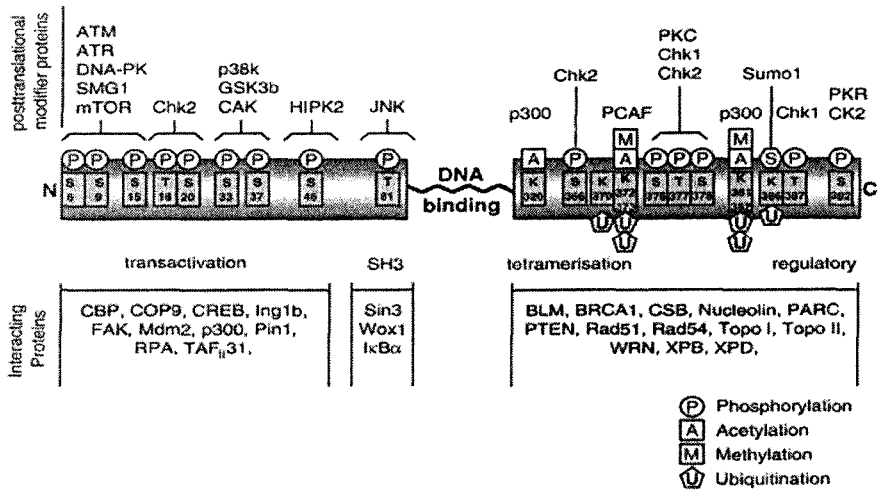


Fig. C. Post-translational modification of p53. (Lavin, 2006).

Phosphorylation of p53 at amino acid residues Ser15, Thr18 and Ser20 also switches the folded transactivation domain to more open conformations that interact with transcription factors such as p300/cAMP-responsive element-binding protein-binding protein, leading to enhancement of gene expression (Kar, 2002). Furthermore the homedomain-interacting protein kinase 2 (HIPK2) phosphorylates p53 on ser46 and may assist in dissociating p53 from MDM2 and also causes the reduced shuttling of p53 from the nucleus (Di Stefano, 2004). JNK is responsible for phosphorylation of p53 at Thr81, which stabilizes and transcriptionally activates the molecule (Buschmann, 2001).

The phosphorylation of p53 is also responsible for promoter recognition by the molecule. In mice, phosphorylation of serine residues 18 and 23 is necessary for apoptosis and tumour suppression, but not cell cycle arrest and senescence (Chao, 2006). Phosphorylation of human p53 on serine 46 has been shown to contribute specifically to the activation of some apoptotic target genes, and mutation of this phosphorylation site reduces the ability of p53 to induce cell

death but not proliferative arrest (Mayo, 2005; Oda, 2000). Phosphorylation of serine 46 can contribute to the interaction of the second transactivation domain of p53 with the p62/Tfb1 subunit of the general transcription factor TFIIH (Mayo, 2005) resulting in the induction of a specific subset of p53-responsive genes. Phosphorylation at serine 46 has also been linked to the ability to p53 to repress expression of galectin-3, an anti-apoptotic protein (Cecchinelli, 2006).

Functions of the Proline Rich Domain

The p53 proline-rich domain (PRD) contributes to the regulation of p53 stability, transactivation ability, and induction of transcription-independent apoptosis (Toledo, 2007). This region was identified as a suppressor of growth by inducing the apoptotis, it is dispensable for transcriptional activation however its deletion results in the impairment of p53's ability to suppress tumor cell growth in culture (Walker, 1996). Further experiments indicated that the proline rich domain is necessary for apoptosis (Baptiste, 2002; Chipuk, 2004; Sakamuro, 1997; Roth, 2000) but not cell cycle arrest (Sakamuro, 1997; Zhu, 1999; Venot, 1998). The p53 protein lacking the proline rich region has altered specificity for endogenous target promoters, there is a decrease in the induction of several apoptotic genes: *PIG3, PIG6, PIG11, p85, and BTG2* (Zhu, 1999), most of which were implicated in the cellular apoptotic response to oxidative stress (Polyak, 1997; Yin, 1998). However, the induction of other apoptotic genes, such as *bax* and *KILLER/DR5,* is unaffected (Zhu, 1999).

The p53 can function as a pro-apoptotic BH3-domain protein that leads to the release of cytochrome *c* from the mitochondria and induction of caspases and cell death (Vousden, 2007). Although this function of p53 is independent of transcription, an elegant model has been proposed in which activation of expression of the BH3-only protein PUMA by p53 is necessary to dislodge cytoplasmic p53 from an inactivating complex it forms with the anti-apoptotic BH3 domain proteins such as BclxL (Chipuk, 2004). This domain might also ensure optimal p53-p300/CBP interactions through PXXP motifs, and the deletion of the proline repeat motif of p53 prevents DNA-dependent acetylation of p53 by occluding p300 from the p53-DNA complex (Dornan, 2003).

The proline-rich region of p53 also plays a role in the regulation of p53 by Mdm2. The prolyl isomerase Pin1 was proposed to affect proline conformational changes that may reduce Mdm2 binding and enable p53 accumulation (Wulf,, 2002; Zacchi, 2002; Zheng, 2002). The Pin1 causes the isomerization of proline 82, enabling the recruitment of CHK2 to phosphorylate serine 20 and consequently reduce MDM2 binding (Berger, 2005). The proline rich region (amino acids 65-71) is the binding site of focal adhesion kinase (FAK) and the mutation in the binding site in p53 reversed the suppressive effect of FAK on p53-mediated transactivation of p21, BAX (Bcl-2-associated X protein) and Mdm2 (murine double minute 2) promoters (Goluboyskaya, 2008). This region was also shown to be required for p53-dependent cell growth arrest through Gas-1, a plasma membrane protein highly expressed during G_0 phase (Ruaro, 1997).

Function of the Repressor Domain

One more functionally divergent region within the N-terminal part of p53 is a repression domain, localized between proline-rich domain and DNA-core domain (residues 100–116) (Curtin, 2005). This domain can function as an independent heterologous repressor, and has been shown to decrease VP16-driven activation up to 20 times in human embryonal carcinoma cells, when being fused to VP16 transactivator (Curtin, 2005). As wild-type p53 is found to be over-expressed in some type of tumors, it has been proposed that the newly identified domain may play an important role in repression of the basal activity of p53 (Laptenco, 2006). Factors that are or can be specifically recruited by this domain, and that might be essential for the repression phenomenon found, are not known at present. Thus, these and other experiments further highlight the likelihood that transcriptional regulation by p53 involves several different regions within its N-terminus.

DNA binding domain

The DNA binding (core) domain of p53 is the largest domain of p53 protein (residues 94–312) and is the location of nearly all of the mutations that inactivate p53 in some 50% of human cancers (Olivier, 2002). The core domain is naturally unstable, with a melting

temperature of 42-44°C (Bullock, 1997), and many oncogenic mutations inactivate the core by either simply destabilizing or distorting it (Bullock, 2001). The structural studies have shown that the p53 core domain consists of an immunoglobulin-like β-sandwich that provides the basic scaffold for the DNA-binding surface (Fig. D). This surface is subdivided into two structural motifs that bind to the minor groove and major groove of target DNA, respectively.

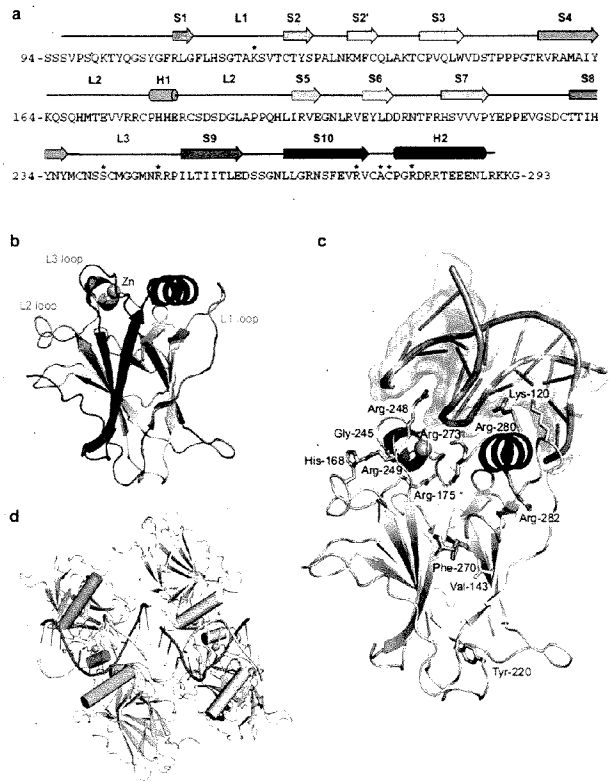


Fig. D. Structure of the p53 DNA-binding domain. (*a*) Sequence and secondary structure of the p53 core domain (p53C). DNA-contact residues are marked with asterisks. (*b*) Structure of DNA-free p53C shown with a rainbow color gradient, from blue at the N terminus to red at the C terminus, Protein Data Bank (PDB) entry 2OCJ (*c*) p53C bound to DNA (PDB entry 2AHI). (*d*) p53 core domains bound to two consecutive DNA half-site motifs (PDB entry 2AHI). (Joerger, 2008).

The loop-sheet-helix motif, which docks to the DNA major groove, includes loop L1, β-strands S2 and S2, parts of the extended a β strand S10,and the C-terminal helix. The other half of the DNA-binding surface is formed by two large loops (L2 and L3), which are stabilized by a zinc ion (Joerger, 2008). The zinc ion is tetrahedrally coordinated by a histidine and three cysteine side chains (Cys-176,His-179, Cys-238,and Cys-242). Zinc loss results in a significant

decrease in thermodynamic stability (Bullock, 2000), increases aggregation tendencies, and is accompanied by structural fluctuations in neighboring loops that cause loss of DNA- binding specificity (Butler, 2006; Duan, 2006). The solution structure of the p53 core domain reveals a number of buried polar groups that are not satisfied by hydrogen bonds, which may be one of the key factors contributing to p53's inherent instability (Canadillas, 2006). Furthermore, less rigid core of the β-sandwich may provide the structural plasticity required for optimal function.

Oligomerization domain

The full-length p53 protein forms tetramers via a tetramerization domain in the C-terminal region of the protein (residues 325 –356) (Veprintsev, 2006). The tetrameric structure of the domain is a dimer of dimers as revealed by X-ray crystallography (Jeffrey, 1995; Mittl, 1998) and in solution by NMR (Clore, 1995). The topology of the tetramerization domain is reflected by its folding pathway, which proceeds via a dimeric intermediate (Mateu, 1999a.). A study of p53 biogenesis in vitro found that dimer formation occurs cotranslationally on the polysome, whereas tetramers are formed posttranslationally by dimerization of dimers in solution (Nicholls, 2002).

Negative regulatory domain

The negative regulatory domain (amino acid residues 360-393) of p53 protein is intrinsically disordered but may undergo local disorder to order transitions upon binding to other proteins or nonspecific DNA (Bell, 2002, Weinberg, 2004 and Friedler, 2005). This region of the p53 protein is subject to extensive posttranslational modifications, including acetylation, ubiquitination, phosphorylation, Sumoylation, methylation, and neddylation, that regulate p53 function and cellular protein levels (Toledo, 2006; Bode, 2004 and Lavin, 2006). The C terminus of p53 binds DNA nonspecifically in vitro via the electrostatic interactions of several lysine residues, albeit with a comparatively low affinity, which inhibits binding of a specific DNA by the core domain in a competitive manner (Weinberg, 2004). Studies with a C-terminally truncated p53 variant lacking the last 30 amino acid residues show that the C terminus is required for efficient binding and transactivation of target genes in the context of large molecules of DNA or chromatin (Espinosa, 2000; McKinney, 2004). Consistent with its capability to bind DNA

nonspecifically, it was suggested that the p53 C terminus promotes linear diffusion on DNA (McKinney, 2004).The sliding of p53 along DNA may facilitate the search for target sequences.

DNA binding activity of p53 protein

Wild type p53 binds to a DNA consensus site containing two or more copies (consecutive or separated by one or two helical turns) of the 10 bp half-site 5'-PuPuPuC(A/T) (T/A)GPyPyPy-3', where Pu and Py represent purines and pyrimidines, respectively (Kern, 1991; El-Deiry, 1992; Funk, 1992) Thus, the consensus site comprises four inverted 5 bp quarter-sites. By mapping p53 target sites in the human genome it was found that more than half of the high-probability binding loci contain consecutive half-sites without insertion (Wei, 2006). X-ray crystallography has revealed that four core domains of the p53 tetramer occupy the four quarter-sites in a full consensus sequence without steric clashes (Cho, 1994). This model is also consistent with solution studies, which shows four p53 core domains can bind cooperatively to a consensus DNA sequence (Balagurumoorthy, 1995; Wang, 1995a.). The p53 tetramer is a dimer of dimers and the two monomers within a dimer bind to a half-site (two consecutive quarter-sites, but not to the alternating quarter-sites) (McLure, 1998). Tetramers bind similarly, with the two dimers within each tetramer binding to pairs of half-sites. Thus the four p53 core domains bind to these response elements in a highly cooperative manner to give a complex with 4:1 stoichiometry (Balagurumoorthy, 1995; Wienberg, 2004).

The architecture of the p53 core tetramers bound to DNA has been revealed by crystal structures of p53 core in complex with 20mer double stranded DNAs containing different half-site motifs (Kitayner, 2006). The core domain dimer interface is formed by residues from the L3 loop and the short helical segment within L2 (177,178,181,243, and 244), and is stabilized by both hydrophobic and water-mediated polar contacts (Rippin , 2002; Klein, 2001). In the crystal structures of all the dodecamer DNA complexes studied, the half-site dimers assemble to form a tetramer through protein-protein and base-pair stacking interactions, mimicking the binding mode of four p53 core domains to a full DNA response element with a two base-pair spacer between the half-sites (Kitayner, 2006). The amino acids Lys-120, Ser-241, Arg-248, Arg-273,

Ala-276, Cys-277, and Arg-280 are the key residues in the p53 core-DNA interface that make direct contact with a DNA half-site (Cho, 1994; Kitayner, 2006).

Loss of p53 protein function

Since the level of p53 is tightly regulated in the cells, the regulation of its stabilization and degradation are the key steps that make the organism healthy. The loss of p53 function is the greatest cause of the human cancer. The p53 function loss is due to the absence of the protein or the presence of inactive mutant protein. The mutation in the p53 gene is very common, even the p53 first discovered was mutant protein. The p53 gene has the features of a recessive tumor suppressor in its wild-type form, and it can be a dominant oncogene in its mutated form. Over 60% of cancer cases have p53 gene mutations and the core domain contains 95% of p53 mutations and 75% of this occur as single missense mutations rather than deletions, insertions or frameshifts (Soussi, 2005; Beroud, 1998).

So, the oncogenic form of p53 is predominantly a full-length protein with a single amino-acid substitution in its core domain (Bullock, 2001). Most of the remaining tumors although containing wild type p53, are defective in the pathway of p53 induced cell cycle arrest or apoptosis due to virus infection, MDM2 overexpression, ARF or ATM deficiency (Wang, 2003). Deficiency of p53 function also confers a growth advantage under hypoxic conditions, which contribute to tumor progression (Pluquet, 2001). The functional status of p53 has been related to prognosis, progression and therapeutic response of tumors. Tumor cells containing wild-type p53 are usually more sensitive than those bearing mutant p53 (Wang, 2003).

Six mutation hot spots cluster to the DNA-binding surface: two contact DNA directly R248 (L3) and R273 (S10 in LSH) and four stabilize the surrounding structure R175 (L2), G245 (L3), R249 (L3) and R282 (H2 in LSH). These give rise to two classes of mutants 'DNA contact' and 'structural' (Bullock, 2001). These mutants can also be distinguished by two polyclonal antibodies, PAb1620 (Milner, 1987) and PAb240 (Gannon, 1990), raised against native and denatured p53, respectively. Contact mutants tend preferentially to bind PAb1620 because they have an intact native fold, whereas structural mutants specifically bind to PAb240, which

recognizes a continuous epitope partially buried and inaccessible in the native state (Milner, 1995). Many of the point mutations in the core domain of p53 produce a change in the global conformation of the protein different from the wild type p53 conformation, which can be recognized by PAb240 antibody (Xirodimas, 1999). Further it was shown that a variety of structural consequences result upon mutation: (i) the removal of an essential contact with DNA, (ii) creation of large, water accessible crevices or hydrophobic internal cavities with no other structural changes but with a large loss of thermodynamic stability, (iii) distortion of the DNA binding surface, and (iv) alterations to surfaces not directly involved in DNA binding but involved in domain-domain interactions on binding as a tetramer (Friedler, 2003).

Many of the core domain mutants are thermodynamically unstable at body temperature, these mutants denature within minutes at 37°C, whereas the half-life ($t^{1/2}$) of the unfolding of wild-type p53 core domain was found to be 9 min. Hot spot mutants denatured more rapidly with increasing thermodynamic instability. The highly destabilized mutant I195T had a $t^{1/2}$ of less than 1 min. (Friedler, 2003). These mutations result in the loss of sequence specific DNA binding, as a result mutant p53 proteins fail to activate transcription of p53 target genes and hence, do not trigger a p53-dependent biological response. The mutant p53 protein also works in a dominant negative manner, the mutant p53 when oligomerizes with wild type p53, drives the conformation of the wild-type protein into a mutant state (Milner, 1991).

Restoration of p53 function

Several studies have demonstrated the ways to restore the normal function to mutant p53 protein in tumors (Bykov, 2003) and these can be divided into three groups: *(a)* design of antagonists for negative regulators of p53 in tumors carrying wild-type p53 (Vassilev, 2007), *(b)* reactivation of mutant p53 (Joerger, 2007; Bullock, 2001), and *(c)* exogenous p53 expression, e.g., via adenovirus-mediated gene transfer (Roth, 2006). Recently, two elegant studies on transgenic mice, in which p53 expression was reversibly switched on and off, have independently shown that restoration of p53 function can lead to tumor regression in vivo, indicating that reactivating p53 is a promising therapeutic strategy (Martins, 2006; Ventura, 2007).

The introductions of second site suppressor mutations in p53 results in the stabilized folding of the mutant protein and also restored sequence specific DNA binding (Nikolova, 2000). Analyses of the effects of second-site mutations that rescue hot spot mutations of p53 imply that it is possible to stabilize many destabilized mutants of p53 in a generic way. Furthermore, specific mutations that distort the native structure might also be reversed (Nikolova, 2000).

A peptide, CBD3 is reported to stabilize mutant p53 core domain and rescue its sequence-specific DNA binding activity (Friedler, 2002). CBD3 binds the native but not the denatured state of p53 core domain increases the half-life of the p53 core domain and thus stabilizes it (Bullock, 2001; Friedler, 2002). It binds to a surface region of p53 that partly overlaps with the DNA-binding surface. The fluorescein-$_{labeled}$ form FL-CDB3 raises the melting temperature of wild-type p53 and the destabilized R249S mutant in vitro (Friedler, 2002). It induces upregulation of wild type p53 and representative mutants in human cell lines (Issaeva, 2003).

Further by screening a chemical library of 100,000 synthetic compounds, using a protein assay two molecules (CP31398 and CP257042) were identified that can preserve wild-type conformation of p53, rescue its transactivation capacity and restore its antitumor activity *in vivo* (Foster, 1999). Wild-type p53 core domain looses the PAb1620-specific epitope within several minutes at 45°C. However the compounds that bind specifically to the native state increase the thermostability and protect the core domain of p53 from denaturation and maintain the PAb1620-reactive conformation for 30 minutes at 45°C (Foster, 1999). In the presence of two compounds CP-257042 and CP-31398 four core domain mutants, V173A, R175S, R249S and R273H, also maintain PAb1620 binding. Furthermore, CP-31398 activates p21 transcription in V173A or R249S transfected Saos-2 osteosarcoma cells that are null for p53 (Foster, 1999). Another compound PRIMA-1 was identified by screening a chemical library, that induces mutant p53-dependent apoptosis and restores native conformation, DNA binding, and transcriptional transactivation to mutant p53, and inhibits tumor growth *in vivo* (Bykov, 2002). Further a maleimide-derived molecule, MIRA-1 was identified that can reactivate DNA binding and preserve the active conformation of mutant p53 protein *in vitro* and restore transcriptional transactivation to mutant p53 in living cells. MIRA-1 induced mutant p53-dependent cell death

in different human tumor cells carrying tetracycline-regulated mutant p53. The structural analog MIRA-3 showed antitumor activity *in vivo* against human mutant p53-carrying tumor xenografts in SCID mice (Bykov, 2005).

Temperature dependent loss of p53 function

The conformation of wild-type p53 is very temperature sensitive. It undergoes irreversible conformational changes after short incubations (10 min) at temperatures in a range from 37°C to 45°C by losing its wild-type (PAb1620-positive) conformation. The loss of the PAb1620-reactive epitope correlates with a loss of the tetrameric nature of p53 and with a loss of the p53 DNA binding activity (Hansen, 1996). The thermal instability of p53 protein is due to its core DNA binding domain, which unfolds with a half-life of 9 min. at 37°C and 10 hrs at 30°C (Friedler, 2003). This unfolding was found to be concentration-dependent and results in the rapid aggregation (Friedler, 2003). The inactivation of p53 DBD at higher temperature is due to the rapid accumulation of misfolded species. Elevated temperatures increase the frequency of cycling between folded and unfolded states and the function of p53 DBD is lost because a fraction of molecules become trapped in misfolded conformations with each folding-unfolding cycle (Butler, 2006). However the folding kinetics do not change appreciably from 5°C to 35°C (Butler, 2006).

Stabilization of p53 functions by antibodies and chaperones

The thermal stability of p53 protein can be increased by several ways. The deletion of C-terminal 30 amino acid negative regulatory region from the p53 showed a dramatically enhanced intrinsic stability to temperature (Hansen, 1996). The *E. coli* heat shock protein DnaK that binds to the C terminus of p53 (Fourie, 1994) protects PAb1620-positive p53 from a temperature dependent loss of DNA binding activity but cannot renature and activate denatured p53 (Hansen, 1996). The DnaK protein also activates the p53 protein for sequence specific DNA binding (Hupp, 1992). Two monoclonal antibodies to the N terminus of p53, PAb1801 and DO-1 also protect the temperature dependent loss of DNA binding at 37°C (Hansen, 1996). The DNA binding activity of mutant p53 is also protected by PAb 1801 and its Fab fragment at 37°C. The

mutant p53 protein binds DNA at 25°C whereas heating at 37°C irreversibly destroyed its ability to bind DNA. Whereas, the addition of amino terminal antibody or the Fab fragments protected the DNA binding ability at 37°C (Friedlander, 1996).

In the last few years several molecular chaperones are reported to protect the sequence specific DNA binding ability of p53 protein at 37°C. The Hsp90 which is abundant in cells, is important for protecting the cells from stress such as high temperature. Hsp90 was found in a complex with several oncoproteins, including v-Src, c-Erb2, Raf-1, Akt, Bcr-Abl, and tumor suppressor protein p53 (Young, 2001; Zylicz, 2001; Picard, 2002; Prodromou, 2003; Wegele, 2004). It has been known from years that genotypically mutant p53 co-immunoprecipitates with members of the Hsp70 and Hsp90 families. However recent studies have shown that Hsp90 associates with the wild type p53 protein. Using purified proteins and applying several independent methods of interaction such as surface plasmon resonance, immunoprecipitation, ELISA, and cross-linking it was demonstrated that Hsp90 directly, in the absence of any other co-chaperones, associates with genotypically wt-p53 but not with mutant p53 protein (King, 2001). The binding of Hsp90 with p53 provides the stability to the p53 protein, and protects the DNA binding ability at 37°C. It also prevents the denaturation and subsequent aggregation of the p53 protein at 45°C (Muller, 2004). Further studies have also shown that the chaperoning activity of Hsp90 towards p53 for DNA binding is ATP dependent and the Hsp90 inhibitors geldanamycin and radicicol, inhibit the activity of p53 as a transcription factor by dissociating it from the target DNA promoter sequence sites (Walerych, 2004).

Recently our group has reported that CHIP (carboxyl terminus of Hsp70-interacting protein) independently binds with the wild type p53 protein and prevents PAb1620-positive conformation as well as DNA binding activity from temperature dependent loss. CHIP also protects p53 from irreversible thermal denaturation and subsequently aggregation at physiological and elevated temperatures. CHIP binds preferentially to the denatured conformation of wild type p53 protein and restores its DNA binding activity in an ATP independent manner (Tripathi, 2007). The MDM2 protein that is mainly responsible for the ubiquitination and degradation of p53 is also reported to work as a chaperone towards p53.

MDM2 protects the DNA binding ability of p53 protein at 37°C in an ATP dependent manner (Wawrzynow, 2007).

Role of the amino terminal domain in p53 DNA interaction

In addition to the transactivation function of the amino terminal domain, it also plays a role in the regulation of p53 binding to its consensus site. Studies have shown that the amino terminus of p53 serves as a self inhibitory region for the DNA binding, it causes the increased dissociation of p53 from the DNA (Cain, 2000). The antibodies that bind in a region from 10 to 55 amino acid residues decrease the dissociation of p53 from DNA and stabilize the interaction. The deletion of 96 amino acid residues further stabilizes the binding (Cain, 2000).

The proline rich region of p53 also plays a role in the regulation of DNA binding by p53, although its role is controversial as different authors have reported contrasting results. The part of proline rich region (amino acids 80-93) of the human p53 is reported to serves as a negative regulator for DNA binding (Muller-Tiemann, 1998). The deletion of this region resulted in the activation of DNA binding by p53 protein, synthetic peptides from this region also activate p53 for DNA binding (Muller-Tiemann, 1998). However it was also reported that, the murine p53 having two deleted proline rich repeats (77-89) binds DNA as efficiently as the wild type p53 protein (Sakamuro, 1997). Interestingly there is another report that shows proline rich domain as a positive regulator for p53 DNA interaction (Roth, 2000). They have studied the binding of p53 protein with three different p53 DBS, the *p21*, *MDM-2*, and of *PIG3* promoters and found that the deletion of proline rich domain causes substantial decrease in the DNA binding by p53 protein to all three promoters (Roth, 2000). Using gel retardation assay it was revealed that this domain did not alter *in vitro* the specific binding to the p53-responsive element of *PIG3*, but plays a critical role in transactivation from a synthetic promoter containing this element (Venot, 1998).

A peptide derived from the amino terminus of the core domain of p53 protein (residues 105-126) is reported to inhibit the DNA binding by the p53 protein (Protopopova, 2003). The peptide interfered the p53 DNA binding activated by PAb.421 in a dose dependent manner and

blocked DNA binding of the constitutively active c-terminal deleted p53 protein(p53Δ30) and it binds with full length p53(1-393), the core domain(100-300) as well as with the C –terminus (320-393) of p53 protein (Protopopova, 2003). Studies have shown that the transactivation domain is responsible for the thermosensitive nature of p53 protein. The binding of N-terminal specific antibodies like DO-1 or PAb 1801 protects the DNA binding ability of wild type and mutant p53 protein at 37°C (Hansen, 1996; Hansen, 1998). Whereas the deletion of 37 amino acid residues from the N-terminus of p53 makes it to be stabilized by every antibody it binds (Hansen, 1998). However further deletion, up to 96 amino acids from amino terminal domain of p53 loses the protection by binding with antibodies, although PAb.1620 protects by 50% (Hansen, 1996).

The amino terminal domain of p53 protein is natively unfolded, the natively unfolded regions of the proteins can be divided into two groups, those with no ordered (secondary) structure and those with some secondary structure; the latter resemble molten globules and lack tertiary structure (Uversky, 2002). The amino terminus of p53 belongs to the second class, which possesses some residual secondary structures but lack a tertiary structure (Bell, 2002; Dawson, 2003). The intrinsically disordered proteins perform different functions including the regulation of transcription and translation, cellular signal transduction, protein phosphorylation, the storage of small molecules, and the regulation of the self-assembly of large multiprotein complexes such as the bacterial flagellum and the ribosome (Dyson, 2005).

Unfolded regions are also present in proteins that function as chaperones for other proteins and for RNA molecules. The protein chaperones like α-synuclein and α-casein are disordered along their entire length (Tompa, 2002), whereas other chaperones like Hsp90, Hsp70, Hsp60, p23, Hsp105 and bacterial GroEL have disordered regions (Tompa, 2004). The structural disorder has been noted as a functional feature of protein chaperones. Removal of the disordered segment by limited proteolysis often abolishes or markedly reduces chaperone activity in other instances, such as for p23 co-chaperone (Weikl, 1999), α-crystallin (Andley, 1996), and Hsp25 (Lindner, 2000). The diminution of chaperone activity by decreasing the flexibility of the disordered segment by a point mutation has been demonstrated for α-crystallin (Smulders, 1996). A reduction in flexibility of the disordered segment upon direct contact with the partially folded

or misfolded substrate protein has been shown by time-resolved fluorescence spectroscopy and NMR in GroEL (Gorovits, 1995) and α-crystallin (Lindner, 1998). These studies indicate that a subtle balance of these regions between structural order and disorder are important in the functional cycle of chaperones. The chaperone function is performed by binding the unfolded regions to misfolded proteins and RNA molecules, such that they function as recognition elements and/or help in the loosening and unfolding of kinetically-trapped folding intermediates including molecular recognition via binding to other proteins or to nucleic acids (Tompa, 2004).

Given the structural flexibility of the amino terminal domain of p53 we have investigated its role in the DNA binding activity of p53 protein. The transactivation function of p53 was found to be responsible for the increased dissociation of p53 to its target DNA and the addition of antibodies against this region or the deletion of this region potentiates the p53 DNA interaction (Cain, 2000). Further the transactivation domain is also responsible for the thermo-sensitive nature of p53 DNA interaction and the addition of antibodies against this region protects the DNA binding activity of p53 protein at physiological temperature (Hansen, 1996; Hansen, 1998). Whereas the role of other sub-domains of amino terminus the proline rich domain and the repressor domain in the DNA binding activity is not known presently. Further the amino terminal domain of p53 protein is intrinsically disordered and as described above the natively unfolded regions are prevalent in protein chaperones and are important in the chaperoning activity. In this study we explored the role of complete amino terminal domain (NTD) on the p53 DNA interaction as well as on the conformation of p53 protein and its DNA binding activity at physiological and elevated temperatures.

AIMS AND OBJECTIVES

1. Cloning of p53 and its amino terminal domain (NTD) in the bacterial expression vector, protein expression and purification.
2. To investigate the effect of NTD on the DNA binding activity of p53 protein.
3. To study the interaction of NTD with p53 under different temperature conditions.
4. To investigate the role of NTD in the conformational modulation of p53 under heat stress conditions.
5. To study the role of NTD in the protection of DNA binding activity of p53 protein from temperature dependent loss and in the recovery of DNA binding activity of heat denatured p53 by NTD.
6. To develop an ELISA based approach to study sequence specific p53 DNA interaction using different p53 DNA binding sites (DBS).
7. To construct the fluorescent tagged p53 and NTD expression plasmids and study their localization.

Materials

MATERIALS

Chemicals:

Ammonium Persulphate, Ampicillin, Aprotinin, Avidin-Alkaline Phosphatase, 5-Bromo 4-Chloro 3-Indolyl Phosphate (BCIP), Boric Acid, Bovine Serum Albumin, Bromophenol Blue, Calcium Chloride, Cellulose Phosphate, Chloroform, Citric Acid, Comossie Brilliant Blue, Dithiothreitol, Ethidium Bromide, Ethylene Diamine Tetracetic Acid (EDTA), Ficoll, Formaldehyde, Glacial Acetic acid, Glutathione Sepharose, Glycine, Glycerol, Guanidium Hydrochloride, HEPES, Imidazole Hydrochloride, Isoamyl Alcohol, Isopropyl Alcohol, Kanamycin, Leupeptin, Luria Bertani (LB) medium, Magnesium chloride, Methanol, Nitro Blue Tetrazolium Chloride (NBT), N, N, N', N'-Tetramethyl-Ethylenediamine (TEMED), Ni^{2+}-NTA, Nonidet P-40, Phenol, Phenyl Methyl Sulphonyl Fluoride (PMSF), Polyvinyl Pyrrolidone, poly(dI-dC),p-Nitrophenyl Phosphate(PNPP), Potassium Dihydrogen Phosphate, Potassium Acetate, Potassium Chloride, Protein A Sepharose, Reduced Glutathione, Salmon Sperm DNA, Sigmacote, Sodium Acetate, Sodium Azide, Sodium Chloride, Sodium Deoxycholate, Sodium Dihydrogen Phosphate, Sodium Hydrogen Diphosphate ,Sodium dodecyl (lauryl) sulphate, Sodium hydroxide, Sodium pyruvate, Tris-Base (Trizma Base), Triton X-100, Tween-20, Trypsin Inhibitor, Xylene Cyanol, Yeast Extract, β-mercaptomethanol and other chemicals were purchased from Sigma Chemical Company, USA, Amersham Pharmacia, UK and Calbiochem, USA. Absolute alcohol was purchased from E. Merck, Germany. Acrylamide, Agarose, Bis-Acrylamide, Luria Bertani (LB) medium, Luria Bertani Agar, Sephadex G-50 was obtained from Amersham Pharmacia Biotech, UK. Diethylaminoethyl cellulose(DE52) was obtained from Whatman Laboratory Divison UK., USA. Concentrated Hydrochloric acid, Concentrated Sulphuric acid, Concentrated Nitric acid, Glucose, Nickel Chloride, Nickel Sulphate, Potassium dichromate, Silver nitrate, Sodium bicarbonate, Sodium carbonate and Sodium thiosulphate were purchased from Qualigens Chemicals, India. Bradford reagent for protein quantitation was obtained from Bio-Rad, USA. Nitrocellulose membrane purchased from Bio-Rad and Millipore, USA. Dialysis tubing, for removing the extra salts from the purified proteins was bought from Sigma, USA. Radioactive chemical such as $\gamma\ ^{32}p$ ATP was purchased from Board of Radiation and Isotope technology (BRIT), Jonaki, Hyderabad, India, X ray films, Fixer and Developer, used for autoradiography were from Kodak, USA.

Enzymes, Reagents and Antibodies:

RNAase A, T4 DNA ligase and T4 polynucleotide kinase, Vent DNA Polymerase were purchased from New England Biolabs UK, Restriction Endonucleases *BamHI, EcoRI, Hind III, Kpn I, Bgl II* and GFX mini-prepreparation kit for plasmid DNA isolation were purchased from Amersham Biosciences USA. *Nhe I* was purchased from Bangalore Genei India. *Taq* DNA polymease was purchased from Binary Gene, India. All the oligonucleotides used were obtained from Microsynth, Switzerland and Sigma Genosys USA. Enterokinase enzyme, medi and maxi prep plasmid DNA isolation kits, and agarose gel elution kit were purchased from Novagen, USA. Anti-p53 antibodies (PAb 1801, PAb 421, PAb 1601 and PAb 240) used for the studies were purchased from Calbiochem, USA. Anti-GST anti-His, PAb FL-393, anti-MDM2, anti-CHIP, anti-Mouse, anti-Rabbit and anti-Goat secondary (AP-conjugated), were purchased from Santa-Cruz, Biotechnology USA.

Bacterial strains and Bacterial expression vectors:

Escherichia coli DH5α strain was used for transforming the ligated product in the cloning experiments and for plasmid DNA preparation, whereas *E.coli* BL-21(DE-3), M15 and B-834 strains were used for the over-expression of the recombinant protein. pET28a and pET32a vectors were used for preparing proteins with 6X-His tag, and pGEX-4T-1 vector system was used for preparing proteins with GST-tag.

ELISA plates:

For doing p53 and DBS interaction and protein -protein interaction on ELISA plate, 96 well flat bottomed Maxisorp ELISA plates were purchased from Nunc, Denmark.

Oligonucleotides used in the study.

No.	**Oligonucleotide sequences**	**No. of bases**	
1.	GGGGTACCCCATGGGATTGGGGTTTTCCCCT CCCATAGATCTTC	**44**	DBSI sense
2.	CCCCATGGGGTACCCTAACCCCAAAAGGGGA GGGTATCTAGAAG	**44**	DBSI anti-sense
3.	GGGGTACCCCTTACTTGCCCTTACTTGTCAGA AGATCTTC	**40**	DBSII sense
4.	CCCCATGGGGAATGAACGGGAATGAACAGTC TTCTAGAAG	**40**	DBSII anti-sense
5.	GGGGTACCCCACAATGCAGGATTCCTCCAAA ATGAAGATCTTC	**43**	DBSIII sense
6.	CCCCATGGGGTGTTACGTCCTAAGGAGGTTT TACTTCTAGAAG	**43**	DBSIII anti-sense strand
7.	GAAAGATCTCGAGCAACATGTTGGGACATGT TCCTCGAGAATTGGTACCCC	**51**	p21 5, promoter sense strand
8.	CTTTCTAGAGCTCGTTGTACAACCCTGTACAA GGAGCTCTTAACCATGGGG	**51**	p21 5, promoter anti-sense strand
11.	ATTCTAGAGGATCCGAATTCATGGAGGAGCC GCAGTCA	**38**	p53cDNA sense primer
12.	ATAAGCTTGCTAGCTCTAGAGTCTGAGTCAG GCCCTTC	**38**	p53cDNA anti-sense primer
13.	CTAGCTAGCATGGAGGAGCCGCAGTC	**26**	p53cDNA sense primer
14	GGCGAATTCCGTGCAAGTCACAGACT	**26**	NTD cDNA anti-sense primer

Methods

METHODS

Bacterial Cultures

All bacterial cultures were grown in LB medium at 37°C with shaking in the presence of appropriate antibiotics. The medium was sterilized by autoclaving at a pressure of 15Ibs/square inch for 15 min. For preserving the bacterial cultures, cells were allowed to grow in LB medium with appropriate antibiotic concentration. When the cells reached log phase, 300µl of culture was added into the micro-centrifuge tube containing 300µl of 100% glycerol solution. The cells were vortexed thoroughly till the solution became homogenous and stored at -80°C.

Preparation of Competent Cells

Competent cells of the *E .coli* stains (DH5 alpha, BL-21DE3 and B834) were prepared with a slight modification in the standard protocol (Sambrook *et al,* 1989). A single colony was picked up from a freshly grown plate and then inoculated in 10 ml of LB broth and the culture was incubated O/N at 37° C. After 16 hrs, 1 ml of this culture was transferred to 100 ml LB broth (1% final concentration). The cells were grown at 37°C till absorbance at 600 (A_{600}) reached 0.4-0.6, then the culture was chilled by putting on ice and rotating it for 20 min, culture was then aseptically transferred to sterile oakridge tubes. Cells were pelleted down at 4000 rpm for 5 minutes at 4°C. Supernatant was discarded and the cell pellet was resuspended in 25 ml of chilled 100mM $CaCl_2$ and incubated on ice for 30 minutes. Again centrifuged at 4000rpm for 5minutes at 4°C, cells were then resuspended in 5 ml of 100mM $CaCl_2$ having 15% glycerol. The cells were left on ice for 4-6 hours, the resuspended cells were then aliquoted in 200 µl batches and were stored at –80°C.

Determination of competency

One aliquot of 200µl competent cells thawed on ice, 10ng of standard plasmid DNA was added to the cells for checking the efficiency of the competent cells per µg of supercoiled

plasmid DNA. The efficiency of competent cells is represented as, the number of colonies obtained per-μg of supercoiled plasmid DNA.

Transformation

Frozen competent cells were thawed on ice, the ligation mixture was then added to the cell suspension, mixed properly and incubated on ice for 30 minutes, then given heat shock at 42°C for 90 seconds in water bath. Incubated again on ice for 2–3 min, added 800 μl of LB broth, mixed and incubated at 37°C for 1 hr. Cells were then pelleted down at 10,000rpm for 30 seconds at room temperature, supernatant was discarded and cell pellet was resuspended in the 25-50 μl media left in the tube and spread on the LB agar plate containing appropriate antibiotic.

Plasmid DNA Isolation:

Small scale plasmid isolation:

The miniprep isolation procedure used was with some modification in original protocol (Birnboim, 1979; Ish-Horowicz, 1981). One ml overnight grown culture was pelleted down and the cell pellet was resuspended in 50 μl of LB media left in the tube. 300 μl of freshly prepared TENS solution was added, mixed and incubated on ice for 5 min, 150 μl of 3M NaOAc (pH 5.2) was then added vortexed vigorously and left on ice for 5 min. Tubes were spun at 4°C for 10 min at 14,000rpm, the supernatant was added with 900 μl of 100% ethanol and incubated at -80°C for 30 min. The tubes were centrifuged for 20 min at room temperature, and the pellet was air dried. 25 μl of TE was added along with 2 μl of RNase A and incubated at 37°C for 30 min. Plasmid DNA was run on 1.2% agarose gel at 70 volts constant voltage and visualized under UV transilluminator.

Agarose gel electrophoresis and DNA quantitation

The agarose gel used varied from 0.8 to 1.2% according to the size of DNA to be resolved. The agarose was resuspended in 0.5X TAE and dissolved by boiling, ethidium bromide was then added to a final concentration of 0.5 μg/ml. The agarose solution was poured in the gel casting mould, further combs were inserted and left it to solidify. Combs were then removed and

gel was kept in the electrophoresis tank containing 0.5X TAE. The DNA sample was mixed with tracking dye and loaded into the wells of the gel and electrophoresis was carried out at constant voltage 5 to 15 volt/cm and then bands were visualized using short wavelength UV transilluminator. For the quantitation of plasmid DNA by spectrometry, DNA was diluted in water and absorbance at 260nm (A_{260}) and at 280 nm (A_{280}) was measured in quartz cuvette. A_{260}=1 is equivalent to 50 µg/ml of plasmid DNA. The ratio of A_{260} / A_{280} was taken to check the purity of the DNA preparation. The ratio of the protein free pure DNA ranges from 1.8-2.0.

PCR amplification

For the amplification of the cDNA of p53 and NTD, PCR amplification was done using the the p53 cDNA cloned in pGEM as template with Vent DNA Polymerase from New England Biolabs. The PCR reaction was set in a 0.2 ml PCR tubes on Gene Amp PCR system 2400(Perkin Elmer). The reaction mixture is as follows

DNA template	100ng
dNTP's	400 µM
Sense Primer	100ng
Anti-sense Primer	100ng
10X Vent Buffer	10 µl
Vent DNA polymerase	5 U
Milli Q water	added to make up the volume 100 µl

The PCR machine was programmed with run for 30 cycles. The different temperatures of the reaction are as

	Temperature	**Time for p53**	**Time for NTD**
Denaturation	95°C	30 sec	30 sec
Annealing	65°C	30 sec	30 sec
Extension	72°C	1.2 min	30 sec

Restriction enzyme digestion of DNA

The DNA samples were digested with restriction endonucleases in the appropriate 1X reaction buffer (supplied with the enzyme). 1-2 µg DNA sample was digested in a volume of 50-100 µl at the incubation temperature of the specified enzyme in the water bath for 1-2 hrs. The digested DNA was ethanol precipitated and air dried. The DNA was dissolved in autoclaved MQ, and if needed second digestion it was further digested with the desired restriction enzyme.

DNA elution from agarose gels

To prepare the insert and vector for cloning these were purified from the other undesired DNA, restriction enzyme and salts. The restriction enzyme digested insert and vectors were agarose gel purified. DNA was run on 1% agarose gel in 0.5X TAE buffer, the desired DNA band was cut and purified using the gel extraction kit of Novagen. The DNA was extracted using the manufacturer's protocol. The eluted DNA was checked on agarose gel, recovery of the DNA was 70-80%.

Ligation

The vector and insert DNA were digested with complementary restriction sites. The plasmid vector and insert were added in ratio of 1:3 in a total reaction volume of 10µl. The reaction mixture was incubated for 16hrs at 16°C. The cohesive end ligation needs the final concentration of 1mM ATP.

Cohesive end ligation	
Incubation temperature	16° C
T4 DNA ligase	1-2 U
ATP concentration	1 mM

After the completion of incubation, samples were kept at -20°C or transformed.

Colony Hybridization

The colony hybridization was performed with few modifications in the standard protocol (Denhrdt, 1966). The bacterial colonies were patched in duplicate on a LB-agar plate containing suitable antibiotic and grown O/N at 37°C. Colonies were then transferred on the nylon membrane and marked for proper alignment. The nylon membrane was put in 5% SDS for 5 min then in denaturation buffer for 5 min and again in neutralizing solution for 5 min. Membrane was then UV crosslinked in Stratalinker to fix DNA, then it was rotated in pre-hybridization buffer for 8-10 hrs at 8-10°C below the melting temperature of the probe DNA with constant rotation, probe was then added (10^7 counts/ml) and incubated further for 12-14 hrs at same temperature. Nylon membrane was then washed in wash buffer for 5 min at room temperature to remove the background and autoradiography was done, the colonies showing positive signals were picked up.

Over-expression of the Recombinant proteins

The expression plasmids containing the desired gene (10 ng) was transformed in competent *E.coli* BL21-DE3 cells. Single colony was picked from plate and inoculated into fresh LB medium and incubated overnight at 37° C. From this overnight grown culture, LB flask was inoculated and incubated at 37° C for 3-4 hours till A_{600} reached 0.8 to 0.9. Culture of p53 in the pET32a as well as in the pET28a was induced with 0.2 to 1.0 mM IPTG (as needed) for over-expression and incubated at 25°C to 30°C for further 3 hours to overnight. Expression was checked by lysis of 1.0 ml cell pellet with lysis buffer and loaded on 10-12 % SDS- PAGE, depending on the size of proteins.

Protein	LB Broth Concen.	IPTG Concen.	Induction Temp.	Induction Time
$p53^{1-393}$	1.5X	0.20 mM	22° C	4-6 hrs
GST-$p53^{1-393}$	1.5X	0.2 mM	30°C	3-4 hrs
GST- $p53^{\Delta 1-140}$	1.5X	0.5 mM	30°C	3-4 hrs
NTD ($p53^{\Delta 126-393}$)	1.5X	0.5 mM	25° C	4 -5 hrs
$p53^{\Delta 1-285}$	1.5X	0.5 mM	25° C	4 hrs

Purification of proteins by Ni-NTA column

For the purification of 6X-his tagged proteins, metal chelating affinity chromatography Ni^{2+}-NTA from Amersham Biosciences was used. The induced cell culture was centrifuged at 7000 rpm for 10 minutes at 4°C to harvest the cells and resuspended the cell pellet in native lysis buffer containing 0.1% Triton -100 and 0.5mM PMSF. Cells were then lysed by sonicating the cell suspension with 20 sec on and 40 sec off cycle, till the lysate become clear. Further the cell lysate was centrifuged at 12000 rpm at 4°C for 30 minutes to separate the soluble protein from inclusion body and membranous fractions. The supernatant was taken and added 20mM-40mM Imidazole (as needed for each protein) and loaded on the prepacked and native lysis buffer equilibrated Ni-NTA column, the flow through obtained was again loaded to the column and passed. The nonspecifically adhered proteins were removed by passing 10-20 bed volumes of wash buffer containing 40mM Imidazole. Protein was then eluted by the 200- 500 mM Imidazole

in native lysis buffer. The purity and yield was determined by running the SDS-PAGE and doing Bradford assay respectively. The high salt present in the native lysis buffer and Imidazole were removed by dialyzing the protein against PBS for 6 hours with three buffer changes at 4°C. The purification as well as the dialysis was done at 4°C.

Protein	Imidazole conc. in Binding buffer	Imidazole conc. in Elution buffer
p53WT	20mM	300-500mM
NTD	40mM	300-500mM
CTD ($p53^{\Delta 1-285}$)	20mM	200-400mM

Purification by glutathione sepharose

For the purification of GST-tagged proteins, glutathione agarose affinity chromatography was used. The Glutathione Agarose beads were from Amersham Biosciences. The induced cell culture containing GST-tagged protein was centrifuged at 7000 rpm for 10 minutes at 4°C to harvest the cells, resuspended the cell pellet in lysis buffer (PBS pH-7.4, 0.1 % Triton and 0.5 mM PMSF) and lysed it by sonicating the cell suspension with 20 sec on and 40 sec off cycle, till the lysate become clear. Cell lysate was centrifuged at 12,000 rpm at 4°C for 30 minutes to separate the soluble protein from the inclusion bodies and membrane fractions. The supernatant fraction was taken and passed it through the pre-packed and lysis buffer equilibrated glutathione sepharose column for binding of the GST tagged protein to the glutathione sepharose beads. To remove the non-specifically adhered proteins, the column was washed with 10 bed volumes of lysis buffer 3-4 times. The elution of the proteins was done by passing the elution buffer (20 mM reduced glutathione in 50 mM Tris-HCl, pH-8.0) to the column and flow through was collected. The eluted protein was dialyzed against PBS (pH7.4) for 4-5 hrs to remove the reduced glutathione from the protein. The purification as well as the dialysis was done at 4°C.

Polyacrylamide Gel Electrophoresis (PAGE)

Native PAGE

Native Polyacrylamide gels are used for running small DNA fragments and to see the difference between annealed double stranded oligonucleotides and single stranded oligonucleotide. The gel was made by adding 30% acrylamide(acrylamide and bis-acrylamide in a ratio of 29:1)in different amounts according to the percentage of gel required in a solution containing 0.5X final concentration of TBE. For 10 ml gel volume, 100 µl of 10 % APS and 10 µl of TEMED were added. TBE (0.5X) was used to run the gel.

SDS-Polyacrylamide (SDS-PAGE) gel

SDS-PAGE gels are used to separate the denatured proteins according to their molecular weight. It was used to study the over expression of recombinant proteins from bacteria, to see the purity of the purified proteins, for the Western blot etc. SDS-PAGE was also used to run the gel in protein-protein interactions by co-immunoprecipitation and GST pull down assay. Different percentage of gels was used according to the size of proteins to be separated. The percentage of resolving gels used in various studies in our experiments were of from 8-12%. Whereas, 4% stacking gel was used in all of our experiments. Samples for the SDS-PAGE were prepared by boiling the protein sample in 1X SDS-sample loading buffer. The buffer used for running the gel to separate the proteins is 1X Tris-glycine.

Coomassie Blue staining

After running the gel, it was transferred to the Coomassie brilliant blue stain solution and incubated for 30 minutes with constant shaking at room temperature. Gel was then destained by placing it in the destain solution and incubating it at room temperature with constant shaking.

Used destain was replaced with fresh destain solution until the background was cleared and the bands become clear.

Western blotting

After running the SDS-PAGE, the gel was transferred to the transfer tank buffer and kept for 20 minutes, followed by the transfer of proteins onto the nitrocellulose membrane by semi dry gel transfer apparatus (Biorad). The transfer was done at 13 volts for 40 min after that the nitrocellulose membrane was put in the blocking buffer (PBS, 1.0%BSA, and 0.05%Tween-20) and incubated at room temperature for one hour with constant shaking. Membrane was then washed thrice, 10 minutes each with wash buffer (PBS, 0.1%BSA and 0.05%Tween-20). Primary antibody was diluted in wash buffer (1:2000) and added, then incubated further for 1hr, again the membrane was washed three times. Secondary antibody conjugated with alkaline phosphatase (1:2000) diluted in wash buffer was added and incubated for 1hr. at room temperature, membrane was then washed thrice 10 minutes each with wash buffer. After washing, the membrane was developed in developing buffer (100 mM Tris-HCl pH-9.5, 50 mM $MgCl_2$, and 100 mM NaCl) containing NBT and BCIP tills the bands appear. Reaction was then stopped by adding 100mM EDTA.

Silver staining: DNA and proteins

Silver staining of the gels to visualize the samples in the polyacrylamide gel is a very sensitive technique and is able to detect as little as 2-3 ng of DNA and 0.1-1 ng of polypeptide. All the buffers required for silver staining are to be prepared freshly at the time of the experiment. The steps after the oxidizer treatment have to be strictly undertaken in dark. The water used in the silver staining was deionised Milli-Q water.

Silver staining of native Polyacrylamide gels for DNA visualization

The gel was placed in Fixative A for 30 minutes, solution was decanted and Fixative B was added and kept for 20 minutes with gentle shaking. The solution was decanted and again fresh Fixative B was added and kept for 10 minutes with gentle shaking. Further the solution was decanted and oxidizer solution was added and incubated for 5-8 minutes with constant shaking. Gel was washed twice with water for 5 minutes and then silver nitrate solution was then added and put on the shaker for 30 minutes in dark. The gel was washed with water for 30 minutes and subsequently the developer was added and incubated with gentle shaking for 2-6 minutes or till the color develops. The reaction was then stopped by washing the gel thoroughly with excess of water.

Silver staining of polyacrylamide gels for visualization of proteins

The gel was kept in Fixative solution for 30 minutes, washed the gel three times for 15 minutes each with water. Hypo solution was added and incubated for 1 minute, again washed two times with water for 1 minute each. Subsequently the gel was placed in silver nitrate solution for 20 minutes, again washed three times with water 1 minute each. Developer was added to equilibrate, then again developer was added and left the developer till the spots become visible, stop solution was then added. The gel was finally put in water.

Kination and purification of oligonucleotides

To perform EMSA the DBS was end labeled with ^{32}P to make the probe, single stranded DNA fragments containing the p53 consensus sequence were end-labeled with ^{32}P-γ-ATP using T4-PNK enzyme and then these were annealed to make radiolabelled DNA probes. Nucleic acids without phosphate group at their 5' end can be radiolabelled with ^{32}P-γ-ATP using T4 Polynucleotide kinase (T4 PNK). T4-PNK catalyzes the transfer of gamma phosphate of ribonucleoside 5'-triphosphate to the 5'-hydroxyl group of DNA. For the kination reaction the following composition was used.

Oligonucleotide	50 ng
10 X buffer	3.0 µl
T4 PNK enzyme	10 U
^{32}P-γ-ATP	2.0 µl
Milli Q water	made up the volume to 30 µl

The reaction mixture was incubated at 37° C for 1hr. Purification of radiolabelled oligonucleotide from unincorporated ^{32}P-γ-ATP was done using Sephadex G-50 column. To make the Sephadex G-50 column, slurry of Sephadex G-50 (50%) was made in sterile water and put it in 1ml syringe blocked with glass wool at the bottom. The column was centrifuged at 3000 rpm for 3 minutes. After packing the column the sample was loaded on it, and collected the eluted DNA in a micro-centrifuge tube kept inside a 15 ml tube by spinning at 3000 rpm for 5 minutes. Counts were taken for 1 µl sample in the beta scintillation counter.

Electrophoretic mobility shift assay

The sequence specific DNA binding activity of p53 was monitored by EMSA. A 51mer oligonucleotide containing the p53 binding site (5'- GGTACCAATTCTCGAGGAA CATG TCC CCAA CATGTTGCTCGAGATCTTTC-3') was derived from 5' site of the p21 promoter, and synthesized it from Microsynth. Both the sense and anti sense oligonucleotides were end labeled with ^{32}p ATP at the 5' end using T4 polynucleotide kinase, annealed and purified by Sephadex G-50 column. To setup the EMSA reaction with KB nuclear extract 5µg nuclear extract was used, the EMSA reaction mixture was made by adding 5µg nuclear extract, 1µg fragmented Salmon Sperm DNA, 2-3ng of radiolabelled probe (25,000 cpm) in 20 µl 1X GMS buffer and incubated the reaction mixture at 4°C for 30 min. For the supershift, the nuclear extract was mixed with 100ng of PAb 421 and incubated at room temperature for 30 minutes before setting up the reaction. The reaction mixture was then loaded on a pre-run 4% Native PAGE containing 2.5% glycerol, and run it at 15 volts/cm until the bromo-phenol dye reached 4/5th of the gel. Gel was then transferred on the Whatman filter paper and dried the gel in vacuum gel dryer at 75°C for 1hr. Autoradiography was done by exposing it against a X-ray film for 20 hrs and then the X-ray film was developed.

To setup the EMSA reaction with bacterially expressed and purified recombinant p53 protein, 100ng of p53 was mixed with 100ng of fragmented Salmon Sperm DNA and 2-3ng of radiolabelled probe (25,000 cpm) was added and the volume of the reaction mixture was made to 20 µl by adding 4 µl of 5X GMS buffer and water. Reaction mixture was mixed thoroughly and incubated it at room temperature for 30 min, the it was loaded on 4% Native PAGE gel containing 2.5% glycerol, run it at 15 volts/cm. Gel was transferred to Whatman paper and dried it in a vacuum gel dryer and autoradiography was done. To study the supershift antibody against p53 C terminus PAb C-19 was added to p53 and incubated at RT for 30 minutes. To study the role of NTD in the p53 DNA interaction NTD was added to the p53 or p53 PAb C-19 mix and EMSA reaction was performed.

To study the role of NTD in the preventing the loss of sequence specific DNA binding activity of p53 during thermal denaturation, the KB nuclear extract or the recombinant p53 protein was incubated for 1hr at 37°C in a water-bath in the presence or absence of NTD. The DNA binding was studied using this thermally treated protein as above. For elucidating the role of NTD in the recovery of DNA binding ability of heat denatured p53, p53 protein was incubated for1hr at 37°C, NTD was then added and incubated at room temperature for 30 min. EMSA reaction was then set up using this p53 protein.

p53 DNA interaction on the ELISA plate

The PAb 421 antibody (0.5µg) was diluted in 50µl PBS and added it in the well of ELISA plate. Plate was incubated at 4°C overnight and washed once with PBS then the remaining sites of the well was blocked by incubating 200 µl of 1% BSA in PBS for 3-4 hrs at 4°C. Subsequently the plate was washed thrice 5 min each, with wash buffer (PBS+0.05% Tween20). After that 0.5µg of p53 protein diluted in 50µl PBS was added to the well and incubated it at 4°C for 1hr., to remove the unbound protein plate was washed thrice 5 min each, with wash buffer. Then 0.5 µg of biotin labeled DBS diluted in 50µl 1X ELISA buffer was placed in the well and incubated at 4°C for 1hr. Plate was washed again three times with wash buffer, and 50µl of 1:400,000 diluted Alkaline Phosphatase conjugated Avidin (Sigma) in PBS was added in the well and incubated for 2 hrs. Washing was again done thrice with wash buffer,

and the color was developed by adding 100 µl of 1mg/ml *p*-nitro phenyl phosphate in alkaline Phosphatase buffer (pH 9.8) and incubating the plate at 37°C. The reaction was stopped by adding 100mM EDTA, and absorbance was taken at 405nm in the ELISA reader (Bio-Rad, Model 550, USA).

To study the binding of Class II DBS with p53, p53 protein (0.5µg) was diluted in the 50µl PBS and added in the well of ELISA plate, and incubated it at 4°C for overnight. The unbound p53 was removed by washing the wells once with PBS, the un-occupied sites of the wells were blocked by putting 200 µl of 1% BSA in PBS and incubating the plate at room temperature for 1hr. Plate was then washed thrice (5 min each) with wash buffer and 0.5 µg of biotin labeled DBS diluted in 50µl 1X ELISA buffer was placed and incubated at 4°C for 1hr. After washing thrice 5 min each with wash buffer the bound DBS was detected by placing 50µl of 1:400,000 diluted Alkaline Phosphatase conjugated Avidin (Sigma) in PBS and incubating the plate at 4°C for 2 hrs. Washing was again done three times, after that the color was developed by adding 100 µl of 1mg/ml para-nitro phenyl phosphate(PNPP) in alkaline Phosphatase buffer (pH 9.8) and incubating the plate at 37°C till the yellow color develops, the reaction was then stopped by adding 100mM EDTA. The absorbance was taken at 405nm in the ELISA reader (Bio-Rad, Model 550,USA).

To study the effect of NTD on the protection of DNA binding activity p53 coated ELISA plate was incubated at 37°C for 1hr. in the absence and presence of NTD and the DNA binding study was performed as above. To study the effect of NTD on the recovery of DNA binding of the heat denatured p53, 0.5 µg of wild type p53 protein was diluted in 50 µl PBS and incubated it at 37°C in the water bath, subsequently the denatured p53 was added in the well and incubated at RT for 2 hrs, the remaining sites were blocked with 1% BSA, then NTD was added to the denatured p53 coated wells and incubated the plate at room temperature for 1hr, after that plate was washed thrice with wash buffer, the DBS was then added and the bound DBS was probed with Avidin alkaline Phosphatase.

Conformation studies of p53 by ELISA

ELISA plate was coated with conformation specific antibodies (PAb 1620 and PAb 240), by adding 0.2μg antibody diluted in 50μl PBS in the wells and incubating the plate at 4° C overnight. Plate was then washed once with PBS and blocking of the remaining sites was done by incubating the plate with 1%BSA in PBS at 4°C for 3-4 hrs. The p53 protein was diluted in 50μl PBS, placed in the wells and incubated the plate at room temperature for 1hr. Plate was then washed thrice 5 min each, with wash buffer and 0.2 μg C-19 antibody was diluted in 50μl blocking buffer, added to the well and incubated 1hr. at room temperature. Washing was again done thrice with wash buffer and 0.2 μg alkaline Phosphatase conjugated anti-goat secondary antibody diluted in 50μl blocking buffer was added to the well and incubated at room temperature for 1hr. The plate was again washed three times 5min each with wash buffer. Color was developed by adding 100μl of 1mg/ml para-nitro phenyl phosphate in alkaline Phosphatase buffer (pH 9.8) and incubating the plate at 37°C till the yellow color developed, reaction was then stopped by adding 100mM EDTA. The absorbance was taken at 405nm in the ELISA reader (Bio-Rad, Model 550, USA). To study the role of NTD in the protection of wild type p53 conformation, p53 was diluted in 50μl PBS and incubated it at different temperatures in water bath for 1hr. in the presence and absence of NTD and placed on the ELISA plate coated with PA1620 and PAb 240 and the bound p53 was detected by goat raised antibody PAb C-19.

Conformation studies of p53 by Immuno-precipitation

The p53(1μg) was diluted in 0.5 ml PBS and incubated at different temperatures for 1hr in the absence and presence of NTD, subsequently PAb 1620 and PAb 240 (0.2μg) separately added and incubated at room temperature for 1hr with constant rotation. Then 10μl of 50% protein A agarose beads were added and further incubated at room temperature for 1hr. The mixture was centrifuged at 2000rpm at 4°C for 5 min, supernatant was discarded and 0.6ml of wash buffer with 0.1% NP-40 was added and further rotated at room temperature for 10 min then centrifuged again at 2000rpm for 5 min at 4°C. The washing step was repeated 5 times and then the beads were boiled in 20μl SDS loading buffer for 5 min. Beads were pelleted down by short

spin and loaded on 12% SDS-PAGE. Proteins were then transferred on nitro-cellulose membrane and immuno-blotted.

Protein-protein interaction

ELISA

To coat the p53 protein on the ELISA plate, 500ng of the GST-p53 protein diluted in the 50 µl PBS pH7.4 was added in the wells of ELISA plate and left the plates at 4°C overnight. Plate was then washed once with PBS to remove the unattached protein from the plate. Blocking of the remaining sites was done by incubating the plate with 1%BSA in PBS at 37°C for 1hr. or at 4°C for 3-4 hrs. Further the plate was washed three times with PBS containing 0.05% Tween20 then the NTD was diluted in the 50 µl of 1% BSA and added to the wells and incubated the plate at room temperature for 1hr. The unbound proteins were washed with wash buffer three times each for 5 minutes. Antibody against the 6X-His tag was diluted in 1%BSA, added in the wells and put the plates at room temperature for 1hr., again plate was washed three times with wash buffer. Then the alkaline phosphatase conjugated secondary antibody was diluted in 1% BSA, added in the wells and incubated at room temperature for 1hr, plate was washed again three times. The color was developed by adding 1mg/ml *p*-nitro phenyl phosphate in alkaline phosphatase buffer pH 9.8 and incubating at 37°C. Reaction was stopped by adding 100mM EDTA and absorbance at 405 nm was taken in ELISA reader (Bio-Rad Model 550 USA).

Immunoprecipitation

For the immunoprecipitation of p53 (3.0 µg) and NTD (1.5 µg) were mixed and incubated at the room temperature for 1hr with constant rotation, then 500µl of PBS and 0.2 µg of anti-p53 antibody was added and further incubated for 1hr at room temperature. Further 15µl of 50% protein A agarose beads was added and again incubated at room temperature for 1hr. The mixture was then centrifuged at 2000 rpm at 4°C for 5 min, supernatant was discarded and 0.6ml of wash buffer with 0.1% NP-40 was added and further rotated at 4°C for 10 min then centrifuged again at 2000 rpm for 5 min at 4°C. The washing step was repeated 4-5 times and

then the properly washed protein A agarose beads were boiled for 5 min in 20µl SDS loading buffer. The beads were pelleted down by short spin and loaded the contents on 12% SDS-PAGE. The proteins of the gel were transferred on the nitrocellulose membrane in a dry blot apparatus (Bio-Rad) for 40 min at 13 Volts, and the membrane was immunoblotted.

Circular Dichroism Spectroscopy

All the measurements were done on a Jasco J-815 Circular Dichroism Spectrometer, in the far ultra-violet range from 200 to 260nm using the cuvette of 0.1cm pathlength. 200µg of p53 or NTD was diluted in the 0.5 ml PBS and the spectra were collected at different temperatures. For each sample an average was taken of the three measurements at a scan rate of 20nm/min. For the interaction studies 200µg each of p53 and NTD were mixed and diluted in 0.5 ml PBS then incubated for 1 hr. at the temperature at which spectra taken. Then the spectra were taken at different temperatures. For all the measurements the spectra of the buffer was deduced from the sample.

Aggregation assay

The thermal aggregation kinetics of wild type p53 in the presence and absence of NTD was monitored at different temperatures on a Varian Eclipse fluorescence spectrophotometer in a thermal cuvette with constant stirring. All the measurements of light scattering were done at the excitation and emission wavelengths of 340 nm with a spectral bandwidth of 2.5 nm. The aggregation kinetics of p53 alone was studied by taking 1µM p53 in PBS and measured the light scattering at 37°C for 60 min, and at 45°C for 40 min. To observe the protection of p53 aggregation by NTD' 2µM and 5µM of NTD was mixed separately with 1µM p53 in PBS and light scattering was measured at 37°C and at 45°C for 60 min and 40 min respectively. The aggregation of NTD was also monitored at 37°C and 45°C which showed no light scattering.

Cell Culture and Transfection

H1299 cells were on grown on 22-mm coverslip (Blue-Star) in DMEM media (Hi-Media) supplemented with 10% fetal calf serum, penicillin, and streptomycin. Transfection of H1299 cells was performed using Effectene Transfection Reagent (Qiagen), according to manufacturer's instructions. After 24 hrs of transfection cells were washed once with PBS and fixation was done by incubating 4% Paraformaldehyde for 20 min at room temperature. Coverslips containing the fixed cells mounted in 50% glycerol in PBS on a slide. Cells were then visualized in the Confocal Laser microscope LSM-510 (Zeiss, Germany).

Results

RESULTS

Specific binding of p53 to its promoter sequence is lost at physiological temperature of 37°C. However in the presence of Hsp90 the p53 binding ability to promoter is retained in an ATP dependent manner both *in vivo* and *in vitro* conditions (Muller, 2004). The p53 protein at 37°C loses its correct fold, which is required for efficient specific binding of p53 to the promoter sequence. However, the ability of p53 to bind to a promoter sequence at 37°C is possible in the presence of the Hsp90 molecular chaperone and ATP both *in vivo* and *in vitro* (Muller, 2004). Recent reports have shown that MDM2 chaperones p53 in an ATP-dependent manner and CHIP in an ATP independent manner, hence maintains the p53 wild type conformation and retains its DNA binding ability to its promoter at physiologically relevant temperature (Wawrzynow, 2007: Tripathi, 2007). Moreover CHIP has been shown to prevent the thermal denaturation and subsequent aggregation of p53 protein. The above mentioned proteins work as chaperones for p53 and protect the stability and activity of the protein in the cell. In the present study we have shown that the amino terminal domain (NTD) of p53 (residues 1-125) protects the wild type conformation of p53 and its sequence specific DNA binding activity at physiological and elevated temperature.

Cloning, expression and purification of p53 and NTD

The cDNAs of human wild type p53 gene and NTD were cloned and the recombinant proteins were overexpressed in *E.coli.* Cloning of p53 was carried out by the PCR amplification using the internal primers and subsequently the PCR product was digested with *Bam*HI and *Hind*III restriction endonucleases and ligated on the same restriction sites in pET-32a (Fig.. 1A) as well as in pET-28a plasmids (Fig.. 1C) (Novagen). Cloning of NTD cDNA was carried out on *Bam*HI and *Hind*III restriction sites in the pET32a plasmid (Fig.. 1B) and on *Bam*HI and *Eco*RI restriction sites of pET28a plasmid (Fig.. 1D). Proteins were expressed in the *E.coli* BL21 (DE3) strain, using the IPTG induction and the purification was done using Ni-NTA metal affinity chromatography.

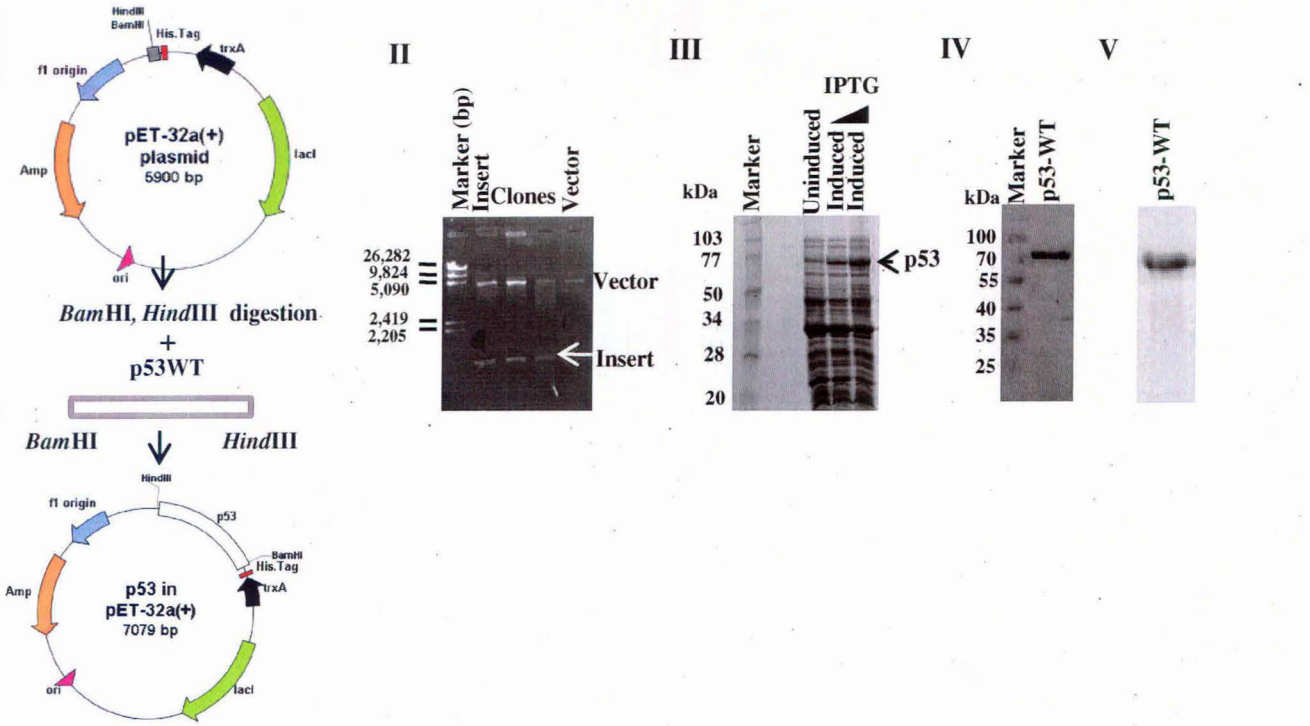


Fig. 1A. Construction of pET32a-p53, protein expression and purification. **(I)** Schematic diagram showing the construction of His-tagged p53 expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Bam*HI and *Hind* III restriction enzymes on 1% agarose gel. **(III)** 10% SDS-PAGE showing the induction of recombinant p53 using increasing concentration of IPTG in *E.coli* BL-21 (DE3) cells. **(IV)** Western blot of p53 using PAb 1801. **(V)** SDS-PAGE showing the purified protein using NI-NTA affinity chromatography.

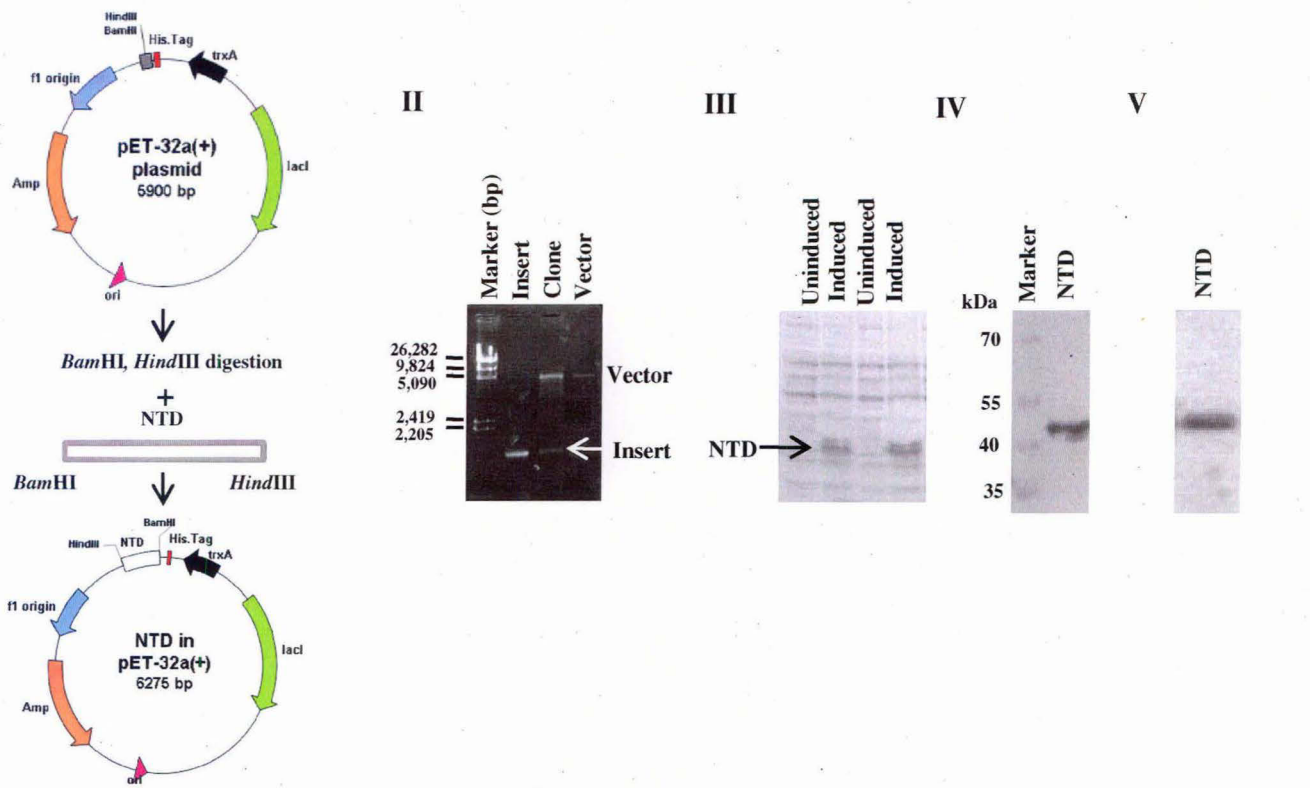


Fig. 1B. Construction of pET32a-NTD, protein expression and purification. **(I)** Schematic diagram showing the construction of His-tagged NTD expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Bam*HI and *Hind*III restriction enzymes on 1% agarose gel. **(III)** 10% SDS-PAGE showing induction of NTD using 0.5 mM IPTG in *E.coli* BL-21 (DE3) cells. **(IV)** Western blot of purified NTD using PAb 1801. **(V)** Ni-NTA purified NTD on 12%SDS-PAGE.

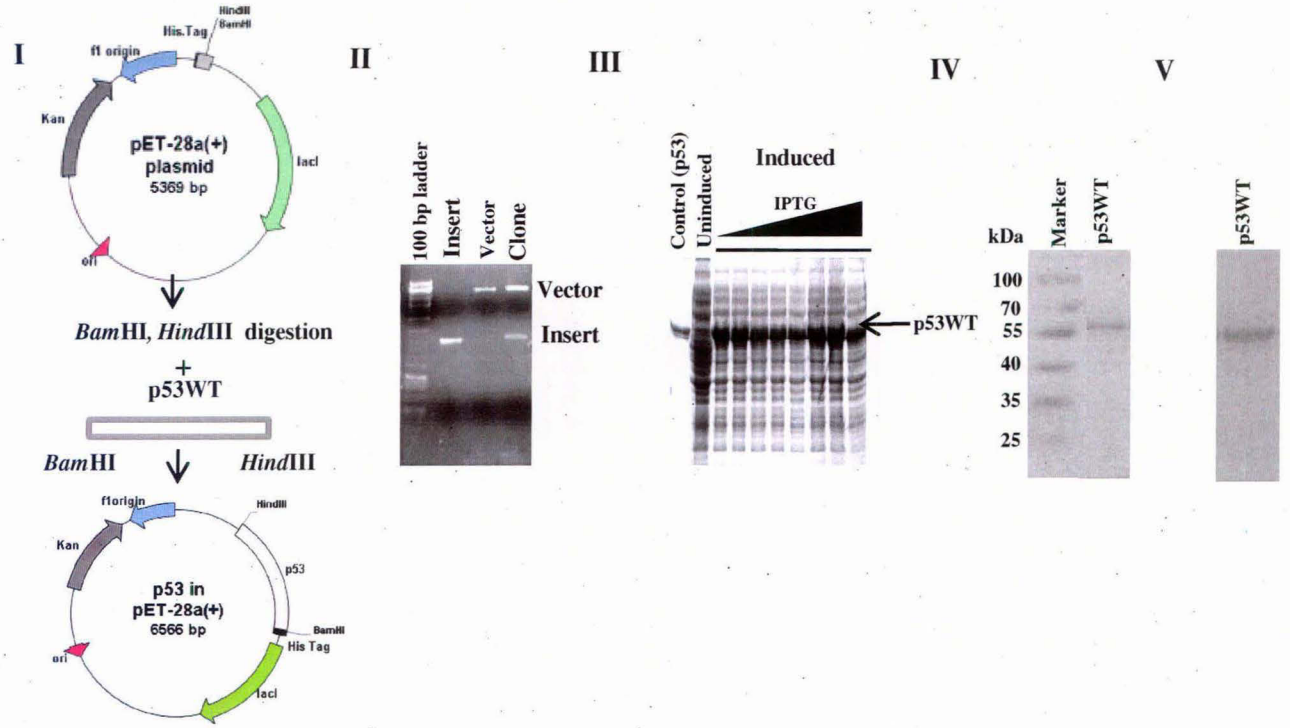


Fig. 1C. Construction of pET28a-p53, protein expression and purification. **(I)** Schematic diagram showing the construction of His-tagged p53 expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Bam*HI and *Hind*III restriction enzymes on 1% agarose gel. **(III)** 12% SDS-PAGE showing the induction of recombinant p53 protein with increasing IPTG concentration (0.1-1.0 mM). **(IV)** Western blot of p53 using PAb 1801. **(V)** Ni-NTA purified protein on 12%SDS-PAGE.

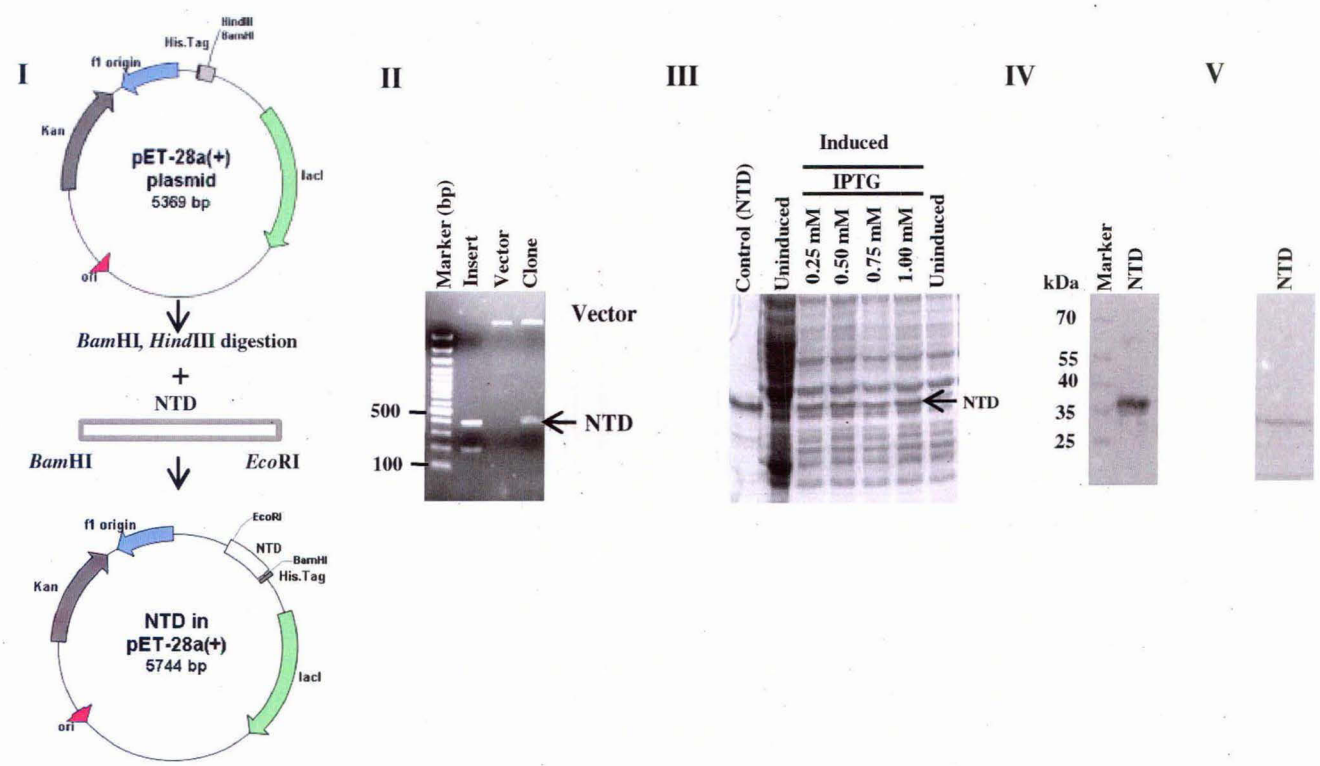


Fig. 1D. Construction of pET28a-NTD, protein expression and purification. **(I)** Schematic diagram showing the construction of His-tagged NTD expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Bam*HI and *Eco*RI restriction enzymes on 1% agarose gel. **(III)** 12% SDS-PAGE showing the induction of NTD with increasing concentration of IPTG in *E.coli* BL-21 (DE3) cells. **(IV)** Western blot using PAb 1801. **(V)** 12% SDS-PAGE showing the Ni-NTA purified protein.

NTD enhances DNA binding activity of p53 protein

To study the effect of NTD on the DNA binding activity of the wild type p53 protein, the bacterially expressed and purified wild type p53 and NTD were used and EMSA was carried out using the P^{32} labeled 5' DBS of p21 promoter. NTD itself does not have any DNA binding property, however to rule out the nonspecific interaction an EMSA reaction was set up using the NTD and p21 5'DBS which didn't show binding (Fig.. 4B, *lane 2*). To observe the effect of NTD on the DNA binding activity of wild type p53 protein, NTD was added in increasing concentration in the reaction mixture containing p53 protein and incubated for 30 minutes at room temperature, subsequently the EMSA reaction was set up using p21 5'DBS in the absence (Fig.. 2A) and the presence (Fig.. 2B) of p53 C terminus antibody PAb C-19. In the presence of NTD we observed an increase in the sequence specific DNA binding activity of p53 protein in a dose dependent manner.

NTD physically interacts with p53

NTD increases the sequence specific DNA binding activity of p53 protein however no supershift was observed which shows that NTD does not interacts with the p53 DNA complex. However there is probability of its interaction with p53 in a reversible manner, which is dissociated after p53 interacts with DNA. It has been shown earlier that Hsp90 interacts with p53 in a reversible manner to its DNA binding domain (Muller, 2004). To investigate whether NTD physically interacts with p53 several independent methods were utilized. To study the interaction using ELISA, His-tagged NTD was added and incubated with GST-p53 already coated on the ELISA plate and the fraction of NTD bound with GST-p53 was detected using the anti-His antibody, which showed interaction between the two (Fig.. 3A, *lane 3*). As a positive control GST-MDM2 was used which showed interaction with NTD (Fig. 3A, *lane 2*), the BSA that is used as a negative control, didn't show binding (Fig. 3A, *lane 1*). Similar results were also observed when the binding reaction was performed vice versa. In this reaction NTD was coated in the well and p53 was added and incubated with it and the bound fraction of p53 was detected with p53 C-terminal antibody PAb C-19. With increase in the p53 concentration the signal of binding was increased (Fig. 3B.).

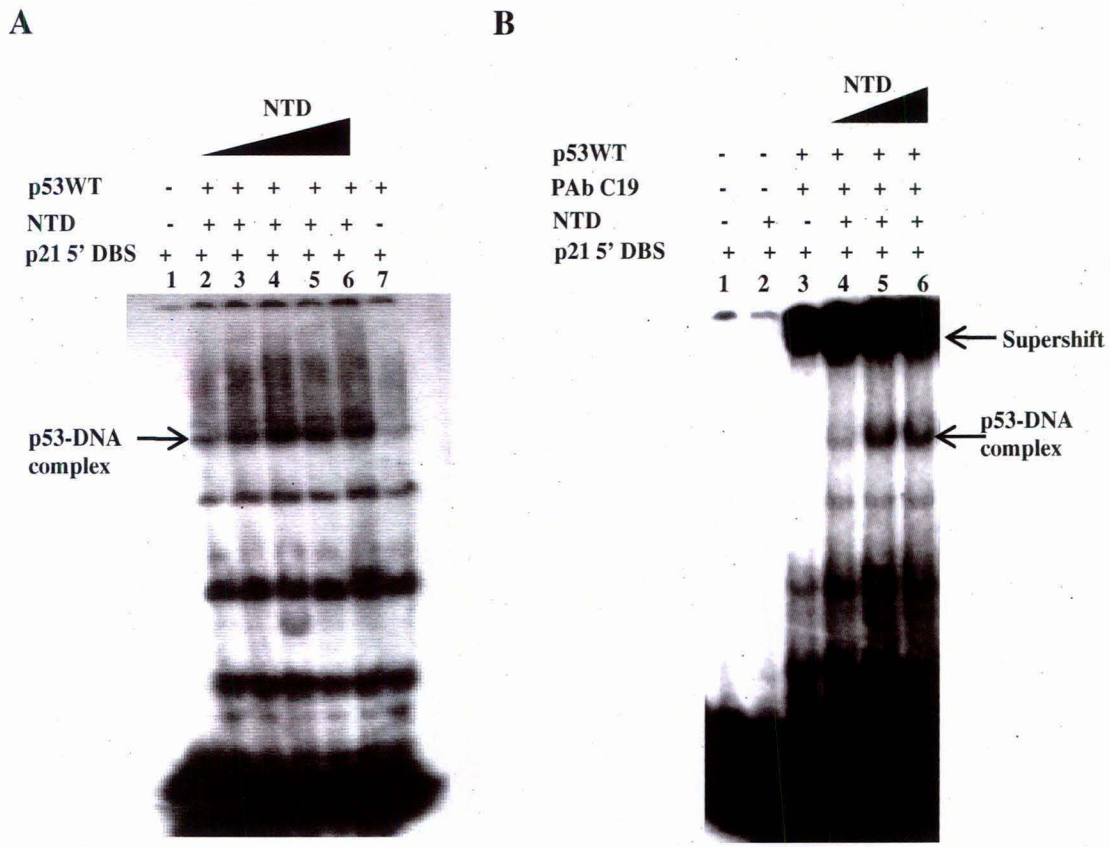


Fig. 2. EMSA showing the increase in the DNA binding activity of p53 protein by NTD in a dose dependent manner. (**A**) 100 ng of recombinant p53 protein was mixed with an increasing concentration of NTD from 0.1 μg (*lane 2*), 0.2 μg (*lane 3*), 0.4 μg (*lane 4*), 0.8 μg (*lane 5*) to 1.6 μg (*lane 6*) and incubated at room temperature for 30 minutes. 2 ng of P^{32}-labeled p21 5'DBS was added and further incubated at room temperature for 30 minutes and electrophoresed was carried out on 4% EMSA gel at 10 V/cm. (**B**) 100 ng of recombinant p53 protein was incubated with PAb C-19 (100 ng) for 30 minutes NTD was then added in an increasing concentration from 0.2 μg (*lane 4*), 0.4 μg (*lane 5*) to 0.6 μg (*lane 6*) and further incubated at room temperature for 30 minutes and subsequently EMSA was performed using P^{32}-labeled p21 5' DBS .

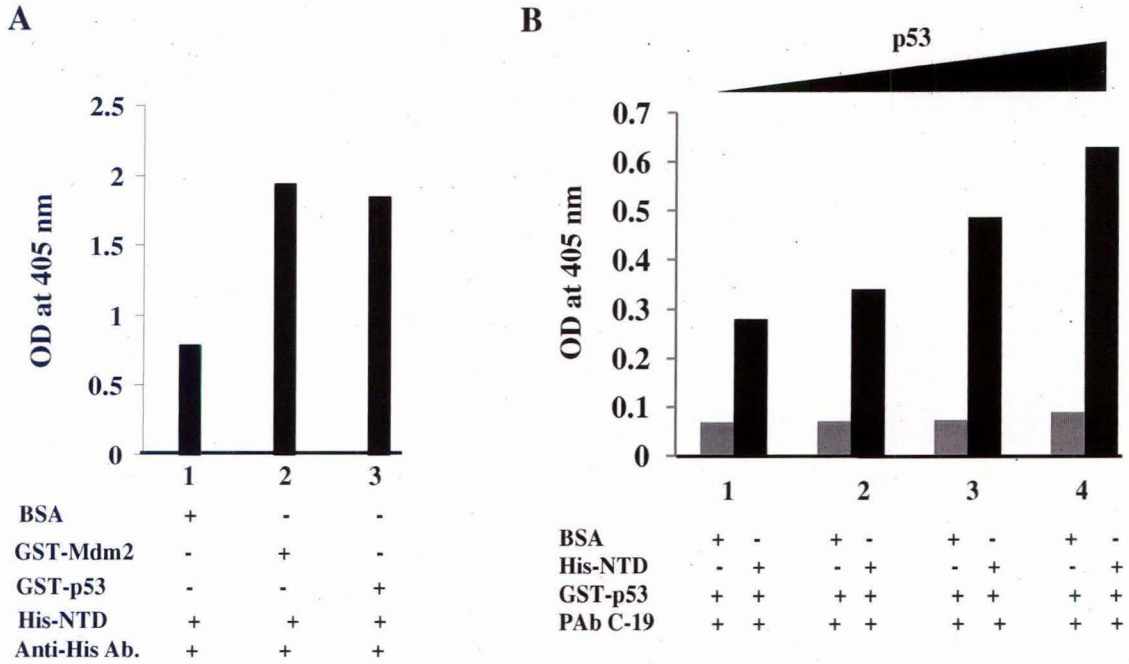


Fig. 3. ELISA showing the interaction between NTD and p53. **(A)** BSA (*lane 1*), GST- MDM2 (*lane 2*), and GST-p53 (*lane 3*) were coated separately on the ELISA plate and His-NTD (0.5 µg) was incubated with these proteins at room temperature for 2 hrs. Subsequently the binding of His-NTD with these proteins was studied using anti-His antibody. **(B)** BSA and His-NTD were coated separately on the ELISA plate and GST-p53 protein was added in increasing concentration from 50 ng to 400 ng (*lane 1* 50 ng, *lane 2* 100 ng *lane 3* 200 ng, *lane 4* 400 ng) onto both the BSA and NTD coated wells and plate was incubated at room temperature for 2 hrs. Plate was washed extensively and the interaction between NTD and p53 was studied using PAb C-19.

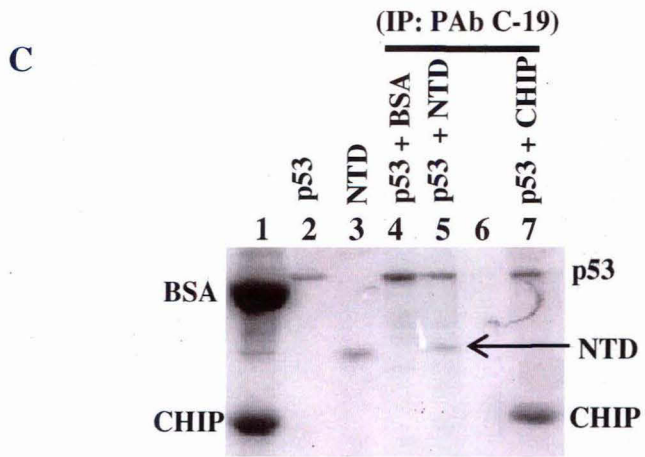


Fig. 3 C. Immunoprecipitation assay showing the interaction between NTD and p53. 1 µg of p53 was mixed separately with 1 µg of NTD (*lane 5*), BSA (*lane 4*) and CHIP (*lane 7*) and incubated the protein mixture at room temperature for 2 hrs, subsequently the PAb C19 (200 ng) was added in protein mixture and further incubated it at room temperature for 1hr then the complex was pulled down using protein A agarose. The protein A agarose beads were boiled and loaded and electrophoresed on 10% SDS-PAGE, and the proteins were visualized by staining the gel with Coomassie Brilliant blue. *Lanes1-3* show the proteins used in immunoprecipitation reaction.

The interaction was further confirmed by immunoprecipitation assay. To perform the assay recombinant p53 and NTD were mixed in equal ratio and incubated it at room temperature for 2 hrs, the p53 was pulled down using PAb C-19 and SDS-PAGE was run, followed by Comassiae blue staining. As a positive control CHIP was taken which has been shown to interact with the p53 amino terminus (Tripathi, 2007) and as a negative control BSA was used. The result showed that NTD was pulled down along with p53 (Fig. 3C, *lane 5*) as was seen in case of CHIP (Fig. 3C, *lane 7*) which served as positive control but not with BSA (Fig. 3C, *lane 4*). To identify the domain of p53 responsible for its interaction with NTD, His tagged NTD was added and incubated with GST-p53, GST-$p53^{140\text{-}393}$ and GST-NTD already coated on ELISA plate and the bound NTD was detected with anti-His antibody. NTD was also incubated in the same way with $p53^{285\text{-}293}$ and the bound fraction was detected with PAb 1801. The signal of binding showed that NTD interacts with GST-p53, GST-$p53^{140\text{-}393}$ and $p53^{285\text{-}293}$ whereas it does not interacts with GST-NTD (Fig. 3D).

Next we studied the role of conformation of p53 on its interaction with NTD. To study the effect of conformation on their interaction, NTD and p53 were mixed in equimolar ratio and incubated at room temperature and the immunoprecipitation was performed with PAb C-19 as well as with conformation specific antibodies PAb 1620 and PAb 240. The PAb 1620 recognizes the native DNA binding conformation of p53 protein whereas the PAb 240 recognizes the structural mutants as well as the denatured p53 protein. Both of these antibodies bind with the DNA binding domain of p53 protein. The results of immunoprecipitation showed that at room temperature the ratio of wild type: mutant conformation of p53 is 2:1. It was observed that the amount of NTD bound with mutant conformation of p53 was two times more than that of wild type conformation of p53 (Fig. 3E, *lane 2* and *lane 3*). This indicated that the binding affinity of NTD towards the mutant conformation of p53 protein is higher in comparison to wild type conformation, however it binds with both the conformations of p53 protein.

NTD protects the unfolding of p53 structure at physiological and elevated temperatures

CD in the far ultraviolet region (178–260 nm) arises from the amides of the protein backbone and is sensitive to the conformation of the protein. Far UV CD spectroscopy of NTD

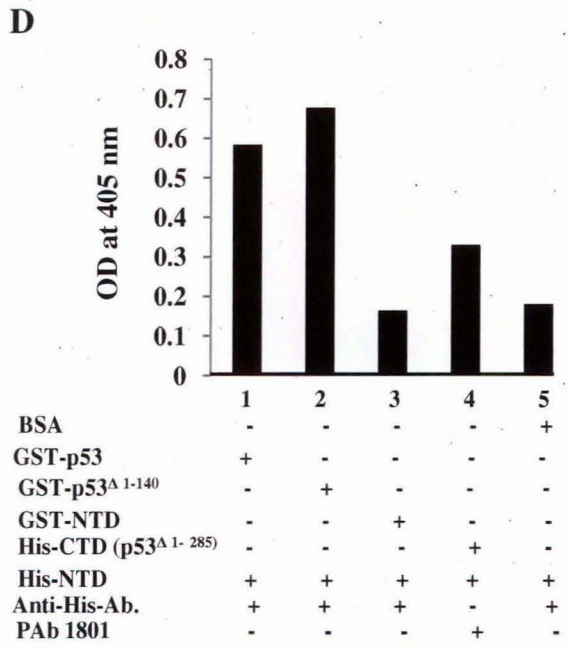


Fig. 3D. ELISA showing the interaction of NTD with different p53 domains. GST-p53 (*lane 1*), GST-p53$^{\Delta 1-140}$ (*lane 2*), GST-NTD (*lane 3*), His-CTD (*lane 4*) and BSA (*lane 5*) were coated separately on the ELISA plate and His-NTD (0.5 μg) was added and incubated with these proteins at room temperature for 2hrs. Subsequently the binding between His NTD and GST-p53, GST-p53$^{\Delta 1-140}$, GST-NTD and BSA was studied by using anti-His antibody (*lanes 1-3*, and *5*). Whereas the binding between His-NTD and His-CTD was observed by using PAb 1801 (*lane 4*).

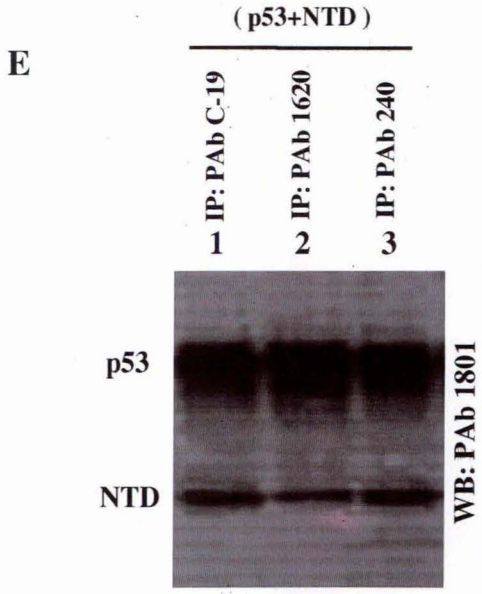


Fig. 3E. Immunoprecipitation assay showing that NTD interacts both with wild type and mutant conformation of p53. p53 and NTD were mixed in equimolar ratio and incubated the protein mixture at room temperature for 1hr then PAb C-19 (*lane 1*), PAb 1620 (*lane 2*) and PAb 240 (*lane 3*)antibodies were added separately to NTD p53 mix and further incubated for 1hr. The antibody bound protein complex was then pulled down using protein A sepharose and the proteins were separated on 12% SDS-PAGE followed by immunoblotting with PAb 1801.

revealed that its secondary structure is reversible. NTD lost its secondary structure when the temperature was raised from 25°C to 95°C, however it gained its original structure when the temperature was lowered to again to 25°C. This shows that NTD is a thermally stable polypeptide and its secondary structure is completely reversible (Fig. 4B). Whereas p53 protein looses its secondary structures with the increase in temperature from 25°C to 95°C and the loss of its secondary structure is irreversible as the lowering of temperature from 95°C to 25°C does not result in the gain of secondary structure (Fig. 4A).

Far UV CD spectroscopy is also a useful technique for studying protein–protein interactions in the solution if the interaction of two proteins results in a change of secondary structure. To investigate if the interaction of NTD with p53 results in secondary structure change, the two proteins were mixed in equal amount and incubated at the room temperature for 1 hr subsequently the CD spectra was taken at 25°C, which showed that the proteins interact as the observed spectra of the protein mixture differs from the sum of individual spectra of NTD and p53 (Fig. 5A). At 37°C (Fig. 5B) and 42°C (Fig. 5C) the observed spectra of the protein mixture also differs from the sum of individual spectra of NTD and p53 which shows that the interaction remains at these temperatures. However at 95°C the difference between observed spectra of protein mixture and the sum of the spectra of p53 and NTD vanished which showed that the two become dissociated at this temperature (Fig. 5D). Further while analyzing the spectra at different temperatures we found that the observed spectra of the NTD and p53 mixture remain constant from 25°C to 42°C, while the sum of the spectra of p53 and NTD continuously decreases. So these results indicate that at 37°C and at 42°C both NTD and p53 starts unfolding, however the mixing of both results in the prevention of their unfolding.

Aggregation of p53 protein is protected by NTD

Many proteins including p53 form aggregates due to unfolding. The CD spectra has shown that unfolding of p53 is prevented in the presence of NTD. To explore whether the thermal aggregation of p53 can be prevented by NTD, we have performed the aggregation assay of p53 in the absence and in the presence of NTD. To study the effect of NTD on the aggregation kinetics of p53 protein, highly purified p53 and NTD were used. First the p53 protein (1μM) was

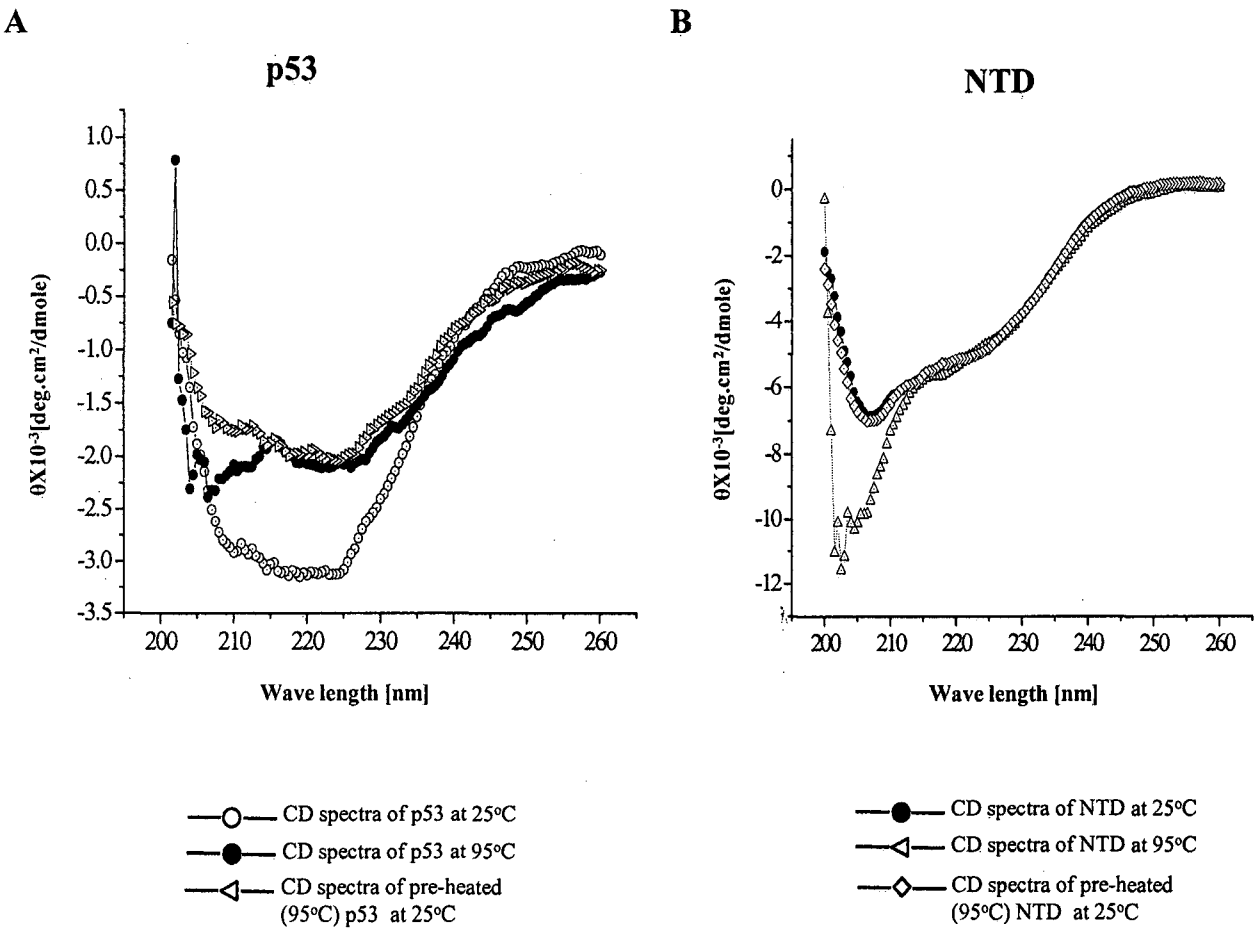


Fig. 4. Far UV CD spectra of p53 and NTD showing the reversibility of NTD structure. (A) CD spectra of p53 (200 μg p53 in 500 μl PBS in a cuvette of 0.1 cm path-length) was collected at 25°C, then the temperature was increased to 95°C and the spectra was collected, subsequently the temperature was again decreased to 25°C and spectra was collected. **(B)** CD spectra of NTD (200 μg in 500 μl PBS in a cuvette of 0.1cm path-length) was collected in the same manner as for p53 protein.

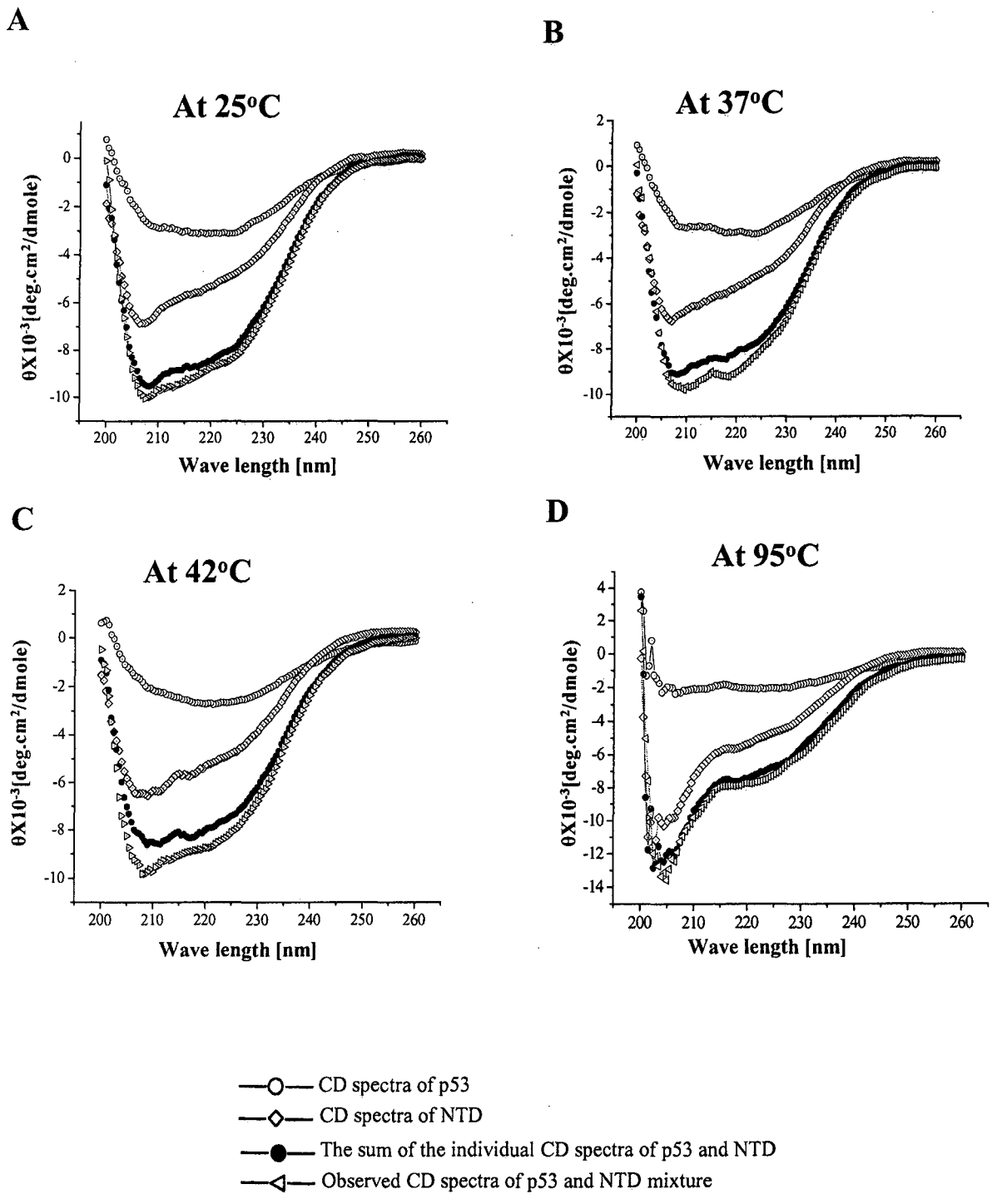


Fig. 5. Far UV CD spectroscopy showing the interaction between NTD and p53 protein. NTD (200 μg) and p53 (200 μg) were mixed in 0.5 ml PBS, incubated separately at different temperature for 1hr and the CD spectra was collected at 25°C **(A)**, at 37°C **(B)**, at 42°C **(C)** and at 95°C **(D)**.

incubated at 37°C with constant stirring and its aggregation kinetics was measured by monitoring the light scattering using fluorescence spectrophotometer at 340 nm on 2.5 nm bandwidth. Aggregation of p53 started immediately after the onset of incubation and a plateau was reached after 40 minutes (Fig. 6A).

Further the NTD was mixed with the p53 protein in a ratio of 2:1, and the light scattering was measured at 37°C, the result of which showed a significant decrease (60-70%), in the light scattering which was further decreased (80%) when the ratio of NTD to p53 was taken 5:1. The aggregation kinetics of p53 protein was also measured at 45°C in the same way as at 37°C and found that p53 aggregates faster and a plateau reached after 10 minutes of incubation (Fig. 6B). Even at 45°C the addition of NTD resulted in the decrease of light scattering in the same manner. This shows that NTD effectively prevents the aggregation of p53 protein in a dose dependent manner both at physiological and elevated temperatures. The aggregation kinetics of NTD was also measured both at 37°C as well as at 45°C, which didn't not show any increase in the light scattering (data not shown). This observation indicated that the NTD is stable at these temperatures and did not aggregate. These results showed that the NTD of p53 protein is very stable and it effectively prevents the aggregation of p53 protein both at 37°C and 45°C, this also indicated that the interaction between these proteins is strong enough at 45°C without which aggregation can not be prevented.

NTD protects the wild type conformation of p53 at physiological and elevated temperatures

Wild type p53 undergoes transient conformational transformations under heat stress similar to that of conformational unfolding caused by structural mutations (Zylicz, 2001). Both the structural mutants of p53 protein and the wild type p53 under heat stress are recognized by the mutant specific antibody PAb 240, whereas the wild type p53 is recognized by PAb 1620. Since the NTD interacts with p53 protein at different temperatures, as well as it prevents the thermal aggregation of p53 protein at physiological and elevated temperatures, we explored the role of NTD in the protection of wild type p53 conformation under heat stress conditions. ELISA as well as the immunoprecipitation assay was used to perform the experiment. In the ELISA conformation specific antibodies of p53 protein PAb 1620 and PAb 240 were coated on the

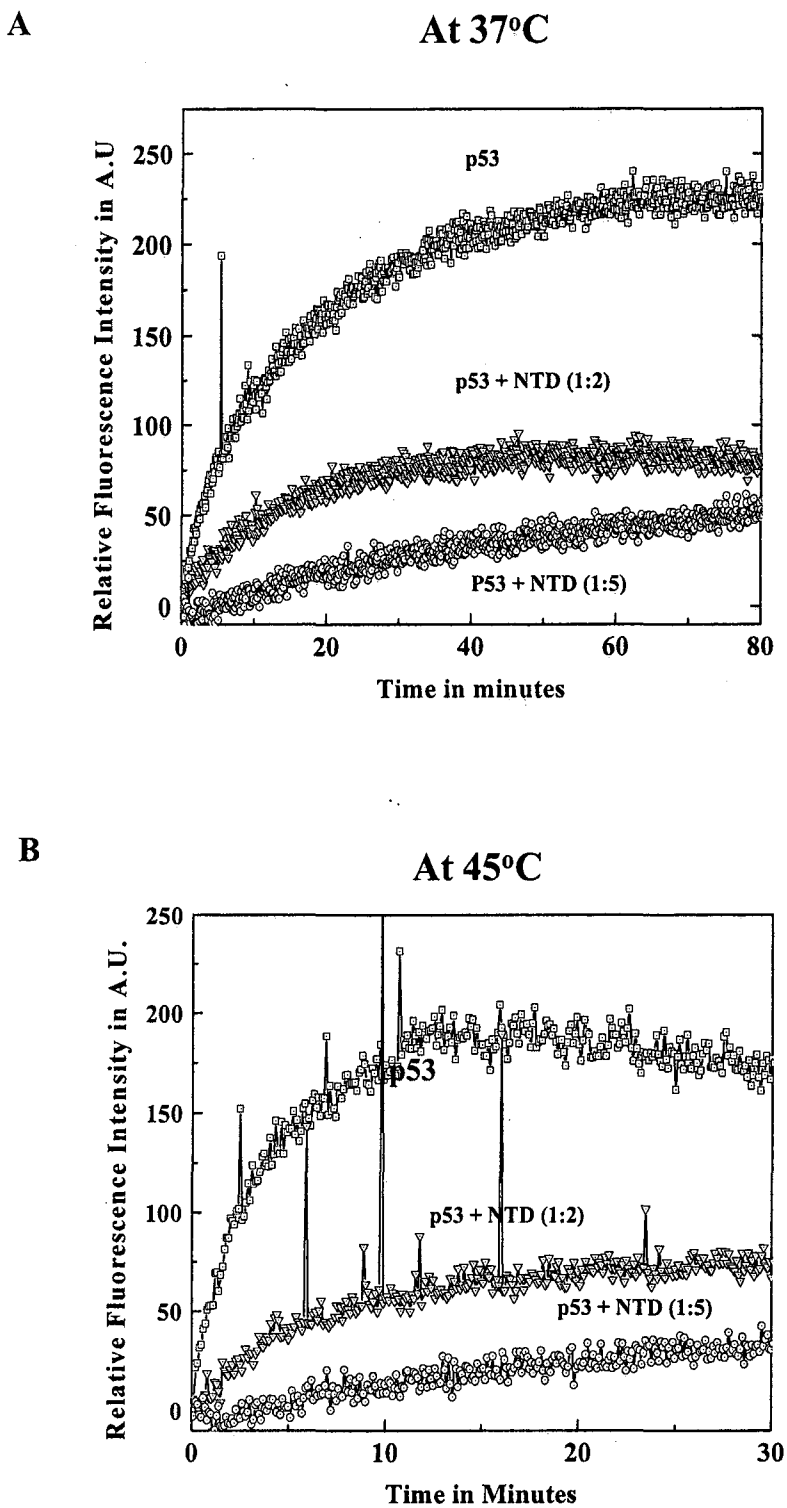


Fig. 6 Light Scattering assay showing the prevention of p53 aggregation by NTD. The aggregation kinetics of p53 protein (1.0 μM) was monitored by measuring the light scattering at 340 nm in the absence of NTD or in the presence of 2.0 μM NTD and 5.0 μM NTD at 37 °C **(A)** and at 45°C **(B)**.

surface of ELISA plate, subsequently the p53 protein was incubated at different temperatures (25°C, 37°C, 40°C, 42°C and 45°C) for 1hr in the absence or in the presence of NTD and added it on the antibody coated ELISA plate. The bound p53 was detected using PAb C-19 raised in goat and anti-goat secondary antibody. The ELISA experiments have shown that the population of p53 protein bearing the wild type conformation decreases as the temperature was increased from 25°C to 45°C, however the p53 population having mutant conformation increases with the increase in temperature. Addition of NTD however resulted in a decrease of p53 bearing mutant conformation as well as an increase of p53 bearing wild type conformation (Fig. 7A).

Further the immunoprecipitation assay also confirms the data obtained using the ELISA. To carry out the experiment the p53 protein was incubated at different temperatures (37°C and 45°C) for 1hr in the absence or in the presence of NTD subsequently the p53 was pulled down with PAb 1620 and PAb 240 and immunoblotted with PAb C-19. At room temperature the ratio of wild type and mutant p53 is equal whereas at 37°C as well at 45°C the amount of wild type p53 decreases whereas mutant p53 increases however the addition of NTD results in the protection of wild type conformation at both of these temperatures (Fig. 7B). These results show that NTD protects the wild type conformation of the p53 protein at physiological and elevated temperatures.

NTD protects the loss of p53 DNA binding activity

The wild type conformation of p53 protein recognized by the PAb 1620 is the conformation that binds with DNA. The conformation of wild-type p53 is very temperature-sensitive and the loss of the PAb1620 reactive epitope correlates with a loss of the tetrameric nature of p53 and the p53 DNA binding activity (Hansen, 1996). The last experiment has shown that the NTD protects the PAb 1620 positive conformation of p53 at physiological as well as at elevated temperature and the PAb 1620 positive conformation of p53 is responsible its DNA binding activity. To explore if NTD is also able to protect the DNA binding activity of p53 in addition to its protection of the wild type p53 confirmation, p53 was heat denatured in the absence and presence of NTD and the DNA binding study was performed. This experiment was carried out using both the cellular p53 protein from KB cells as well as recombinant p53 and

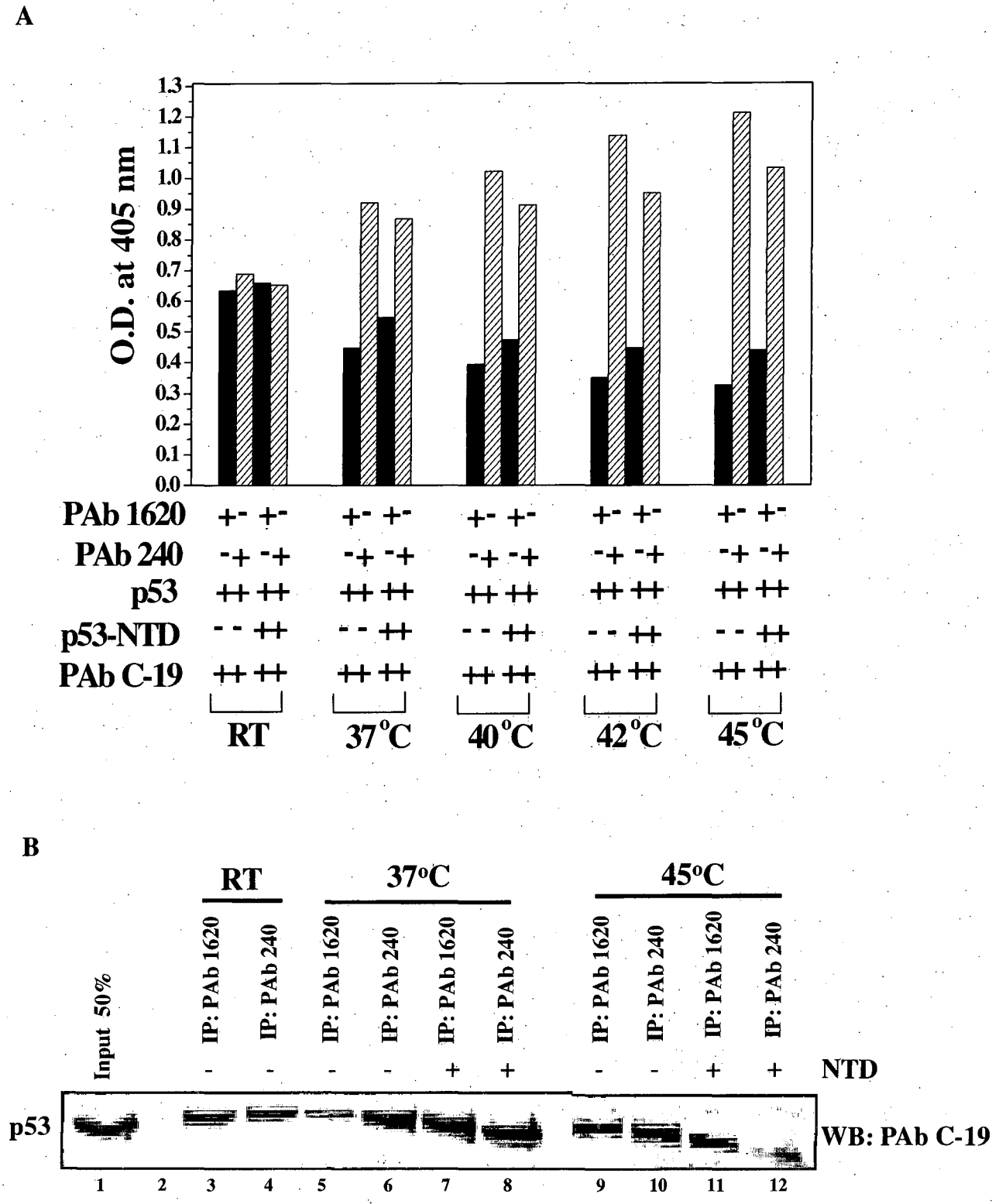


Fig. 7. ELISA and immunoprecipitation assay showing the role of NTD in the protection of wild type conformation of p53 at physiological and elevated temperatures. (A) Conformation specific antibodies of p53, PAb 1620 and PAb 240 were coated on the ELISA plate. The p53 (100 ng) protein was heat denatured separately at different temperatures for 1hr (37°C, 40°C, 42°C and 45°C) in the absence or presence of 1.0 μg of NTD and the conformation of p53 protein was detected by placing these samples on ELISA plate coated with PAb 1620 and PAb 240 followed by detecting the bound p53 with PAb C-19 (goat raised). (B) p53 protein (1.0 μg) was incubated at 37°C and 45°C separately for 1hr in the absence or presence of 10 μg of NTD, subsequently the amount of wild type and mutant conformation of p53 protein was detected by immunoprecipitation with PAb 1620 and PAb 240 followed by immunoblotting with PAb C-19.

P^{32} labeled p21 5'DBS. The wild type p53 protein present in the KB nuclear extract looses its DNA binding activity by more than 95% when incubated at 37°C for 1 hr (Fig. 8A , *lane 4*) whereas addition of NTD with KB nuclear extract before the heat treatment resulted in the protection of its DNA binding activity in a dose dependent manner (Fig. 8A, *lanes 5-7*). Further the NTD also protects the DNA binding activity of recombinant p53 protein in the same manner as that of cellular p53 (Fig. 8B, *lanes 3-5*). These results showed that NTD protects the DNA binding of p53 protein from temperature dependent loss.

NTD restores the DNA binding activity of heat denatured p53

The role of NTD was also investigated in the restoration of the DNA binding activity of heat denatured p53 protein. Since the NTD binds with both the wild type and mutant conformation of p53, we wished to investigate whether NTD by binding with mutant conformation of p53 converts it to the wild type conformation and thereby activates the DNA binding ability of thermally denatured p53. Earlier it was shown that CHIP activates the DNA binding activity of denatured p53 protein (Tripathi, 2007). MIRA-1 also reactivates the DNA binding activity of mutant p53 protein *in vitro* and restores transcriptional transactivation to mutant p53 in living cells (Bykov, 2005). To perform the study the cellular p53 present in the KB nuclear extract was incubated at 37°C that resulted in the loss of its DNA binding activity. However the addition of NTD in the heat denatured KB nuclear extract and incubation at room temperature for 30 minutes resulted in recovery of DNA binding activity of p53 protein (Fig. 8A, *lanes 8* and *9*). These results might indicate that NTD by binding with the wild type p53 protects its conformation and by binding to the denatured p53 NTD converts it to the wild type conformation.

ELISA based method for p53 DNA interaction

EMSA reaction was utilized to investigate the role of NTD in the protection of DNA binding activity of p53 and its role in the recovery of DNA binding activity of heat denatured p53. To further confirm its role in the protection and recovery of p53 DNA interaction, the DNA

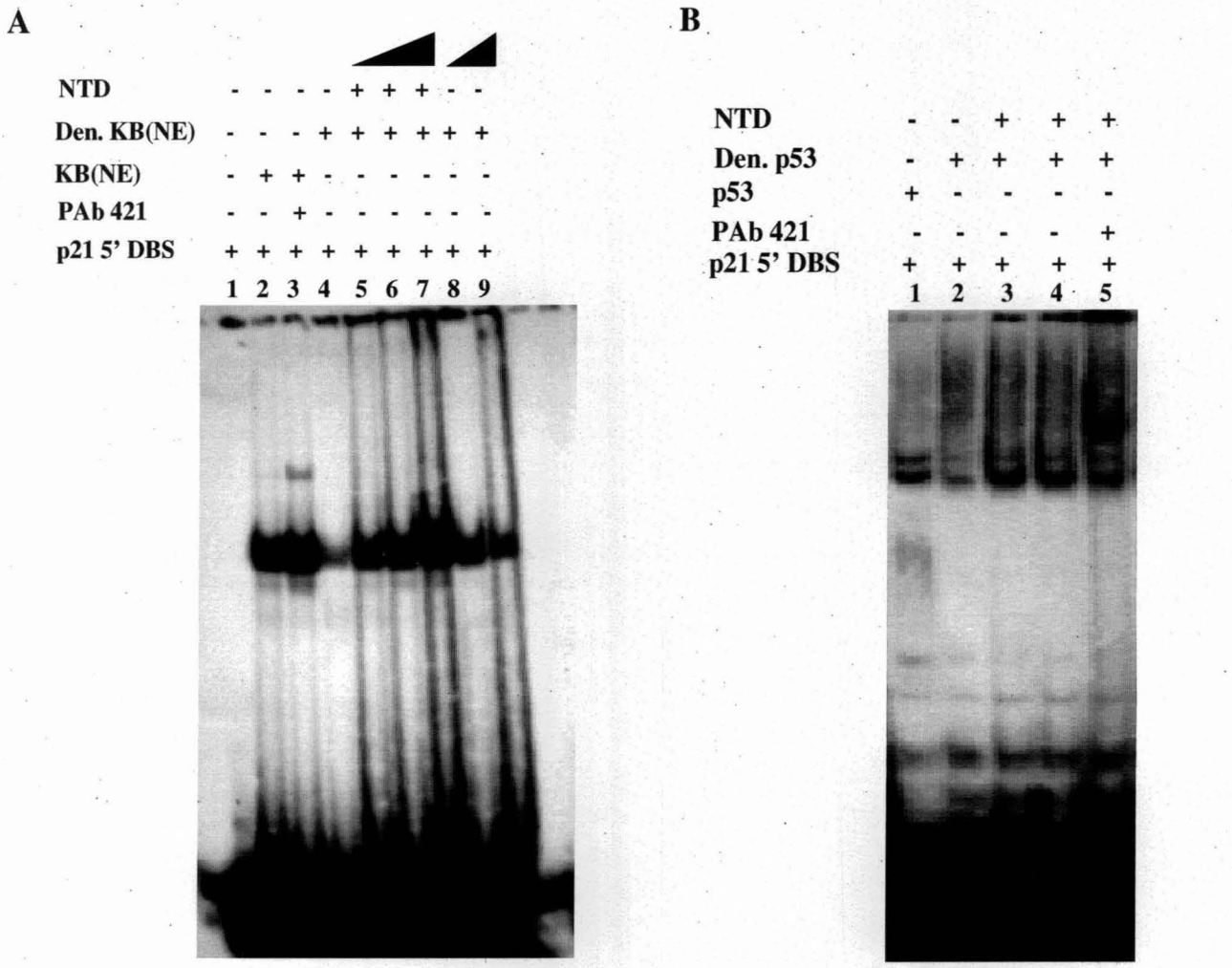


Fig 8. EMSA showing the protection of DNA binding activity of p53 at 37°C as well as restoration of DNA binding activity of heat denatured p53 by NTD. (A) The KB nuclear extract (2.0 μg) was incubated at 37°C for 1hr in the absence (*lane 4*) or in the presence of increasing concentration of NTD from 2.5 μg (*lane 5*), 5 μg (*lane 6*) to 10 μg (*lane 7*) and DNA binding was studied using the P^{32} labeled p21 5'DBS. *Lane 1* shows the radiolabelled p21 5'DBS in the absence of p53 whereas *lane 2* shows the binding of native p53 to p21 5'DBS and *lane 3* shows the binding of native p53 in the presence of PAb 421. To study the NTD mediated restoration of DNA binding activity of heat denatured p53, KB nuclear extract (2 μg) containing p53 was incubated 37°C for 1hr subsequently the NTD was added in an increasing concentration (*lane 8,* 5 μg ; *lane 9,* 10 μg) and incubated at room temperature for 30 minutes subsequently EMSA was performed using P^{32} labeled p21 5'DBS. **(B)** Recombinant wild type p53 (100 ng) was incubated at 37°C for 1hr in the absence or presence of 1 μg NTD and the sequence specific DNA binding activity of p53 was observed using P^{32} labeled p21 5'DBS as above. *Lane 1* shows the binding of native p53, *lane 2* of heat denatured p53, *lane 3* and *lane 4* are same and show the DNA binding activity of p53 heat denatured in the presence of NTD and *lane 5* is same as *lane 4* except PAb 421 was also used in the reaction.

binding experiments were performed on the ELISA plate. ELISA has been used to determine the interaction of p53 protein with its consensus DNA sequence (Jagelska, 2002). However while using the above method we got increased background that hampered in the detection of specific signal of DNA binding. The occurrence of background probably resulted from the non-specific interaction of p53 or antibody on the ELISA plate. To solve this problem an alternative method was developed to perform the interaction of p53 to its DNA binding sites (DBS) on the ELISA plate. For which recombinant p53 protein and four biotin tagged p53 DNA binding sites (DBS) were utilized. The p53 DNA binding sites (DBS) have been divided into two classes based on the binding of p53 protein with the two p53 DBS present in the p21 promoter, in the presence of p53 C-terminal antibody PAb 421 (Resnick-Silverman, 1998). Class I DBS has a conserved C at fourth position in the pentamer PuPuPuC(A/T) the binding site for single p53 molecule, and its binding is enhanced by PAb 421, whereas class II DBS has a mutation at position C and its binding is inhibited by PAb 421. However in the absence of PAb 421 the binding of p53 protein with both the classes of DBS is similar (Resnick-Silverman, 1998). We have taken four p53 DNA binding sites (DBS), one is the 5' p53 DBS from the p21 promoter, and three were from the p53 promoter called DBSI, II and III. The p21 5'DBS and p53 DBSII belong to the Class I DBS, whereas the p53 DBSI and p53 DBSIII belong to the Class II DBS.

Since the binding of p53 protein to the Class I DBS is increased by the addition of p53 C-terminal antibody PAb 421, to study the p53 binding with Class I DBS we used PAb 421 to perform thee binding reaction. The wells of the ELISA plate was coated with the PAb 421 and then p53 protein was added to the wells, subsequently the biotin labeled DNA containing the p53 binding site was added and the p53 bound DNA was detected by the addition of alkaline phosphatase conjugated avidin and its substrate *p*-nitrophenyl phosphate (PNPP). The alkaline phosphatase conjugated avidin binds with the biotin tagged DNA and upon the addition of PNPP alkaline phosphatase hydrolyses PNPP to *p*-nitrophenol, a chromogenic product with absorbance at 405 nm which was measured using ELISA reader. The binding was performed using all the four DBS (0.5 µg each) and the signal intensities of their binding was compared which showed that only the DNA containing the p21 5'DBS was able to interact with p53 in this system (Fig. 9A, *lane 2*). The other three DBS (p53 DBSI, DBSII and DBSIII) were unable to bind in spite of the fact that p53 DBSII belongs to the Class I DBS (Fig. 9A, *lane 3-5*). However when the DNA

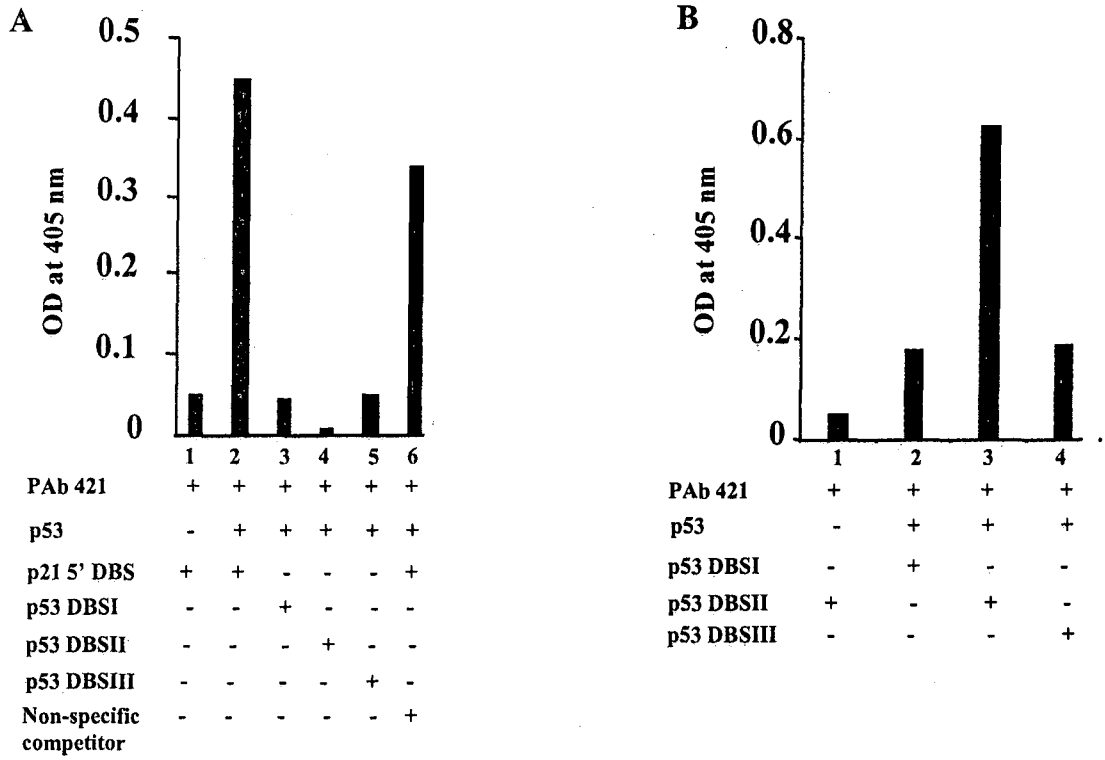


Fig. 9. ELISA showing the interaction of p53 to DBS in the presence of PAb 421. (A) PAb 421(0.5 μg) was coated on the ELISA plate by overnight incubation on the ELISA plate and subsequently p53 protein (0.5 μg) was added and incubated at room temperature for 1 hr, subsequently 0.5 μg of biotin labeled DNA containing the p53 DNA binding site was placed on it and the bound DNA was detected using alkaline phosphatase conjugated avidin. *Lane 6* shows the effect of 5 μg of salmon sperm DNA on the DNA binding activity of p53 protein. **(B)** The experiment was performed exactly as in A except the amount of DNA was raised to five times (2.5 μg).

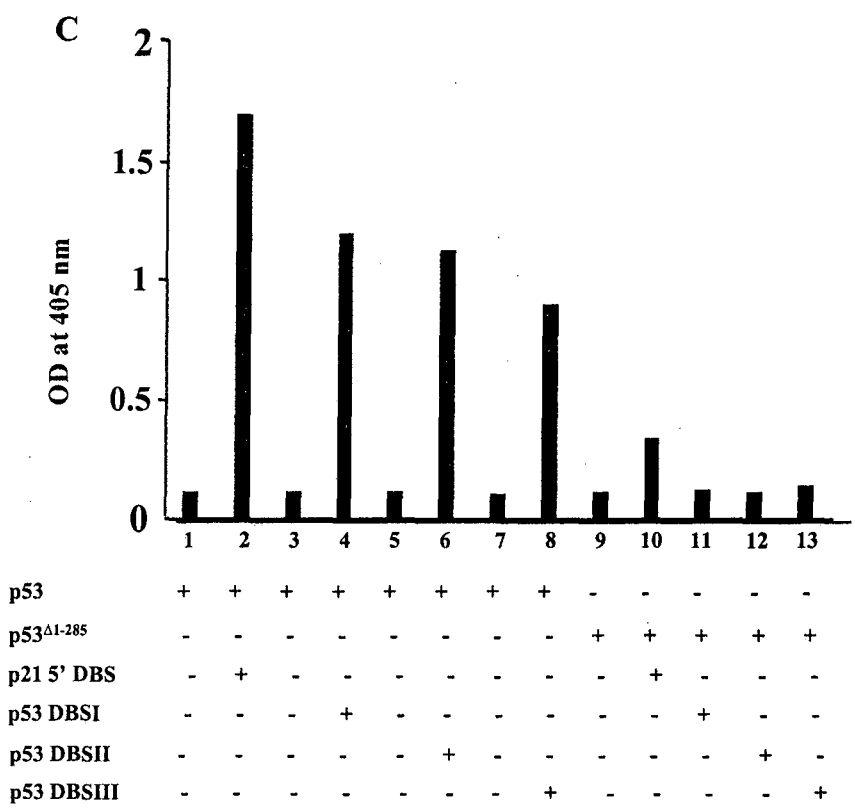


Fig.9 C. ELISA showing the interaction p53 to DBS on p53 coated plates. 0.5 μg of p53 protein or the p53 C-terminus ($p53^{\Delta 1\text{-}285}$) was coated on the ELISA plate and subsequently 0.5 μg of biotin labeled DNA containing the p53 DBS were incubated with p53 and $p53^{\Delta 1\text{-}285}$ separately for 1 hr and the bound DNA was detected using alkaline phosphatase conjugated avidin.

amount was increased by 5 times the p53 DBSII also showed the interaction (Fig. 9B, *lane 3*) whereas p53 DBSI and p53 DBSIII didn't show binding (Fig. 9B, *lane 2* and *4*). Further the comparison of the binding intensities has shown that p21 5' DBS has five times more affinity to p53 protein than p53 DBSII. The specificity of the binding reaction was confirmed by setting the competition experiments with non-specific DNA (salmon sperm DNA) which did not remove the binding reaction (Fig. 9A, *lane 6*).

For the binding of p53 protein with the Class II DBS an alternative approach was developed since the PAb 421 is reported to inhibit the binding of p53 with Class II DBS (Resnick-Silverman, 1998). Therefore the p53 protein was directly coated on the surface of the ELISA plate and subsequently the biotin tagged DNA containing the p53 DBS was added and the bound DNA was detected by adding alkaline phosphatase conjugated avidin, which binds with the biotin attached with DBS. The binding was performed using recombinant p53 and the four DBS as described above. The signals of interaction indicated that the p53 protein binds with all the four p53 DBS, however the binding affinity of the p21 5'DBS was the highest (Fig. 3C, *lane 2*).

Several methods have been applied to confirm the specificity of binding. First the non-specific adherence of the DNA molecules in protein coated wells was ruled out by placing the p21 5'DBS on the ELISA plate coated with non DNA binding proteins like BSA, CHIP and Thioredoxin which didn't show interaction (Fig. 9D). Further, to rule out the possibility of non-sequence specific DNA interaction with the C-terminus of the p53 protein, the p53 C-terminus ($p53^{285-393}$) was interacted with all the four p53 DBS. The signal of DBS binding with p53 C-terminus was only 5-15% in comparison of the binding of p53 with DBS which indicated that the binding signal obtained was not due to non-sequence specific DNA binding with the C terminus (Fig. 9C, *lanes 9-13*). Incubation of p53 at 37°C for 1 hr resulted in the loss of its DNA binding activity by >90% (Fig. 9E, *lanes 6-9*) which also indicates that core domain is involved in the binding. Since the core domain of p53 is heat labile and loses its DNA binding activity as well as wild type conformation rapidly upon heat denaturation (Hansen, 1996). The 53BP1 protein is reported to bind with the DNA binding domain of the wild type p53 protein and compete with DNA for binding with p53 (Iwabuchi, 1994). The effect of p53 binding domain of 53BP1 was also studied on the p53 DNA interaction which shows that 53BP1 inhibits the binding of p53

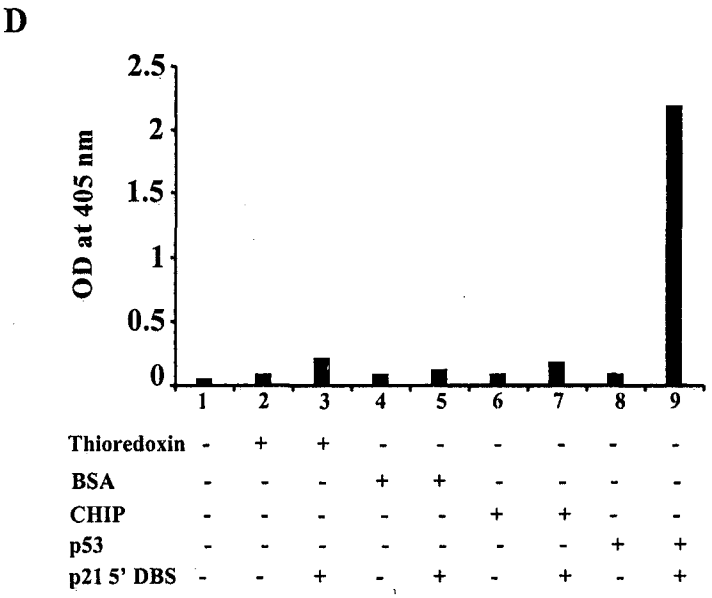


Fig.9 D. ELISA showing the specificity of p53 DNA interaction. p53, CHIP, Thioredoxin, and BSA (0.5 μg each) were coated separately on the ELISA plate, subsequently the biotin labeled p21 5' DBS (0.5 μg) was incubated with these proteins for 1hr and subsequently the bound DNA was detected using alkaline phosphatase conjugated avidin.

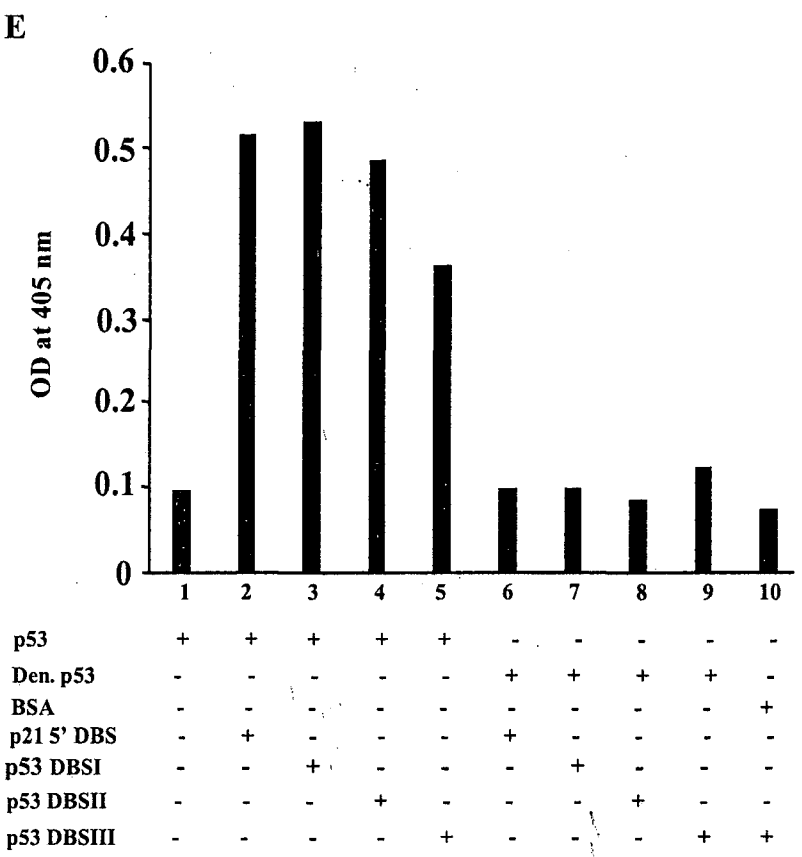


Fig. 9 E. ELISA showing the loss of DNA binding activity of p53 upon heat denaturation. The native and heat denatured (37°C/1hr.) p53 protein (each 0.5 μg) were separately coated on the ELISA plate, subsequently the biotin labeled DNA containing different DBS were added and incubated for 1 hr at room temperature and the bound DNA fraction was detected by using alkaline phosphatase conjugated avidin.

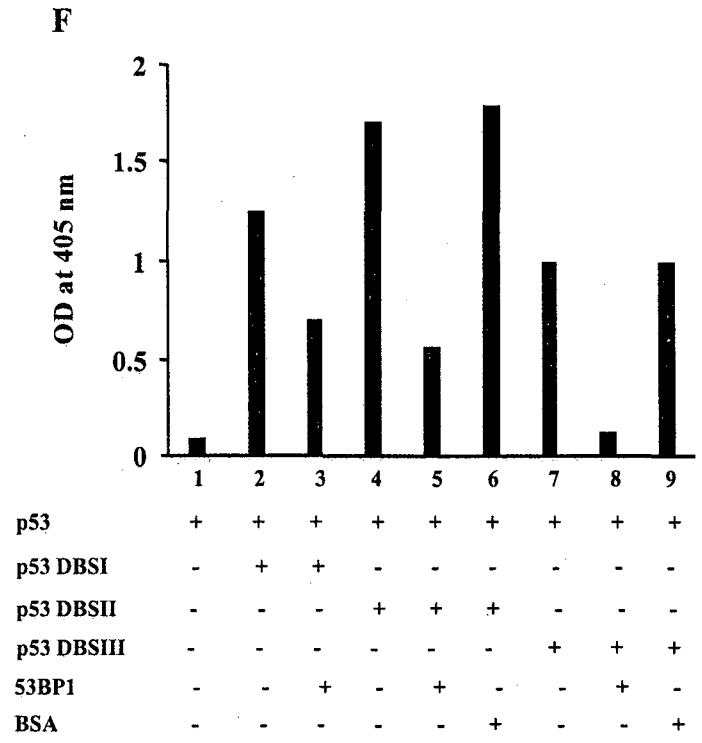


Fig. 9 F. ELISA showing the decreases of p53 DNA binding activity by 53BP1. The p53 binding domain of 53BP1 (1 μg) or BSA (1 μg) was added on the p53 coated ELISA plate and incubated at room temperature for 1 hr. Subsequently the plate was washed extensively and the p53 DNA interaction was done using biotin labeled DBS.

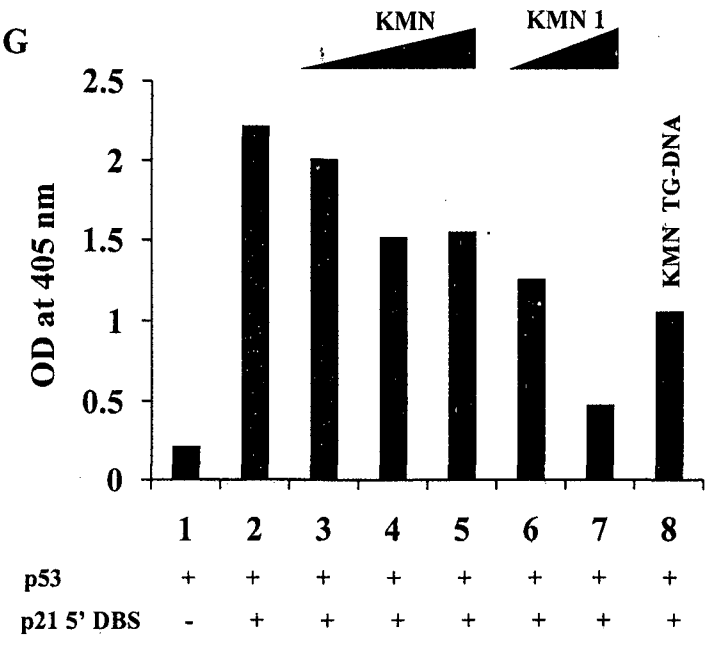


Fig. 9 G. ELISA showing the effect of curcumin and its derivatives on the p53 DNA interaction. Curcumin or its derivatives were added on p53 coated ELISA plate, incubated for 1hr. at room temperature subsequently the plate was washed and DNA binding was done using p21 5'DBS.
KMN= curcumin, KMN1= 4,4'-di-(O-glycinoyl)(O,O-Cysteinoyl) curcumin, KMN TG-DNA = Curcumin tetraglycine -oligonucleotide (5'-GTTAGGGTTAG-3') conjugate.

with DNA. 53BP1 decreased the p53 binding with p53 DBSI by 50% and to p53 DBSIII by 90% (Fig. 9F), whereas addition of BSA didn't affect the binding. Although the variation in the 53BP1 mediated decrease of DNA binding activity of p53 towards different DBS is not fully understood, but the decrease in binding is substantial which indicated that the binding is specific. By using gel shift assay curcumin has been reported to inhibit the binding of p53 to its target DNA (Moos, 2004). Using the ELISA method we have studied the effect of curcumin and its two derivatives 4,4'-di-(O-glycinoyl)(O,O-Cysteinoyl) curcumin, and curcumin tetraglycine-oligonucleotide(5'-GTTAGGGTTAG-3') conjugate on the p53 DBS interaction and found that all of these compounds decrease the interaction of p53 with p21 5'DBS (Fig. 9G). Further the addition of p21 5'DBS in 10 times excess as a competitor removes the p53 binding to the p21 5'DBS which also conforms the specificity of binding (Fig. 9H).

Study of the protection and recovery of p53 DNA binding by ELISA

After standardizing the ELISA method to study the p53 DNA interaction we applied it to investigate the role of NTD in the protection of DNA binding activity of wild type p53 protein as well as the role of NTD in the recovery of DNA binding activity of heat denatured p53. To study the role of NTD in the protection of DNA binding activity of p53, the p53 protein was coated on the ELISA plate and incubated at 37°C for 1hr in the absence as well as in the presence of increasing concentration of NTD. Incubation of p53 protein at 37°C resulted in the loss of its DNA binding activity and the addition of NTD results in partial protection in a dose dependent manner, whereas BSA which was used as a negative control didn't show protection (Fig. 10A). For the investigation of the role of NTD in the recovery of DNA binding activity of p53 protein, p53 was incubated at 37°C for 1hr and coated on the ELISA plate, NTD was then added on the plate and incubated for 30 min at room temperature and then the DNA binding was performed using biotin tagged DBS, results of which showed that the DNA binding activity of heat denatured p53 was recovered for p21 5'DBS, p53 DBSI and p53 DBSIII whereas not for p53 DBSII (Fig. 10B). Further the effect of NTD on the recovery was also studied by adding increasing concentration of NTD, the results of which showed that NTD caused the recovery in a dose dependent manner whereas addition of BSA didn't result in recovery of binding signal (Fig. 10C, *lane 6-7*). The ELISA reactions showed that NTD protects the DNA binding activity of

H

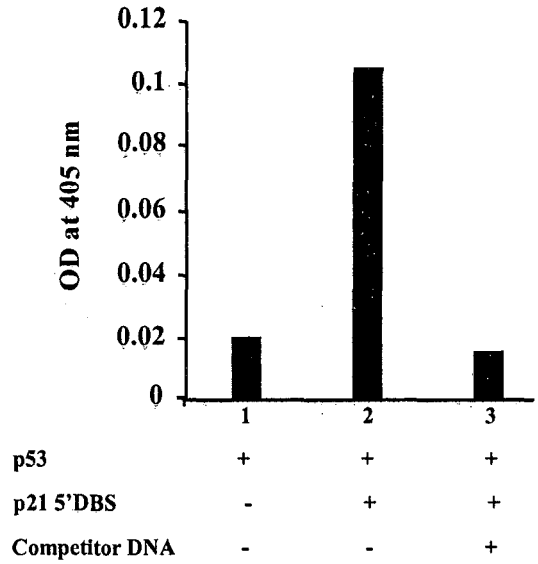


Fig. 9 H. Specific competitor removes the p53 DNA binding. Biotin labeled p21 5'DBS was added to the p53 coated ELISA plate and incubated it at pla10 times excess unlabelled p21 5'DBS was added to the ELISA plate well containing biotin labeled p21 5'DBS bound with p53 protein and incubated for 1hr at room temperature. Plate was then washed extensively and bound DNA was detected using alkaline phosphatase conjugated avidin.

A

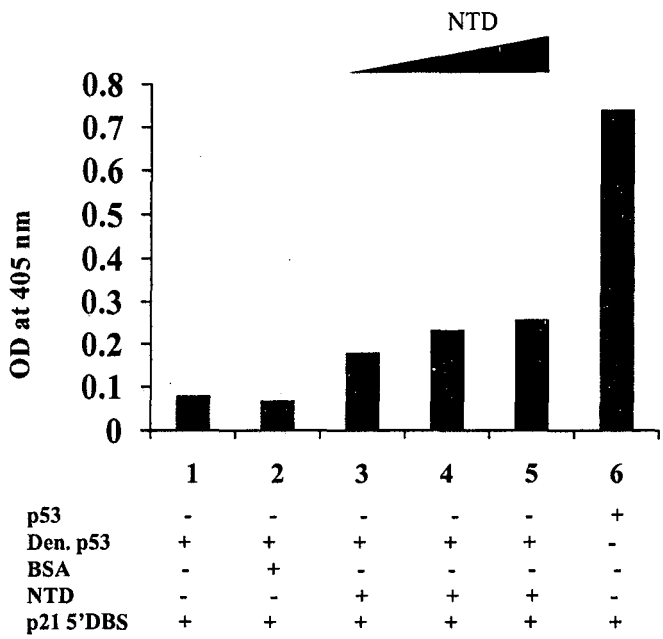
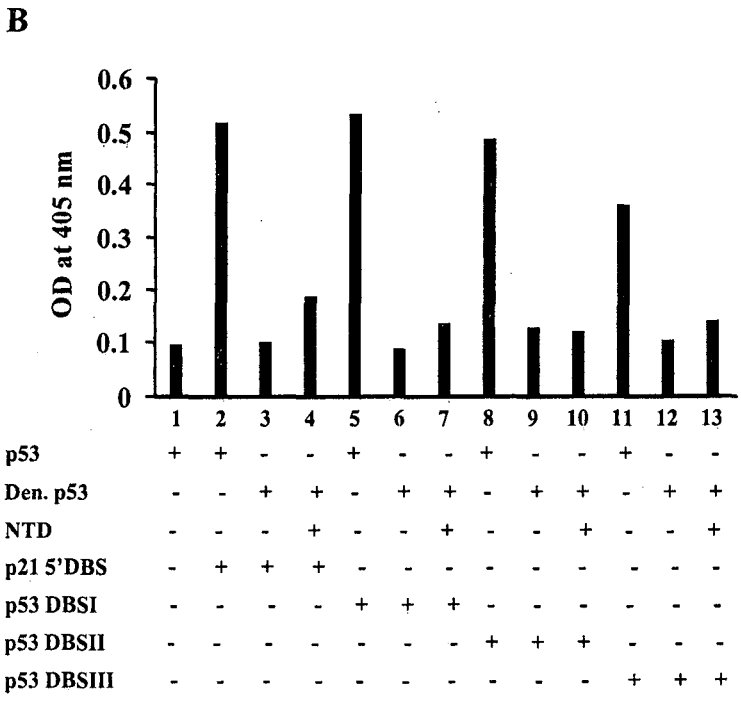


Fig. 10 A. ELISA showing the protection of DNA binding activity of p53 protein by NTD. (A) Wild type p53 protein (0.5 μg) was coated on the ELISA plate and denatured(37°C/1hr.) alone (*lane 1*) or in the presence of increasing concentration of NTD; 2.5 μg (*lane 3*), 5 μg (*lane 4*) and 10 μg (*lane 5*) and 5.0 μg BSA (*lane 2*) and then p21 5'DBS DNA was added and the bound DNA was detected using alkaline phosphatase conjugated avidin . Lane 6 shows the binding of native p53 to p21 5'DBS.



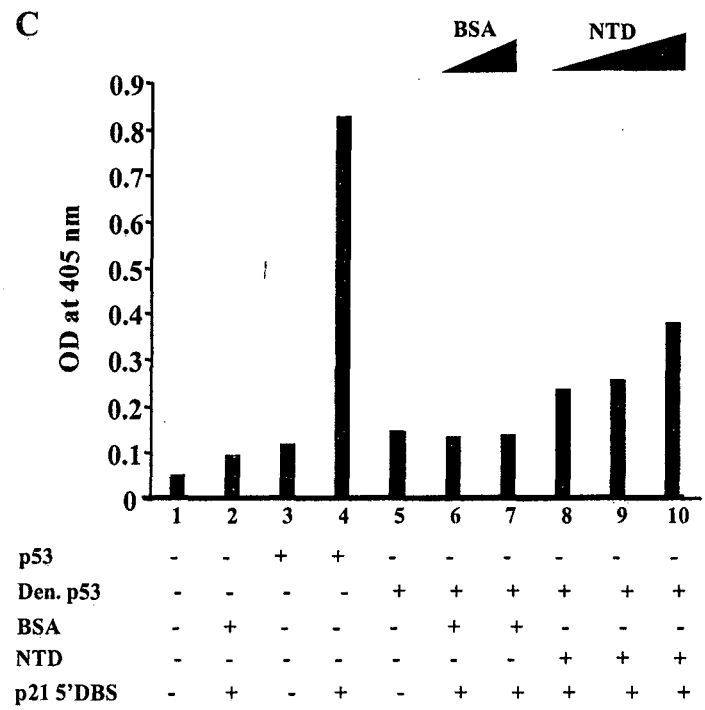


Fig.10 (B and C). ELISA showing the restoration of DNA binding activity of heat denatured p53 by NTD. **(B)** Purified p53 protein (0.5μg) was denatured (37°C/1hr.) and coated on the ELISA plate, subsequently 5μg NTD was added and incubated at room temperature for 30 minutes. The biotin labeled p21 5'DBS (*lane 4*), p53 DBS I (*lane 7*), p53 DBS II (*lane 10*) and p53 DBS III (*lane 13*) were added separately. Subsequently quantitated the bound DNAs using alkaline phosphatase conjugated avidin. **(C)** Increasing concentration of NTD (*lane 8,* 2.5 μg; *lane 9,* 5 μg; *lane 10,* 10μg) or BSA (*lane 6,* 5 μg; *lane 7,* 10μg) were incubated for 30 minutes with heat denatured p53 coated plate and incubated at room temperature for 30 minutes and added biotin labeled p21 5'DBS and its binding was detected using alkaline phosphatase conjugated avidin.

3 from temperature dependent loss as well as it causes the recovery of DNA binding activity p53. However the level of protection and the recovery was less when compared to the EMSA ction, this discrepancy might be due the difference in the nature of two methods. In the EMSA ction the p53 is unbound to any solid surface and hence NTD can bind to p53 from all the es whereas in the ELISA p53 is attached to the surface of the ELISA plate which hinders NTD m interacting with p53 from all the sites as a result there is a less protection or recovery seen ng this method.

nstruction of fluorescent tagged p53 and NTD and their localization

The in vitro data has shown that NTD interacts with p53 and protects its function from nperature dependent loss. To investigate whether NTD colocalises with p53 in the stress nditions and protects its function from thermal denaturation, we have constructed fluorescent ged p53 and NTD proteins. The p53 cDNA was cloned on the *Nhe*I and *Eco*RI sites of the CFP-N1 (Fig.. 11 A), pEYFP-N1 (Fig.ure 11b.) and pEGFP-N1 (Fig.ure 11E), and the cDNA NTD (residues 1-125) was cloned in pEYFP-N1 (Fig. 11C). Further the transactivation nain (NTD-56) of p53 (residues 1-56) was also cloned on *Nhe*I and *Eco*RI sites of the YFP-N1 (Fig.ure 11B.) and pEGFP-N1 vectors. All of the above clonings were performed by R amplification of the cDNA using internal primers by Vent DNA polymerase. The orescent tagged expression plasmids were constructed by placing p53, NTD and NTD-56 NAs separately on the amino terminus of the fluorescent tag. The cloning was confirmed by cking the insert fall and sequencing with Sanger's dideoxy method. To study the localization fluorescent tagged p53 and NTD, these constructs were transfected in p53 null lung cancer l line H1299 and their localization studied separately. The p53-EGFP fusion protein was nd to localize in the cytoplasm and nucleus. Surprisingly, the NTD-EYFP fusion protein also alizes to the cytoplasm and nucleus and the amount of NTD present in nucleus is high in nparison to the cytoplasm. The p53 is a nuclear protein and it contains three nuclear alization signals (NLS I- 312-322, NLS II- 366-372, NLS III- 376-382) in its C-terminal ion (Shaulsky, 1990). The presence of NTD-EYFP fusion protein in the nucleus indicates that night contains a nuclear localization signal which directs it to the nucleus. To investigate the localization of p53-ECFP and NTD-EYFP, the constructs were transfected in the H1299 cells.

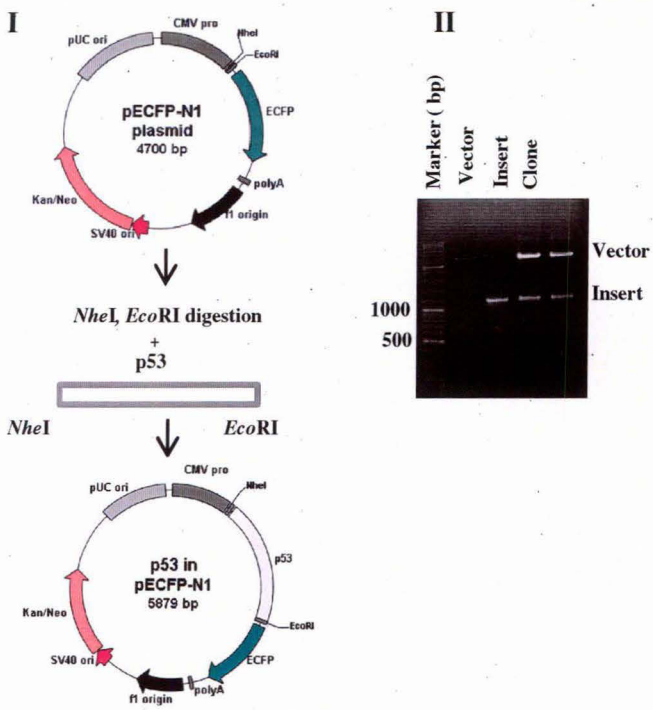


Fig. 11A. Construction of pECFP-N1 p53 plasmid. **(I)** Schematic diagram showing the construction of ECFP tagged p53 expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Nhe*I and *Eco*RI restriction enzymes on 1% agarose gel.

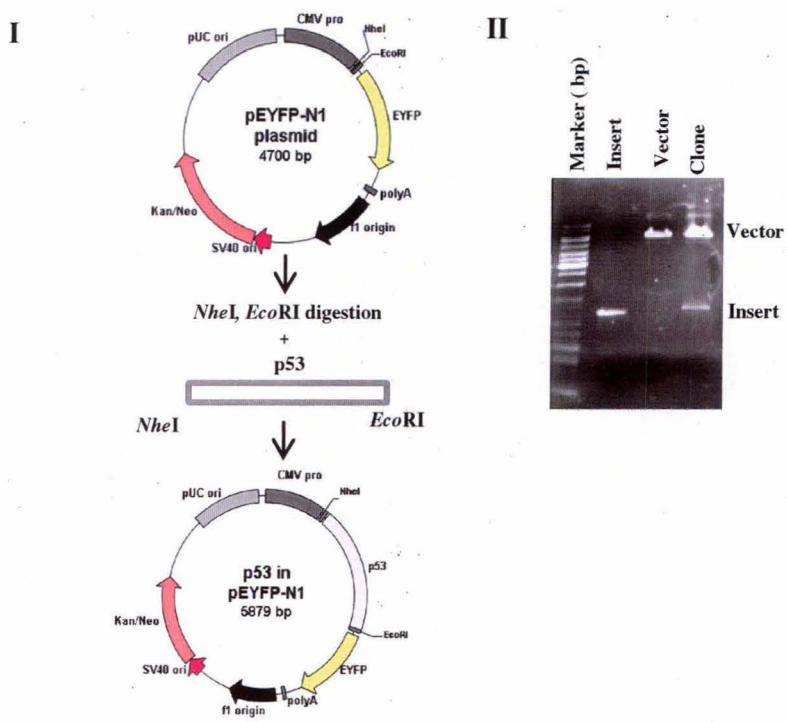


Fig. 11B. Construction of pEYFP-N1 p53 plasmid. **(I)** Schematic diagram showing the construction of EYFP tagged p53 expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Nhe*I and *Eco*RI restriction enzymes on 1% agarose gel.

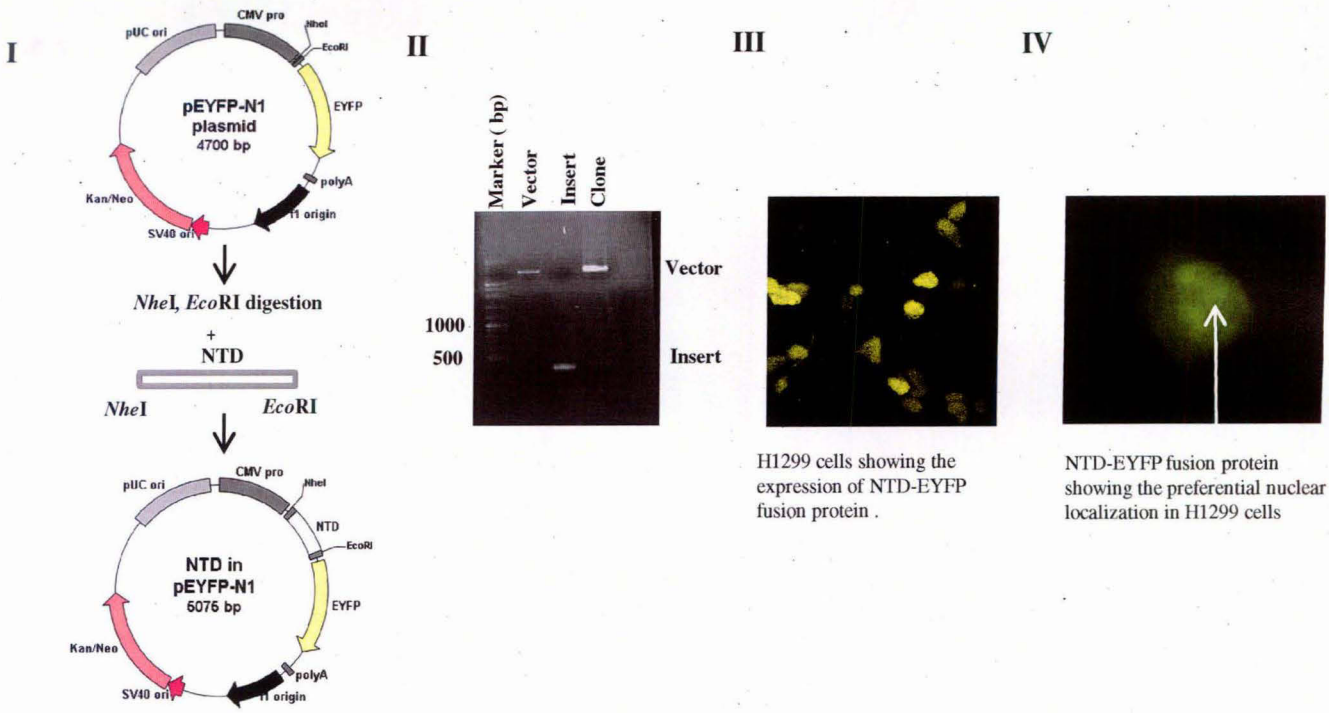


Fig. 11C. Construction of pEYFP-N1 NTD plasmid. **(I)** Schematic diagram showing the construction of EYFP tagged NTD expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Nhe*I and *Eco*RI restriction enzymes on 1% agarose gel. **(III)** and **(IV)** pEYFP-N1 NTD transfected H1299 cells showing the expression of NTD-EYFP fusion protein and its localization in the cell.

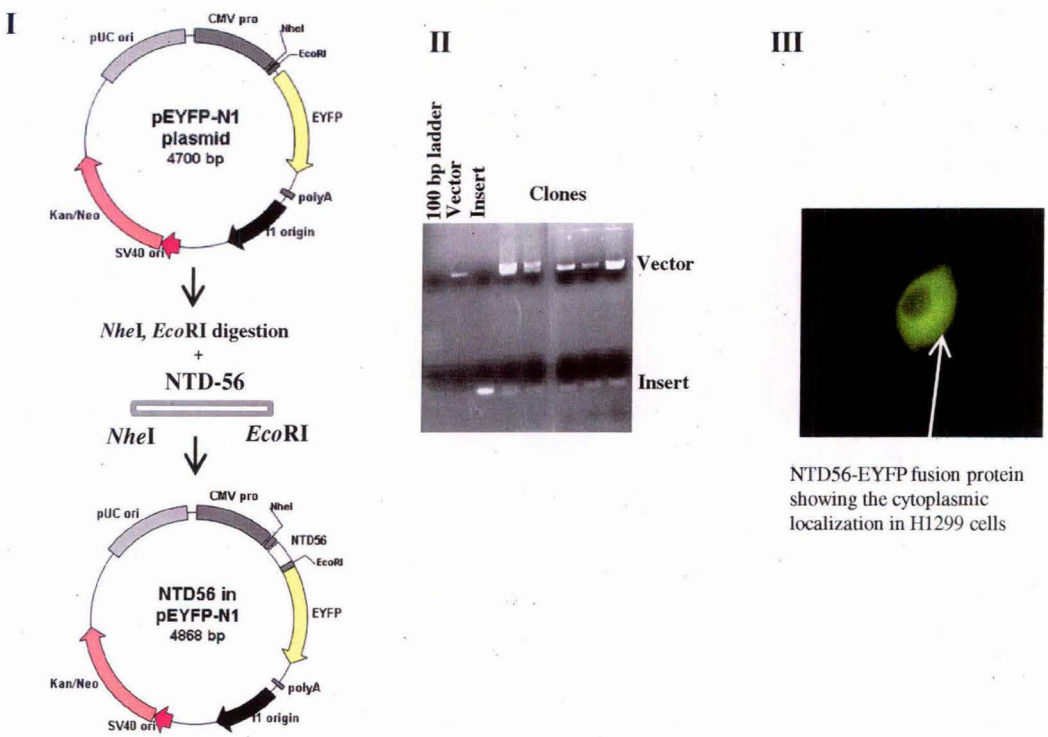
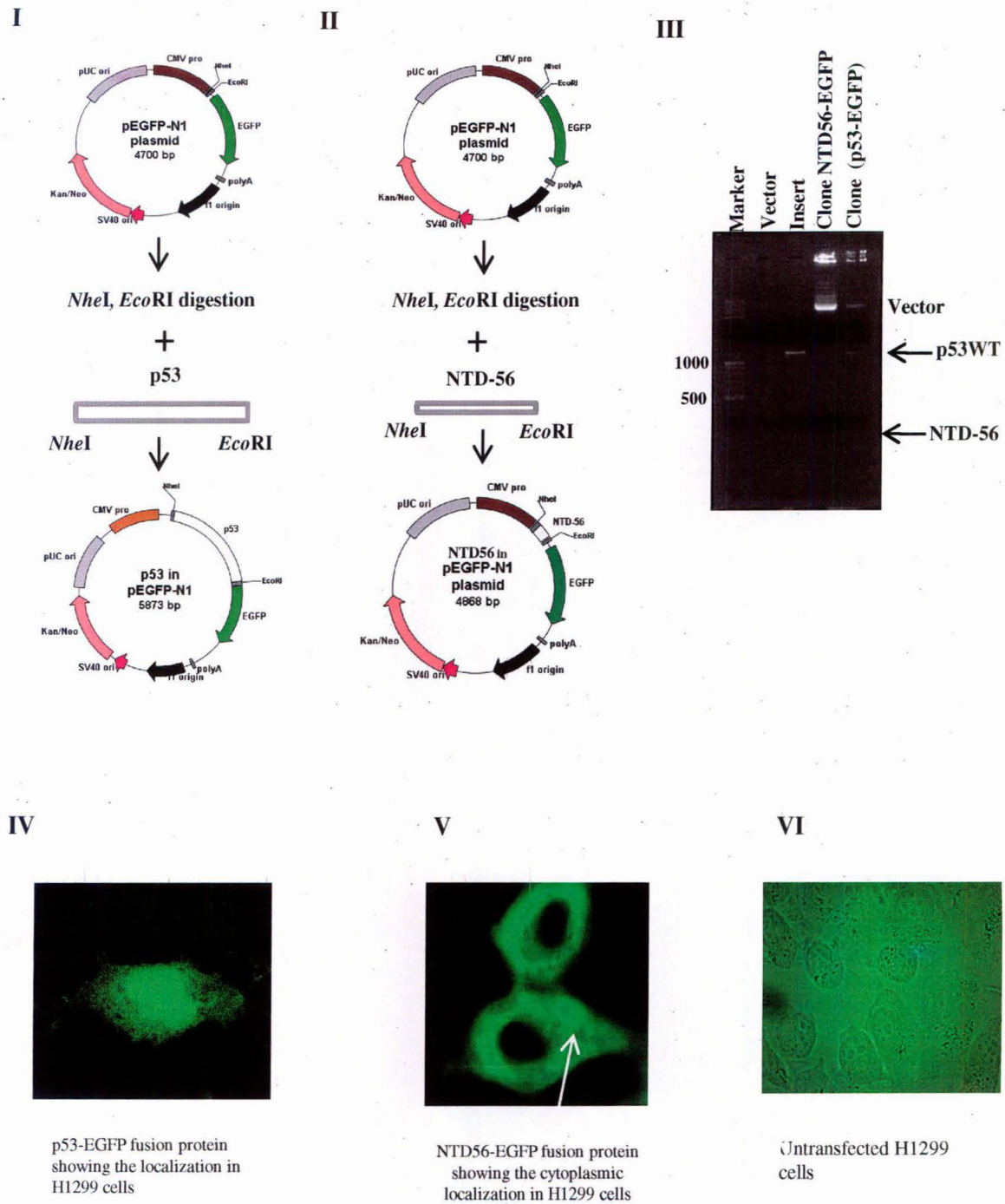


Fig. 11D. Construction of pEYFP-N1 NTD-56. **(I)** Schematic diagram showing the construction of EYFP tagged NTD-56 expression plasmid. **(II)** Cloning confirmation by checking insert fall using *Nhe*I and *Eco*RI restriction enzymes on 1% agarose gel. **(III)** pEYFP-N1 NTD-56 transfected H1299 cells showing the expression of NTD56-EYFP fusion protein and its localization in the cytoplasm .



p53-EGFP fusion protein showing the localization in H1299 cells

NTD56-EGFP fusion protein showing the cytoplasmic localization in H1299 cells

Untransfected H1299 cells

Fig. 11E. Construction of pEGFP-N1-p53 and pEGFP-N1 NTD-56. **(I)** Schematic diagram showing the construction pEGFP-N1 p53 and **(II)** pEGFP-N1 NTD-56. **(III)** Cloning confirmation by checking insert fall using *Nhe*I and *Eco*RI restriction enzymes on 1% agarose gel. **(IV)** pEGFP-N1-p53 transfected H1299 cell showing the expression of p53-EGFP fusion protein and its localization in the nucleus as well as in the cytoplasm. **(V)** pEGFP-N1 NTD-56 transfected H1299 cells showing the expression of NTD56-EGFP fusion protein and its localization in the cytoplasm. **(VI)** Untransfected H-1299 cells.

However the data was not interpretable due to very weak fluorescence signals and lots of background signal in the ECFP. Further we also showed that NTD-56 EGFP and NTD-56 EYFP fusion proteins localize precisely in the cytoplasm. This confirms the potential role of amino acid residues lying between 57aa and 125aa are prerequisite regions for nuclear localization. The amino terminal domain of p53 is also a nucleo-cytoplasmic protein showing an ability to protect p53 conformation in stress conditions which is in corroboration with other nucleo-cytoplasmic chaperones Hsp90, CHIP and MDM2.

Discussion

DISCUSSION

The stability and activity of the tumor suppressor protein p53 has a significant influence on the cellular defense against cancer. It has been reported that wild type p53 is a labile protein and the thermal unfolding of p53 results in an irreversible loss of its native conformation and the DNA binding activity (Friedler, 2003). The purified p53 protein is already inactivated at physiological temperatures, which shows that p53 is stabilized by interacting with other proteins (Muller, 2004). Hsp90 is the major chaperone protein that associates with the DNA binding domain of wild type p53. This results in the stabilization of p53 at physiological temperatures that prevents it from irreversible thermal inactivation (Muller, 2004; Walerych, 2004). Recently CHIP and MDM2 are found to be acting as chaperone. These proteins bind to p53 and protect the DNA binding activity and native conformation at physiological temperature (Tripathi, 2007; Wawrzynow, 2007).

Our results show that the exogenously provided complete amino terminal domain (NTD) increases the DNA binding activity of wild type p53 at room temperature. At physiological temperature it protects the DNA binding activity and the native conformation of p53. NTD also prevents thermal aggregation of p53 at physiological and elevated temperatures. The amino terminal domain is responsible for binding to number of proteins including its negative regulator MDM2 (Vise, 2007). The N-terminal region of p53 is natively unfolded in its functional state (Dawson, 2003). The amino terminal domain of p53 is reported to modulate the DNA binding activity of p53 protein. The transactivation domain was shown to destabilize the p53 DNA interaction (Cain, 2000) and was also shown to be responsible for the thermo-sensitive nature of p53 DNA interaction (Hansen, 1996; Hansen, 1998). While different authors have reported contrasting results for the role of proline rich region in the DNA binding activity of p53 protein (Sakamuro, 1997; Muller-Tiemann, 1998; Venot, 1998 and Roth, 2000).

The DNA binding by the wild type p53 protein using EMSA showed that the NTD increases the DNA binding activity of p53 protein. Addition of NTD prior to the DNA binding by p53 results in the enhancement of DNA binding activity. The p53 DNA binding activity was enhanced by NTD is in a dose dependent manner, however no-super-shift was observed after the addition of NTD. This indicates that the enhancement in the DNA binding activity of p53 is not

due to a stable complex formation between the NTD and p53 protein, however there is probability of weak interaction between the NTD and p53 or the reversible interaction that dissociates when p53 interacts with DNA. Similar interactions have been reported earlier between p53 and Hsp90 (Muller, 2004), p53 and CHIP (Tripathi, 2007) as well as p53 and MDM2 (Wawrzynow, 2007), all of which acts as chaperone for p53 protein. To investigate the reason for the enhancement of DNA binding activity of p53 protein by NTD, we performed binding reactions between NTD and full length p53 using several independent techniques all of which confirms that NTD interacts with p53. Using ELISA the interaction between NTD and full length p53 was studied and its specificity was confirmed by adding MDM2 as positive control and BSA as negative control. The co-immunoprecipitation assay also confirmed the interaction between NTD and p53. The ATP does not play any role in the interaction between NTD and p53. NTD interacts with both wild type and mutant conformation of p53 however the binding affinity of NTD to mutant conformation of p53 is high. The increment of temperature from room temperature to 45°C does not alter the interaction between NTD and p53.

Further the interaction studies also showed the interaction of NTD with the DNA binding domain and the C terminus of p53. Earlier it was reported that a peptide derived from p53 protein (residues 105-126) interacts with the core domain and the C-terminal domain of p53 which inhibits the DNA binding by the p53 protein (Protopopova, 2003). Near UV CD spectroscopy was also used to confirm the interaction between p53 and NTD and their interaction results in a gain of secondary structure. The binding of p53 amino terminal domain with MDM2 results in a gain of secondary structure that can be detected by far UV CD spectroscopy (Matt, 2004). The UV CD spectroscopy shows that NTD interacts with p53 at 25°C, 37°C and 42°C. The secondary structure of individual protein p53 and NTD is decreased at 37°C and at 42°C but the two proteins can still recognize each other because the structure is not completely unfolded. Upon binding of NTD to p53 at 37°C and at 42°C, the secondary structure increases equal to NTD-p53 complex at 25°C.However further increase in temperature results in the dissociation and at 95°C there is no association and hence the CD spectra of individual proteins is equal to the CD spectra of NTD and p53 mix. The UV CD spectroscopy shows that NTD interacts with p53, further the change in secondary structure of p53 and NTD shows that the affinity of their interaction increases with increase in temperature from room temperature to

42°C, further increase in temperature results in the dissociation and at 95°C there is no association. The difference in secondary structure at room temperature indicates that NTD gains structure while interacting with p53. While increase in secondary structure at elevated temperature indicates that the denaturation of p53 is protected by NTD. The thermal unfolding of p53 protein which causes the loss its DNA binding activity is shown to be due to the loss of its secondary structure (Muller, 2004). The p53 protein gets denatured with increase in temperature which results in the loss of its secondary structure however addition of NTD prior ho incubation resulted in the protection of p53 secondary structure from thermal denaturation. CD spectroscopy also revealed that NTD is a very thermostable polypeptide as the change in secondary structure at 95°C gets back to normal after decreasing the temperature to 25°C.

The purified p53 protein is temperature sensitive and it rapidly aggregates due to the unfolding at elevated temperatures or due to the loss of zinc from p53 (Friedler, 2003; Butler, 2003). The thermal sensitivity of p53 protein is due to its DNA binding domain, which gets denatured at 37°C with a half-life of 9 minutes (Friedler, 2003). The binding experiments have shown that NTD interacts with full length p53 and CD spectra further reveals that their interaction results in a change of secondary structure. The significance of interaction was further studied in the aggregation kinetics of p53 protein by using light scattering assay. Incubation of p53 at 37°C and 45°C results to a rapid aggregation, however the addition of NTD prevents p53 aggregation in a dose dependent manner. This shows that NTD binds to full length p53 protein and prevents its denaturation at elevated temperature which consequently prevents p53 aggregation. This shows that NTD keeps the p53 protein in a native like conformation at physiological and at elevated temperatures. Earlier it has been reported that the thermal aggregation of p53 is prevented by Hsp90 and CHIP. The chaperones function by shielding the exposed hydrophobic surfaces from aberrant interaction with other nearby proteins and folding intermediates in the concentrated milieu of the cell (Muchowski, 2002). The results of aggregation studies indicate that NTD functions as a chaperone for wild type p53 protein by physical interaction.

The p53 protein is a conformationally dynamic molecule and its incubation at physiological or elevated temperatures results in irreversible change (Bell, 2002). Increase in temperature causes the change in conformation of p53 protein from wild type (PAb 1620

recognizable) to mutant (PAb 240 recognizable) (DeDecker, 2000) and it also resulted in the loss of sequence specific DNA binding activity (Hansen, 1996). An important question arises whether NTD protects the p53 protein at structural and functional level, since it interacts with p53 and the interaction resulted in the protection of p53 secondary structure loss and subsequent aggregation. To address this we performed the ELISA and immunoprecipitation of p53 in the absence as well as in the presence of NTD at 37°C or at 45°C. Incubation of p53 protein at 37°C or at 45°C at 1hr. resulted in the change of conformation from wild type (PAb 1620 recognizable) to the denatured (PAb 240 recognizable). Addition of NTD prior to incubation resulted in the protection of wild type conformation both at 37°C and at 45°C. The prevention of p53 aggregation by NTD and its protection of p53 wild type conformation indicate that NTD protects the p53 protein at structural level. Further it also confirms that the p53 protein which was not aggregated in the presence of NTD at physiological and elevated temperatures bears the wild type conformation.

Several small molecules like CP-31398, ellipticine,PRIMA-1 and MIRA are reported to activate the DNA binding activity of mutant p53 protein by binding to the newly translated mutant p53 and prevent it from going to the mutant conformation (Sugikawa, 1999; Foster, 1999; Bykov, 2002; 2005). A nine amino acid long peptide, CDB3 (REDEDEIEW), binds to and stabilizes the p53 core domain *in vitro* (Friedler, 2002). It binds to p53 and mutants during biosynthesis, raises it melting temperature to above body temperature so it can fold, and then transfers p53 to its natural binding partners in the cell that would take over the stabilizing function (Friedler, 2002; Friedler, 2003). The protection of DNA binding activity of p53 protein at physiological temperature by NTD without being part of the p53 DNA complex indicates that NTD functions in a chaperone like manner by binding with p53 DNA binding domain and then transferring the p53 protein to DNA. Hsp90, CHIP and MDM2 are also reported to protect the DNA binding activity of the wild type p53 protein from temperature dependent loss (Muller, 2004; Tripathi, 2007 and Wawrzynow, 2007). Bacterial heat shock protein DnaK by binding with the C terminus of p53 protects its DNA binding activity at 37°C, whereas it is not able to recover the DNA binding activity of heat denatured p53 protein (Hansen, 1996). However CHIP was found to protect the DNA binding activity of p53 as well it causes the recovery of DNA

binding activity of p53 protein (Tripathi, 2007). Our results also showed that NTD was able to mediate the recovery of DNA binding activity of heat denatured p53 protein.

Our results also indicate that NTD acts in a chaperone like manner as the incubation of the recombinant wild type p53 protein as well as the p53 protein from the nuclear extract of KB cells at 37°C for 1hr. resulted in the loss of DNA binding activity. Whereas addition of NTD before the heat denaturation resulted in the protection of DNA binding activity that also indicates that NTD functions like a chaperone like manner. At functional level, to study NTD role in p53 protection NTD was added prior to denaturation that results to a significant protection of DNA binding activity. This shows that NTD interacts with p53 and maintains the DNA binding competent state and consequently protects its DNA binding function from temperature dependent loss.

To study the role of NTD in the protection and recovery of DNA binding activity we implemented EMSA using p32 labelled oligos. Further ELISA based approach was also carried out using biotin labeled oligos as reported earlier (Jagelska, 2003). However in our experiments due to non-specific signals the detection of p53 DNA complex was not feasible. The probable reason for increased background might be due to non-specific interactions between the p53 or antibody with the coated streptavidin. In order to minimize the background we have developed an ELISA method for p53 DBS interaction in which biotin labeled DNA was applied on the fixed p53 molecule.

To study the interaction of p53 with DBS, p53 was incubated on the PAb421 coated wells and the biotin labeled DBS was added later and we found that it preferentially binds to Class I DBS (having a conserved C at 4^{th} position in the quarter site), but not with the Class II DBS (having a mutation at 4^{th} position in the quarter site). This result agrees with an earlier published EMSA data showing the binding of p53 protein with Class I DBS increases while that of Class II DBS decreases in the presence of PAb 421, whereas in the absence of PAb 421 the binding of p53 with both the DBS is comparable (Resnick-Silverman, 1998). The binding affinity of p53 to the p21 DBS is five times more than that of p53 DBSII, although both belong to the Class I DBS. This discrepancy in the affinity is probably due to the difference in the

flexibility in two DNA molecules. It has also been reported that the p53-binding sites containing d(CATG) sequences at the junction of two pentamers are intrinsically more flexible than d(CTTG) and forms a more stable p53–DNA complex (Balagurumoorthy, 2002). The p21 DBS contains the d(CATG) sequences at the junction of two pentamers whereas the p53DBSII contains d(CTTG) which makes the binding affinity of p21 DBS to p53 more in comparison to the DBSII.

To optimize for the binding of p53 with Class II DBS, the p53 protein was directly coated on the ELISA plate and incubated with the biotin labeled DBS. The results have shown that p53 binds to both Class I and Class II DBS. Specificity of binding was validated by ruling out the non specific interactions of p21 DBS with CHIP, Thioredoxin and BSA as well as with p53 C-terminus. Further the specificity was confirmed by competing the binding with specific competitor. Competition of DBS binding by 53BP-1 and the loss of binding by heat denatured p53 also validates the method. In addition to this the decrease of DBS binding by curcumin using this method also validates the specificity of the procedure. In conclusion it can be stated that this method can be used for studying the p53 binding to both classes of DBS whereas the earlier approach can be used for studying the interaction of p53 with class I DBS.

The role of NTD in the protection of DNA binding activity was further confirmed by ELISA by coating p53 directly on ELISA plate, and incubating p53 a which showed that NTD protects the DNA binding activity. While using ELISA both Class I and Class II DBS were used that showed NTD protects p53 DNA binding activity. The role of NTD was further confirmed by using BSA which didn't show any protection. The data obtained by ELISA substantiates the results obtained by EMSA in the protection of p53 DNA binding activity by NTD. However the level of protection seen in this method is less in comparison to the EMSA. This might be due the fixation of p53 protein on the ELISA plate. The effect of NTD on the recovery of DNA binding activity of heat denatured p53 was also studied by this method. Incubation of p53 at 37°C loses its DNA binding activity, whereas the addition of NTD and incubation at room temperature partially restores its DNA binding activity. The specificity was further confirmed by adding BSA in the place of NTD which didn't show any protection. The level of recovery seen while using this method is less in comparison to the EMSA which could be due to the limited movement of attached p53 protein on the ELISA plate.

Our results show that the NTD of p53 protein acts like a chaperone for the wild type p53 protein. The NTD of the p53 protein is intrinsically disordered and the results shown here indicate that it acts like a chaperone for the p53 protein. Intrinsic disorder is a common feature in the molecular chaperones, both RNA and protein chaperones are found to have intrinsically disordered regions (Tompa, 2004). There are some molecules which are intrinsically disordered along their entire length and works as chaperone, α-casein (Bhattacharyya, 1999) and α-synuclein (Park, 2002) are few examples that act like chaperones. Recently two plant proteins ERD10 and ERD14 (for early response to dehydration) that are intrinsically disordered proteins act as chaperones in vitro conditions (Kovacs, 2008). Further the molecular mechanism of their chaperone function must be linked with their unstructured nature (Bhattacharyya, 1999; Kim, 2002; Park, 2002) as the removal of the disordered segment by limited proteolysis often abolishes or markedly reduces chaperone activity of p23 co-chaperone (Weikl, 1999) α-crystallin (Andley, 1996) and Hsp25(Lindner, 2000). The diminution of chaperone activity by decreasing the flexibility of the disordered segment by a point mutation has been demonstrated for α-crystallin (Smulders, 1996).

It has also been shown that these disordered molecular chaperones have exposed hydrophobic surfaces and work like cageless chaperones in an ATP independent manner. These cageless chaperones bind to a single substrate protein and increase folding yields by reducing the time the substrate spends in an aggregation-prone state in a dual manner: (a) by competing for aggregation-prone hydrophobic sites on the surface of a protein, hence reducing the time the protein spends unprotected in the bulk and (b) by accelerating folding rates of the protein (Jewett, 2006).

Our results indicate that the stabilization of p53 protein at physiological and elevated temperatures by the exogenously provided amino terminal domain is due to its function as a cageless chaperone in an ATP independent manner. Furthermore the amino terminal domain of p53 might act like an intra-molecular chaperone that is neither dissociated nor cleaved (Ma, 2000). This work also explains that the DNA binding domain of p53 molecule is very temperature sensitive and the stability of p53 might be due its amino terminal domain. We also propose that the chaperone function of amino terminal domain can also be employed in the

stabilization of the hotspot p53 mutants by increasing their melting temperature in a manner similar to the CBD3 peptide, since the decreased stability of the mutant p53 is the main reason behind the loss of their function. The increase in the stability of p53 protein by NTD can be used for the activation of p53 protein resulting in the enhancement of cell cycle arrest and/or apoptotic function by p53. Further the stabilizing function of NTD for the p53 protein also shows that it can also be used in the activation of DNA binding activity of mutant p53 by decreasing the melting temperature of the DNA binding domain in a manner similar to the CBD3 peptide. Thus the amino terminal domain also functions like a chaperone in addition to its function in transactivation and transactivation independent apoptosis.

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Appendix

APPENDIX

Preparation of Culture Media

LB Medium

Dissolve 20 gm of LB powder (Sigma) in double distilled water. Add ddH_2O to make up the volume to 1 litre. Sterilize the media by autoclaving for 15 minutes at 121°C/15 lb/sq. in.

LB Agar Plate

Dissolve 35 gm of LB Agar powder (DIFCO) in double distilled water. Add ddH_2O to make up the total volume to 1 litre. Sterilize the media by autoclaving for 15 minutes at 121°C/15 lb/sq. in. Allow LB Agar to cool and just before pouring the plates, add appropriate antibiotic to the autoclaved media in the required final concentration to be used as a selection marker.

Ampicillin Solution

Dissolve ampicillin salt in sterile water to make the stock solution of 100 mg/ml and sterilization of the sock solution was done by filtration through a 0.22 micron disposable filter. Store the solutions at -20°C. The working concentration was 100 µg/ml for both broth and plates.

Kanamycin Solution

Powder of kanamycin salt was dissolved in sterile water to make the stock solution of 50 mg/ml and sterilized by filtration through a 0.22-micron disposable filter. Store the solutions at -20°C. The working concentration of the antibiotic was 50 µg/ml for both broth and plates.

Stock Solutions of Commonly Used Reagents

30% Acrylamide

Acrylamide	29 gm
N, N'-methylene-bis-acrylamide	1 gm

Add ddH_2O to make up the total volume of 100 ml for filter the solution and keep at 4°C.

10% Ammonium persulphate

Add 10 gm ammonium persulphate powder in 100 ml of sterile ddH_2O and mix. Keep the solution at 4°C. The solution is stable for 2-4 weeks.

10 M Ammonium Acetate

Dissolve 385.4 gm of Ammonium acetate in 150 ml of water. Make up the volume to 500 ml. Sterilize the solution by autoclaving for 15 minutes at 121°C/15 lb/sq. in.

1 M $CaCl_2$

Dissolve 147 gm of Calcium Chloride ($CaCl_2.2H_2O$) in 1 litre water and sterilize the solution by filtration with a 0.22-micron filter membrane.

1 M DTT (Dithiothreitol)

Dissolve 3.09 gm of Dithiothreitol in 20 ml of 0.01 M Sodium acetate, pH 5.2 and store at -20°C after filter sterilization with 0.22-micron filter.

0.5 M EDTA

Dissolve 186.1 gm of $Na_2EDTA.2H_2O$ powder in 700 ml of water. Keep on shaking vigorously. EDTA will not dissolve completely until the pH of the solution reaches 8.0. Adjust pH to 8.0 with 10M NaOH. Finally, add water to 1 litre and autoclave the solution.

70% Ethanol

Mix 70% of pure ethanol and add 30 ml of sterile water to make up the total volume to 100 ml. Store it at 4° C.

Ethidium Bromide

Dissolve 100 mg Ethidium bromide tablet in 10 mL of water. Leave overnight at 37° C shaker to dissolve the tablet completely and store in dark at room temperature.

50% Glycerol

Add 50 ml of glycerol to 50 ml of sterile water and mix thoroughly and sterilized it by autoclaving.

1 M HEPES Buffer (pH 7.9, 8.0)

Dissolve 23.83 gm of solid HEPES salt into 80 ml of sterile water. Adjust the pH to the desired value accordingly with the help of 1 M NaOH. Make up the final volume to 100 mL with water. Filter the solution with a 0.45 micron disposable filter. The buffer should be stored at 4°C.

1 M IPTG (Isopropylthio-β-D-galactoside)

Dissolve 2.3 gm of IPTG powder in 8 ml of sterile distilled water. Make up to total 10 ml with water to a final concentration to 200 mg/ml and sterilize the solution by filtration through a 0.22-micron disposable filter. Dispense the solution into small aliquots and store them at -20°C.

1 M KCl

Dissolve 74.6 gm of Potassium Chloride in 1 litre of water and autoclave. Store at room temperature.

1 M $MgCl_2$

Dissolve 20.3 gm of Magnesium Chloride (Hexahydrate) dry powder and make up the total volume to 1 litre with water. Sterilize the solution by autoclaving.

5 M NaCl

Dissolve 292.2 gm of Sodium Chloride in 800 ml water and make up the total volume to 1 litre with water. Finally, sterilize the solution by autoclaving.

10% NP-40 (Nonidet P-40) (v/v)

Make 10% solution of NP-40 detergent in sterile water as a by diluting from 100% stock solution. This solution can be diluted as per requirement.

dNTP's Mix (dATP, dCTP, dGTP, dTTP)

Prepare 10 mM each dNTP in $T_{10}E_1$ buffer, pH 7.5. Combine all the four dNTP's at a final concentration of 2.5 mM each and store in small aliquots at -20° C.

PhenolChloroformIsoamyl alcohol

Mix 25 parts (v/v) Phenol (previously equilibrated in 150 mM NaCl/50 mM Tris-Cl, pH 7.5 and 1 mM EDTA) with 24 parts (v/v) Chloroform and 1 part (v/v) of Isoamyl alcohol. Store in a dark colored glass bottle at 4°C.

Protease Inhibitors

All the protease inhibitor solutions were made as 100X concentration stock. They should be added to the pre-cooled solutions just before use. All the protease inhibitor solutions are active for 3-4 weeks at a storage temperature of -20° C. The required concentration of the solutions is 1X.

Leupeptin	100 μg/ml in water
Aprotinin	100 μg/ml in water
Trypsin Inhibitor	100 μg/ml in water

100mM Phenyl methyl sulphonyl fluoride (PMSF)

Dissolve 174 mg of PMSF powder in 10 ml of isopropanol. Divide th solution in aliquots and store at -20° C. Wear gloves while handling the chemical. The half-life of PMSF in aqueous solution in 20 minutes. So, it should be added to the solution just before use.

Salmon Sperm DNA (denatured)

Dissolve 100 mg Salmon Sperm DNA in 1 ml of water. Pass vigorously through an 18-guage needle 20 times to shear the DNA. Place in boiling water bath for 10 minutes, then chill it. Store at -20°C in aliquots.

Sephadex G-50

Add 1 gm of Sephadex G-50 powdered medium to 35 ml of sterile water. Wash the swollen resin with sterile water several times to remove soluble dextran, which can create problems by

precipitating during ethanol precipitation. Finally, autoclave (121°C/15 lb/sq. in. for 15 minutes) and store at room temperature.

10% Sodium Dodecyl Sulphate (SDS)

Dissolve 10 gm of SDS (electrophoresis grade) in 70 ml of sterile ddH_2O. Heat at 55°C for 2 minutes. Make up the final volume to 1 litre with sterile ddH_2O.

1 M Tris (pH 6.8, 7.0, 7.2, 7.4, 7.5, 8.0, 8.8, 9.1, 9.5)

Dissolve 121.1 gm of Tris base in 800 ml of ddH_2O. Adjust pH to desired value by adding concentrated HCl. Make up the final volume to 1 litre with water and sterilize by autoclaving

10% Triton X-100 (v/v)

Add 10 ml of Triton X-100 detergent solution into 90 ml of water to make up the final volume to 100 ml to get a final concentration of 10%.

10% Tween-20 (v/v)

Add 10 ml of Tween-20 detergent solution into 90 ml of water to make up the final volume to 100 ml to get a final concentration of 10%.

BUFFERS

5X EMSA Buffer

Tris-Cl (pH 7.5)	50 mM
NaCl	250 mM
$MgCl_2$	5 mM
EDTA	0.5 mM
DTT	5mM
Glycerol	25%

10X ELISA Buffer

Tris-Cl (pH 7.5)	100 mM
NaCl	500 mM
$MgCl_2$	10 mM
EDTA	1.0 mM
DTT	10 mM

Annealing Buffer

Tris, pH 7.4	60 mM
$MgCl_2$	10 mM
DTT	5 mM
Spermidine	1 mM

TENS solution

Tris	10 mM
EDTA	1 mM
NaOH	0.1 N
SDS	0.5%

2X Sample buffer (SDS gel loading buffer)

Tris-Cl, pH 6.8	100 mM
β-mercaptoethanol	2%
or	
Dithiothreitol	200 mM
SDS	4% (w/v)
Bromophenol Blue	0.2% (w/v)
Glycerol	20% (v/v)

6X DNA Loading Buffer

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol	30% (v/v)

Add ddH2O to make up the total volume. Store at 4°C.

50X TAE buffer

Tris Base	242 gm
Glacial acetic acid	57.1 ml
$Na_2EDTA.2H_2O$	37.2 gm

Add sterile water to make up the total volume to 1 litre.

10X TBE buffer

Tris base	107.8 gm
Boric acid	55 gm
Disodium EDTA.$2H_2O$	7.44 gm

Add sterile water to make up the total volume to 1 litre.

5X Tris glycine buffer

Tris base	15.1 gm
Glycine	94.0 gm
SDS	5.0 gm

Add ddH_2O to make up the total volume to 1 litre.

10X T4 PNK buffer

Tris-Cl, pH 7.5	500 mM
$MgCl_2$	100 mM

DTT	50 mM
BSA/Gelatin	0.5 mg/ml

Solution for Colony Hybridization

Denaturation Buffer

NaCl	17.55 gm
NaOH	4.0 gm

Dissolve in water to make the volume 200 ml and autoclave.

Neutralization Buffer

NaCl	17.55 gm
Tris	12.10 gm

Dissolve in water to make the volume 200 ml and autoclave.

Pre-Hybridization Buffer

SSC	6X
Denhardt solution	5X
SDS	0.2%

Buffers for Proteins Purification

Purification with Ni-NTA column

Lysis buffer (1 liter)

50 mM NaH2PO4	6.90 g (MW 137.99 g/mol)
300 mM NaCl	17.54 g (MW 58.44 g/mol)
20 mM Imidazole	0.68 g (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

Wash buffer (1 liter)

50 mM NaH2PO4	6.90 g (MW 137.99 g/mol)
300 mM NaCl	17.54 g (MW 58.44 g/mol)

40 mM Imidazole	1.36 g (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

Elution buffer (1 liter)

50 mM NaH2PO4	6.90 g (MW 137.99 g/mol)
300 mM NaCl	17.54 g (MW 58.44 g/mol)
250 mM Imidazole	17.00 g (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

Purification with Glutathione Sepharose

Lysis Buffer

PBS (pH 7.4)	1X
Triton X100	0.1 %
PMSF	0.5 mM

Wash Buffer

PBS	1X
Triton X-100	0.1 %
PMSF	0.5 mM

Elution Buffer

Tris-HCl pH-8.0	50 mM
Reduced Glutathione	20 mM
PMSF	0.5 mM

Buffers for Western Blotting

Transfer Buffer

Tris	48 mM
Glycine	39 mM
SDS	0.037%

Methanol	20%

Buffer 3

Tris-Cl pH-9.5	0.1 M
NaCl	0.1 M
MgCl2	0.05 M

Buffer 4

Tris-Cl pH-9.5	0.1 M
NaCl	0.1 M
MgCl2	0.05 M
NBT	0.32 mg/ml
BCIP	0.16 mg/ml

It should be prepared just before use and kept in falcon tube wrapped with aluminum foil.

Solution for silver staining

Fixative A

Methanol	50%
Acetic acid	10%

Add water to make up the total volume.

Fixative B

Ethanol	10%
Acetic acid	5%

Add water to make up the total volume.

Oxidizer solution

Potassium dichromate	3.4 mM
Nitric Acid	3.2 mM

Staining Solution

Sliver nitrate	12 mM

Developer

Sodium Carbonate	0.28 mM
Formaldehyde (Formalin)	7.5 µl (0.05%)

Solution for Coomassie stain

Destain

Methanol	45 ml
Water	45 ml
Acetic Acid	10 ml

Coomassie stain

0.25 gm of Coomassie brilliant blue was dissolved in 100 ml destain solution.

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CHIP Chaperones Wild Type p53 Tumor Suppressor Protein*

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Wild type p53 exists in a constant state of equilibrium between wild type and mutant conformation and undergoes conformational changes at elevated temperature. We have demonstrated that the co-chaperone CHIP (carboxyl terminus of Hsp70-interacting protein), which suppressed aggregation of several misfolded substrates and induced the proteasomal degradation of both wild type and mutant p53, physically interacts with the amino terminus of WT53 and prevented it from irreversible thermal inactivation. CHIP preferentially binds to the p53 mutant phenotype and restored the DNA binding activity of heat-denatured p53 in an ATP-independent manner. In cells under elevated temperatures that contained a higher level of p53 mutant phenotype, CHIP restored the native-like conformation of p53 in the presence of geldanamycin, whereas CHIP-small interfering RNA considerably increased the mutant form. Further, under elevated temperatures, the levels of CHIP and p53 were higher in nucleus, and chromatin immunoprecipitation shows the presence of p53 and CHIP together upon the DNA binding site in the p21 and p53 promoters. We propose that CHIP might be a direct chaperone of wild type p53 that helps p53 in maintaining wild type conformation under physiological condition as well as help resurrect p53 mutant phenotype into a folded native state under stress condition.

p53 is a transcription factor that is responsible to maintain the integrity of the genome and is mutated in over 50% of human cancers (1). Wild type (WT)[2] p53 is a structurally unstable protein, which undergoes conformational changes at elevated temperatures (2, 3). p53 is normally expressed at low levels in a latent form that is unable to bind specifically to DNA, and several *in vivo* experiments suggest that WT p53 may exist in a constant state of equilibrium between the wild type and mutant conformation. In its mutant form, it could inactivate the protein from the wild type allele, and the formation of hetero-oligomers of wild type and mutant proteins could drive the wild type protein into mutant conformation (4). It was suggested that chaperone-mediated actions might decrease the probability for the formation of kinetically trapped, mutant-like intermediates that would allow a shift in the conformational equilibrium toward the active, wild type p53 conformation (5). Hsp90 was recently shown to bind to a folded, native-like conformation of p53 *in vitro* that was essential to stabilize p53 at physiological temperature (6). Hsp90 stabilized WT p53-DNA complexes at 25 °C as well as partially protected the WT p53-DNA binding conformation during long term exposures at 37 °C in an ATP-dependent manner (7) and might be involved in regulating the shift between wild type and mutant p53 conformation (8), whereas other human chaperones Hsp70 and Hsp40 failed to efficiently substitute Hsp90. The function of Hsp90 may be modulated by association with co-chaperones, such as Hsc70, Hsp40 (9), and Hop (10). A drug CP-31398 (11, 12) as well as a plant alkaloid ellipticine (13) have also been shown to maintain p53 in active conformation and can drive some mutant p53s into wild type conformation. Further, MDM2 binding to the p53 amino terminus could induce a conformational change in wild type p53, and this change was opposed by Hsp90 (14).

CHIP (carboxyl terminus of Hsp70-interacting protein) is a dimeric 35-kDa ubiquitin ligase (15) comprising three functional domains: a tetratricopeptide repeat (TPR) at the amino terminus, a U-box domain at the COOH terminus, and a highly charged region separating the two (16). The TPR domain mediates its interaction with Hsp90 and Hsp70 during the regulation of signaling pathways and during protein quality control (17) targeting Hsp70 (15) and its substrates, such as p53 (18) and GR (19) and for proteasomal degradation. Overexpression of CHIP in fibroblasts increased the refolding of proteins after thermal denaturation and inhibition of Hsp70 chaperone activity abolished the effects of CHIP on protein folding, indicating that the CHIP-mediated events were Hsp70-dependent (20). Although the full range of cellular substrates of CHIP remains to be explored, misfolded CFTR (17, 21), tau (22, 23), and polyglutamine aggregation (24) are suppressed by CHIP-assisted quality control.

In this study, we have analyzed in detail the interaction of p53 with CHIP and have discovered that the co-chaperone CHIP regulates p53 conformation and activity under physiological and elevated temperatures both *in vitro* and *in vivo* independent of Hsp90 function. CHIP prevents p53 from irreversible thermal inactivation and restores the DNA binding activity of heat-denatured p53 in an ATP-independent manner. In cells under elevated temperatures, CHIP helps resurrect the p53 mutant conformation into a folded native state. CHIP was shown to co-associate with WT p53 upon DBS in chromatin. We have proposed that CHIP might be a direct chaperone of WT p53 both under physiological and elevated temperatures.

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[2] The abbreviations used are: WT, wild type; TPR, tetratricopeptide repeat; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; DBS, DNA binding site(s).