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 BamHI and HindIII 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 BamHI and HindIII restriction sites in the pET32a plasmid (Fig. 1B) and on BamHI and EcoRI 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 BamHI and HindIII 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 BamHI and HindIII 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 *BamHI* and *HindIII* 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 *BamHI* and *EcoRl* 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.
**Results**

**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 P32-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 P32-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. Lanes 1-3 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 Comassia 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^{140-393} 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^{285-293} and the bound fraction was detected with PAb 1801. The signal of binding showed that NTD interacts with GST-p53, GST-p53^{140-393} and p53^{285-293} 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\(^{1-140}\) (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\(^{1-140}\), 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.
Results

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 0.1 cm 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.
P32 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-I 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 P32 labeled p215' 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 P32 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 P32 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.
Results

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 the 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<sub>81-285</sub>) 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<sub>81-285</sub> separately for 1 hr and the bound DNA was detected using alkaline phosphatase conjugated avidin.
Results

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<sup>285-393</sup>) 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.
**D**

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 1 hr and subsequently the bound DNA was detected using alkaline phosphatase conjugated avidin.

**E**

Fig. 9 E. ELISA showing the loss of DNA binding activity of p53 upon heat denaturation. The native and heat denatured (37°C/1 hr.) 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'-GTTAGGTTAG-3') conjugate.
Results

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 tetracycline-oligonucleotide(5′-GTAGGGTAGGTTAG-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
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 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.

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 (0.5 μg) was denatured (37°C/1 hr.) and coated on the ELISA plate, subsequently 5 μg 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, 10 μg) or BSA (lane 6, 5 μg; lane 7, 10 μg) 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.
Results

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 action, this discrepancy might be due the difference in the nature of two methods. In the EMSA action the p53 is unbound to any solid surface and hence NTD can bind to p53 from all the sites whereas in the ELISA p53 is attached to the surface of the ELISA plate which hinders NTD in interacting with p53 from all the sites as a result there is a less protection or recovery seen in this method.

Construction 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 temperature dependent loss. To investigate whether NTD colocalises with p53 in the stress conditions and protects its function from thermal denaturation, we have constructed fluorescent tagged p53 and NTD proteins. The p53 cDNA was cloned on the NheI and EcoRI sites of the CFP-N1 (Figure 11 A), pEYFP-N1 (Figure 11b) and pEGFP-N1 (Figure 11E), and the cDNA NTD (residues 1-125) was cloned in pEYFP-N1 (Figure 11C). Further the transactivation main (NTD-56) of p53 (residues 1-56) was also cloned on NheI and EcoRI sites of the YFP-N1 (Figure 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 fluorescent 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 checking 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 line H1299 and their localization studied separately. The p53-EGFP fusion protein was found to localize in the cytoplasm and nucleus. Surprisingly, the NTD-EYFP fusion protein also localized to the cytoplasm and nucleus and the amount of NTD present in nucleus is high in comparison to the cytoplasm. The p53 is a nuclear protein and it contains three nuclear localization 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 it 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 NheI and EcoRI 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 NheI and EcoRI 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 Nhel and EcoRI 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 Nhel and EcoRI 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.
**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 NheI and EcoRI 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 H1299 cells.
Results

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.