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Three assays show differences in binding of wild-type and mutant p53 to unique gene sequences

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Abstract

Cancer-associated mutations in the *p53* gene often change amino acids in the protein's DNA binding domain. We used three different binding assays specifically gel shift, DNA binding scintillation proximity assay and a streptavidin magnetic bead assay to analyze the DNA binding of the tumor suppressor p53 from 4 human cell lines with different DNA sequences from the *mdm2*, *p21* and *cyclin G* genes and a mutant form of the *cyclin G* sequence. Treatment of MCF-7 cells having wild-type p53 with hydrogen peroxide increased the binding of p53 to DNA as detected using all three assays, but to different extents. The p53 proteins from the thyroid cancer cell lines with different *p53* mutations (ARO, WRO and NPA) have comparable binding reactions in the three assays, but show different specificities for the sequences. Here we show that multiple different binding assays allow us to generate a more complete picture of the function of DNA transcription factors in diseases such as cancer.

Keywords

DNA affinity immunoblotting; DNA binding; electrophoretic mobility shift assay (EMSA); scintillation proximity assay (SPA) beads; streptavidin magnetic beads

Introduction

Knowledge of the functional status of cell cycle regulatory proteins is important as it helps to understand the control of cell division and events that lead to cancer. Several DNA binding proteins have been studied as they help to elucidate the series of events resulting in cell transformation. One of the most widely studied DNA binding proteins in relation to cancer is the p53 protein (1,2). The p53 protein is called the guardian of the genome (3) because it is activated in case of cellular stress and transcribes the genes responsible for DNA repair, apoptosis, cell cycle arrest as well as others and helps in reducing uncontrolled growth of cells (4,5). Most human cancers have a mutation in the *p53* gene (6) and 95% of the mutations in this gene lie in the DNA binding domain of the p53 protein (7,8,9,5).

The p53 protein is known to regulate many genes such as the *p21* gene which is involved in growth arrest (10,11,12), the *cyclin G* gene which regulates the cell cycle (13,14) and the *mdm2* gene which produces a protein that binds to p53 and promotes its degradation in a negative feedback loop (15,16). The p53 protein is activated when cells are exposed to genotoxic stress such as UV radiation or chemicals, like hydrogen peroxide (17,18). Not only the wild type p53 protein but also some mutant p53 proteins are reported to specifically bind to DNA and transactivate reporter genes having the control regions from some of the

cell cycle regulatory genes like *p21* or apoptosis related genes like *PIG-3* and *Bax*. Some mutant p53 proteins have also been shown to bind to the p53 consensus DNA sequence (19,20,21).

There are different assays developed to test the DNA binding of the p53 protein. The electrophoretic mobility shift assay (EMSA) or gel shift is the most commonly used method to analyze sequence-specific binding of proteins to DNA (21). In this assay, the mobility of a labeled DNA fragment in a gel is retarded when a binding protein is associated with the fragment. The DNA binding ability of the mutant p53 protein from thyroid cancer cells has been studied using EMSA (21). They found that some of the p53 mutants still bind to a p53 consensus DNA binding motif. There are certain assays like chromatin immunoprecipitation (ChIP) and the yeast based functional assay that measure the *in vivo* p53 activity. ChIP requires the use of specific antibodies against the DNA binding protein of interest to pull out those proteins bound to chromatin, and then uses techniques like PCR to then analyze the sequences that are isolated (22,23). Researchers have used this assay to analyze the DNA binding properties of the p53 protein to p53 responsive genes like *mdr-1*, *survivin*, *pcna* and *p21* (24,25). The yeast based functional assays express p53 in the presence of a reporter driven by a p53 responsive promoter. This work shows that the p53 mutants are transcriptionally more active with the *p21* promoter as compared to the *Bax* promoter (26,19). Jagelská and colleagues (27) describe an enzyme linked immunosorbant assay (ELISA)-like technique to analyze the DNA binding properties of the p53 protein. This assay uses streptavidin coated microtiter plates to bind the biotin labeled DNA containing the p53 recognition site. An extract containing the p53 is added and then binding is detected by p53-specific antibodies in an ELISA-like method. This technique is useful when analyzing p53 protein DNA binding from many samples as the assay is done using a microtiter plate. This is similar to the DNA binding scintillation proximity assay (SPA) method (28), except this latter technique uses radioactive DNA. This method was used to analyze the interaction of recombinant wild-type p53 protein to the *cyclin G* promoter sequence. There is also a DNA binding assay for the p53 protein using fluorescence anisotropy which monitors the change in movement of a fluorescently labeled DNA immobilized by a binding protein (29,30). This assay has shown differential interaction of the recombinant wild type p53 and truncated p53 to unique gene sequences. Lastly, there is a technique that uses streptavidin magnetic beads to analyze the DNA binding properties of the p53 protein using biotinylated gene sequences (31,32). Liu and colleagues (32) measured the DNA binding of the p53 protein from tissue lysates from frozen breast cancer specimens and found that the p53 protein from some specimens bound to the consensus sequence while others did not indicating that this assay could be used to detect the functional state of the p53 protein from tumor samples. They also detected DNA binding of the mutant p53 from cervical carcinoma cells to a consensus p53 binding sequence. Buzek and colleagues (31) used a similar approach to detect the DNA binding ability of the p53 protein to various gene sequences. One of their results showed that some mutant p53's did not bind to the modified *GADD45*, a cell cycle regulator gene, while the wild type p53 protein and other mutants did. Their research uses p53 protein produced from expression vectors transfected into SaOS-2 (human osteosarcoma) cells.

Few research articles use multiple techniques to look at DNA binding. This research article compares the DNA binding of p53 detected using three different DNA binding assays. We have chosen EMSA/ gel shift as this is the classic method to study DNA protein interactions and compared this method to two newer ones to measure DNA binding. The DNA binding SPA is a relatively new, quantitative method that can be done in a high throughput manner. The other method utilizes streptavidin magnetic beads which are useful because the protein(s) bound via biotinylated DNA can be analyzed. We analyzed the binding of different wild-type and mutant p53 proteins to four different DNA sequences. We used the

p53 protein from a breast cancer cell line (both in stressed and unstressed conditions) which has the wild-type p53 protein and from three thyroid cancer cell lines which contain mutant p53 protein. We have used three different native gene sequences i.e. *cyclin G*, *mdm2* and *p21*. These gene sequences were chosen as they are from different pathways regulated by the p53 protein. We also used one mutated version of the *cyclin G* gene sequence as a test of the binding reaction specificity. We wanted to determine whether there is the same binding of these different p53 proteins to specific p53-regulated gene sequences. We found some interesting similarities and differences in the binding activity detected by the different assays.

Material and Methods

Cell culture

Thyroid cancer cell lines ARO, NPA and WRO (provided by Fran Carr now at the University of Vermont) were grown in RPMI-1640 (Lonza, Walkersville, MD) with 10% fetal calf serum (FCS). The breast cancer cell line MCF-7 obtained from the American Type Culture Collection (ATCC, Manassas, VA) was grown in Minimal Essential Medium (ATCC) with 10% FCS. The MCF-7 cells were treated with 0.2 mM hydrogen peroxide (H₂O₂) three hours prior to harvesting to induce the p53 protein.

Nuclear extracts and determination of the p53 and total protein concentration

The method for preparing nuclear extracts was based on the one used by Jagelská and colleagues (27). The total protein concentration of the cell extract was determined using the bicinchoninic acid assay (BCA, Sigma St. Louis, MO). To determine the concentration of the p53, ELISA's (p53 ELISA Kit Pantropic, Calbiochem-EMD, La Jolla CA) were performed. The WRO cell extract had p53 that was not detectable by ELISA, but western blots done using the WRO cell extract showed the presence of this protein. Hence for this extract, the concentration of the p53 protein was determined by comparing the intensities of the p53 protein from the WRO cell extract on western blots (see below) to that in other cell extracts that had been quantitated using ELISA.

Electrophoretic mobility shift assay (EMSA) for p53 DNA binding

Double-stranded biotinylated oligonucleotides for the *cyclin G* wild type promoter sequence (top strand 5' AGGCCAGACCTGCCCCGGGCAAGCCTTGGCA 3') (13), a *cyclin G* mutant promoter sequence (top strand 5' AGGCCAGACCTGACCGGGAAATCCTTGGCA3') (14) the *mdm2* promoter sequence (top strand 5' CGGAACGTGTCTGAACCTGACCAGCTC 3') (16) and the 5'-*p21* promoter sequence (top strand 5' CGAGGAACATGTCCCAACATGTTGCTCGAG 3') (10,12) were obtained from Integrated DNA Technologies (Coralville, IA) with the biotin label at the 5' end of one of the strands. Oligonucleotides without any label were used in the competition experiments. The nuclear extracts containing the p53 protein (100 pg) from the different human cell lines was incubated with 20 pmole of biotin labeled DNA, 1 µg of non-specific DNA (poly-dAdT; Roche, Indianapolis, IN) in the binding buffer (20 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 10 mM (NH₄)₂SO₄, 10 mM KCl, 0.2% Tween-20) in a total volume of 20 µl. For the competition experiments, 10× unlabelled DNA was added. This reaction was incubated at room temperature for 30 min then the samples were separated on 5% TBE polyacrylamide gels (Biorad, Hercules, CA) and transferred to positively charged nylon membranes (Roche) using a semi dry transfer system (Bio-Rad). This membrane was incubated after blocking in a solution containing 1:100,000 dilution of streptavidin horseradish peroxidase (Pierce, Rockford, IL) and visualized using a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Pierce). Super shift reactions were done using the extracts from ARO and WRO cell lines. Here along with the other

components, either 0.1 μ g of the pAb421 (Oncogene Research Products-EMD Chemicals) or the DO-1 (Calbiochem-EMD) antibody was added. The p53 protein from WRO and NPA cell extracts was immuno-depleted using a polyclonal biotinylated p53 antibody (R&D Systems, Minnesota, MN). Here the cell extracts from WRO or NPA cells (100pg of p53 protein) were incubated with 100 μ g of streptavidin magnetic beads (Roche) which were pre-incubated with 0.1 μ g of biotinylated p53 antibody. The unbound fraction was collected (see section on streptavidin bead assay for details). This unbound fraction with reduced p53 (confirmed using western blots) was used for the gel shift reactions.

Scintillation proximity assay (SPA) to measure DNA binding

SPA DNA binding was measured with 0.5 pmole ^{3} H-labeled *cyclin G* DNA fragment (185 Ci/mmol) using MCF-7 untreated (39 pg p53, 56 μ g total protein), MCF-7 H_2O_2 treated (39 pg p53, 74 μ g total protein), ARO (465 pg p53, 68 μ g total protein), WRO (20 pg p53, 75 μ g total protein) and NPA (30 pg p53, 71 μ g total protein) nuclear cell extracts in binding buffer with 100 ng anti-p53 monoclonal antibody (pAb421), 1 μ g non-specific DNA (poly dAdT) in 20 μ l as previously described (28). For the competition experiments, 10 pmole (20 $\times$) of the unlabeled nucleotide was added in the binding reaction at the beginning of the experiment. The counts per minute (cpm) after the indicated time period were determined, and the background counts (with buffer instead of cell extract) were removed to obtain specific cpm. The percent of specific cpm in the sample with competitor was calculated based on the specific cpm without competitor being made to equal 100%.

Streptavidin magnetic bead DNA binding assay

For the assay, 20 pmoles of the biotinylated DNA was incubated with 100 μ g of streptavidin magnetic beads with 1 μ g of polydAdT in the binding buffer. To the binding reaction mixture, nuclear extracts from ARO, WRO, NPA, H_2O_2 treated and untreated MCF-7 cells containing approximately 100 pg of p53 were added in a total volume of 20 μ l. The binding reaction was done for 30 min at room temperature on a shaker. The beads were separated using a magnet (Roche) and the unbound fraction removed. The beads were then washed using 50 μ l binding buffer three times. The bound fraction from the beads was then isolated by boiling the beads in 20 μ l H_2O for 5 min. The bound and unbound fractions were then analyzed using western blots. For the western blots, the antibody against p53, DO-7 (Calbiochem-EMD) (1:1000 dilution) was incubated with the membrane for 2 hr at room temperature. As a secondary antibody, anti-mouse IgG, horse radish peroxidase (HRP) linked (1:1000 dilution; Cell Signaling, Danvers, MA) was used. The signal was then visualized using a chemiluminescent substrate. Signal intensities were quantified by means of densitometry and analyzed using the Quantity One software (Bio-Rad). The Global background subtraction method was used. The percentages of p53 in the bound and unbound fractions were calculated by taking the sum of the two intensities and then calculating the percentage in each fraction. Statistical difference between two binding reactions was determined by the Students *t*-test utilizing the MS Excel data analysis tool pack. A probability (*P*-value) of <0.05 was considered as a significant difference between the two reactions.

Results

Electrophoretic mobility shift assay (EMSA) to measure DNA binding of p53

To analyze the DNA binding properties of wild-type and mutant p53 proteins, three different types of assays were used with extracts from 4 different human cell lines. The cells included the human breast cancer cell line MCF-7 that has wild-type p53 (33,34). These cells were treated with hydrogen peroxide (H_2O_2) which is known to increase the level of the p53 protein (17). Three human thyroid cancer cell lines were used which each have a different

p53 gene mutation. The ARO cells have an Arg to His mutation at codon 273, the WRO cells have a mutation at codon 223 (Pro to Leu) and the NPA cells have a Gly to Glu mutation at codon 266 (35). The NPA cells are heterozygous for this mutation, the WRO cells homozygous at the *p53* locus, while in one paper the ARO cells are listed as being homozygous (35) and in another heterozygous (21) for this alteration. All of these mutations are in the DNA binding domain of the p53 protein (36). The first assay for DNA binding that we used was the classical technique electrophoretic mobility shift assay (EMSA). Here we tested the mobility shift of the *cyclin G* wild-type and mutant sequences with the 5 different cell extracts and found clear shift of the free DNA in the gel (Figure 1A). Interestingly, there were two bands with NPA and MCF-7 cell extracts (see lanes 4, 5 and 8–11) but only the lower band (marked with an arrow) was competed completely with 10× unlabeled DNA (lanes 12–17; and data not shown). Thus, we presume the upper band (marked with a star) is a sequence non-specific shifted band. Since a similar amount of p53 protein from the different cell lines was used, a more intense band on this gel should indicate higher affinity of the protein for the DNA. The p53 protein from the WRO and MCF-7 treated and untreated cells had a higher affinity for the wild-type sequence than the protein from the ARO and NPA extracts (Figure 1B). Treatment of the MCF-7 cells with H₂O₂ caused an increase of 40% in the binding to the wild-type and mutant *cyclin G* sequences. Interestingly, these proteins do not clearly distinguish between wild-type and mutant *cyclin G* DNA sequences as all appear to bind with comparable intensities (Figure 1). From the competition experiments, binding to the protein in both MCF-7 treated and untreated extracts was competed very efficiently by the *cyclin G* (wild-type and mutant) and *p21* DNA sequences (Figure 1). A similar competition was seen with the extracts from the ARO, WRO and NPA cells (data not shown). We attempted supershift experiments using monoclonal anti-p53 antibodies pAb421 and DO-1, but did not detect a shifted band from ARO extracts using the *cyclin G* wild-type sequence (data not shown). To provide evidence that it is the p53 protein that interacts with these DNA sequences, we reduced the level of p53 from the extracts from WRO and NPA cells using a biotinylated polyclonal antibody against the p53 protein (confirmed using a western blot, data not shown). Using these extracts, there was a less intense shifted band seen with the *cyclin G* sequence (data not shown).

Scintillation proximity assay (SPA) to measure p53 DNA binding

SPA beads have been used to analyze the DNA binding of p53 from baculovirus infected insect cells (28). Here, we wanted to test this assay using the p53 derived from human cell lines. The same 5 cell extracts were incubated with the ³H-labeled *cyclin G* sequence and the amount of binding after 2 hours determined (Table I). Specific binding to the p53 was detected in all of the extracts but at different levels. When the counts detected were divided by the amount of p53, the extracts from the MCF-7 H₂O₂ treated cells and from the WRO cells had an approximately 10-fold higher amount of binding to the *cyclin G* sequence than the p53 from ARO and NPA cells (Table I). This level of DNA binding is comparable to the results of the EMSA which showed more shifted DNA in these extracts when the same amount of p53 was used (Figure 1). The treatment of the MCF-7 cells with H₂O₂ increased the binding detected by about 40% (when looking at the cpm normalized for p53; Table I). Competition experiments were then performed using the labeled *cyclin G* gene sequence and 20× unlabeled DNA from the *cyclin G*, *p21* and *mdm2* genes as well as the mutant *cyclin G* sequence. The mutant DNA shows very little binding to the wild-type p53 produced in insect cells (28). Interesting, the mutant *cyclin G* sequence does not compete with the DNA binding of the p53 from the H₂O₂ treated MCF-7, ARO and NPA cells, but does compete for binding with the untreated MCF-7 and WRO cells (Table II). The three native sequences appear to compete equally with all of the extracts indicating that the p53 binds equally well to all three of these DNA sequences. The overall competition is however less with the NPA

cell-derived p53 compared to the other two thyroid cancer cells, ARO and WRO (~55% versus 20–30% binding left in the presence of competitor) (Table II).

Streptavidin magnetic bead assay to measure DNA binding by p53

We used a similar assay as Liu and colleagues (32) where we incubated the p53 protein from the nuclear extracts with biotinylated DNA along with the streptavidin magnetic beads. The unbound and bound fractions were isolated and then subjected to western blot to measure the p53 protein. Using the nuclear extracts from the MCF-7 treated and untreated cells, there is a differential interaction of the wild-type p53 protein to the unique gene sequences used for binding (Figure 2). The results show that the binding affinity of the p53 protein from the MCF-7 H₂O₂ treated cells is highest to the wild type *cyclin G* DNA sequence (about 65% of the protein bound). As a control, the mutated *cyclin G* DNA sequence was used and there was minimal binding detected to this sequence (Figure 2). The binding affinity for the *mdm2* and *p21* DNA sequences is lower (about 50%). Hence some variation is seen when binding is done with different DNA sequences. Compared to the untreated MCF-7 cells, there was a much higher amount of binding when these cells were treated with H₂O₂ by about 6.5-fold which did not vary with the gene sequence used for the binding reaction (Figure 2). This indicates differences in the DNA binding capacity of the p53 protein from stressed and unstressed cells.

The western blots showed a differential interaction of the mutant p53 proteins with the gene sequences that were used for binding (Figure 3). We observed that the p53 protein from the ARO cell line shows two bands on western blots, but only the upper band binds to the DNA as it is found in the bound fraction. We are not sure of the difference between these two protein bands, but similar banding was seen in the breast cancer cell line MDA-MB-468 which has the same mutation (Arg to His at 273) (37). The p53 protein from all three nuclear extracts binds to the *p21* DNA sequence although the amount of p53 protein detected in the bound fraction from the WRO cell extract is very low. The p53 from the NPA cells appears not to distinguish the different sequences; all of the reactions showing about 10 to 18% binding (Figure 3). The p53 from ARO cells can not bind to the *cyclin G* mutant sequence and only 20% of the protein from both ARO and NPA cells bound to the *mdm2* sequence. There was no interaction of the p53 from WRO to the *cyclin G* wild type and mutant sequence. We next tested the effect of adding unlabelled DNA to the binding reactions. Binding reactions were done using the *cyclin G* wild type biotin-labeled DNA sequence in the presence of 10× unlabeled competitors. From the western blots, there is competition when unlabeled *cyclin G* wild type and *p21* DNA sequences were used with the ARO, NPA, and MCF-7 H₂O₂-treated cells (Figure 4). There was minimal competition seen with the *cyclin G* mutant sequence using all three extracts.

Discussion

In this research, we used three different DNA binding assays to study the interaction of wild-type and mutant p53 proteins to unique gene sequences. The three different assays were expected to show the same results although the assays provide different means for detecting this activity. Previous work has been published using these different assays separately, but this is one of the few articles to compare all three with the same cell extracts and sequences. The SPA is the only assay that requires the presence of the p53 for the assay to work as the antibodies that localize the protein to the SPA beads are mono-specific. The other two assays use the labeled DNA for the distinction of bound and unbound proteins but then have different ways of detecting the signal. These differences in the set-up and monitoring of the DNA binding activity may explain the somewhat unexpected differences in the results observed with the different assays.

All three assays showed an increase in the DNA binding with extracts from MCF-7 cells treated with H₂O₂ compared to extracts from those cells without treatment consistent with this compound inducing production and DNA binding of p53 (17). But the increase was to different extents; with the EMSA and SPA, the increase was about 40% but with the streptavidin bead assay, more than a 6-fold induction of DNA binding activity was measured. The specificity of the binding also appeared slightly different in the three assays. Using EMSA, binding was competed completely in both extracts by the wild-type and mutant *cyclin G* sequences. With the SPA, the p53 from untreated cells could bind to all sequences (apparent through a competition assay), but the p53 from the treated cells did not bind the mutant *cyclin G* sequence. For both direct binding and competition experiments using streptavidin magnetic beads, MCF-7 cell extracts produced a p53 that poorly bound the mutant *cyclin G*. Previous research has shown that the MCF-7 cell line carries the wild-type *p53* gene (33,34). The wild-type p53 protein was initially analyzed using the SPA after the protein was produced in insect cells (28). There, the mutant *cyclin G* sequence bound with a much lower affinity than the wild-type sequence. These results are consistent with other research where binding to *cyclin G* and *p21* DNA sequences by the wild type p53 protein was measured using fluorescence anisotropy. In that research, they compared binding of p53 to the cell cycle regulatory and apoptosis genes and showed that there is more binding of the p53 protein to the former. Their results also show that the interaction of the p53 protein to the *cyclin G* sequence is 1.5 times more than the *p21* sequence (30). Thus, our results appear to be consistent overall with data using other assays for DNA binding by wild-type p53 protein.

We also analyzed the DNA binding of mutant p53 proteins isolated from 3 different thyroid cancer cell lines. Despite the fact that the three mutations are in different regions of the DNA binding domain of the p53 protein, all three cell lines produced p53 that could bind to the *cyclin G* wild-type sequence. Although the ARO cell line produced the most p53 protein per µg total nuclear protein, it was the extract from the WRO cell line that showed the most binding per pg p53 protein. This was easily visualized with the EMSA and SPA DNA binding assays, but was not seen with the streptavidin magnetic bead assay. Because the streptavidin binding assay uses the western blot for p53 to visualize the DNA binding and there are very low levels of detected p53 in the WRO cells, a relatively large volume of that extract was used for this assay. We tested the possibility that the WRO cell extracts contained some material that blocked the binding of the biotinylated DNA to the streptavidin magnetic beads, but we found no evidence for this (Pluff, Chandrachud and Gal, unpublished observation).

Using various direct binding and competition formats, differences were seen in the specificity of binding by the p53 from the thyroid cancer cell extracts. With the EMSA, all three extracts bound to both mutant and wild-type *cyclin G* sequences and to the *p21* gene sequence (as evidenced from the competition experiments). With both the SPA and the streptavidin magnetic bead assay, the p53 extract from ARO cells showed significant preference for the wild-type *cyclin G* sequence over the mutant. The p53 from and NPA cells showed similar binding to all sequences for all assays. But there was a difference in the binding seen for WRO (discussed above). We are unsure of the significance of the apparent binding of these p53 proteins to the mutant *cyclin G* sequence in some of the assays, but we feel it is not likely due to non-specific binding as polydAdT DNA is always included in the assay (see Materials and Methods). Interestingly, Buzek and colleagues (31) using the streptavidin magnetic bead assay tested binding of the wild type and mutant p53 proteins to different gene sequences. In one of their experiments, they used a modified *GADD45* promoter sequence and found that the p53 protein having the mutation Arg to His at codon 273 could not bind to this sequence while, the wild-type protein and some other mutated p53 proteins could bind. We found significant binding of this same mutant p53 (from ARO cells)

to the three gene sequences in all three assays, although we have not tested the *GADD45* promoter sequence. The fact that in their work (31), the genes were transfected into cells may have resulted in differences in binding as in our assay, the sources of the p53 protein were cell lines with their innate level of the p53 protein.

This study gives a comparative overview using three different DNA binding assays with human cell lines carrying wild-type and mutant p53 proteins. It is important to remember when comparing these assays that the DNA binding SPA and EMSA monitor the DNA used for binding while the streptavidin magnetic bead assay monitors the p53 protein although the DNA is used in the isolation. The SPA is the only assay that requires a p53-specific antibody in the assay. Although it is possible that the monoclonal antibody we used had a positive influence on the DNA binding of the p53 as seen in other work (38,39), this antibody (pAb421) did not enhance the binding or cause a supershift of the band in the EMSA for the p53 protein from WRO and NPA cell lines. The reason for this is not completely clear based on the results with the SPA and the fact that this antibody did show supershifting of the p53 from the baculovirus infected cells (data not shown; (28)). This lack of recognition of the DNA-protein complex by the antibody may be in part because the antigens were hidden in the DNA bound complex.

Binding by the p53 related proteins, p63 and p73 could also explain the shifted bands as they demonstrate sequence-specific DNA-binding like p53 and regulate both shared and distinct subsets of downstream target genes (40). Although it has been reported that both ARO and NPA have p73 (41), nothing has been reported, to our knowledge, about p63 in these cells. The p63 protein was not detected in MCF-7 cells (42), but the p73 protein was not analyzed in these cells in this publication. Although the p63 and p73 proteins have some of the same transcriptional targets as the p53 protein, the binding detected using the SPA and streptavidin bead assays is not likely due to the presence of these other proteins since the antibodies used are specific to the p53 protein and detect a ~53kDa peptide. It is also possible a completely unrelated protein is causing the gel shift bands detected in these extracts.

These three assays are not the only ones appropriate for studying protein interactions with DNA strands. Chromatin immunoprecipitation (ChIP) can measure the interactions of proteins with genome sequences in the living cell. This method reveals that there are about 65,572 unique p53 ChIP DNA fragments (43). Although ChIP can quantitate the portion of a specific gene that is bound by a transcription factor, it is not really able to determine what portion of that transcription factor binds to DNA. That is measurable using the streptavidin magnetic bead assay used in this study. The DNA binding SPA used here is able to measure both on- and off-rates of DNA binding in a quantitative way (28), although, in some cases, kinetic experiments measuring the *in vivo* p53 DNA binding following certain treatment have been done with ChIP (22,23). However, for a variety of reasons, assays with cell extracts provide additional information about the status of the p53 protein. These latter assays can be more quantitative on the DNA binding measurements than PCR based ChIP protocols. Many of these assays can be done with extracts from tumors that have been frozen (32), and done on multiple samples at a time in 96-well microtiter plates. A few of these other assays can measure the association and dissociation rates of DNA binding as they are continuous assays. Although gel shift is the classical approach, the streptavidin magnetic bead assay is useful since it may be able to isolate DNA binding protein complexes that are important for gene regulation (44). For instance, ChIP at various times after treatment of cells with ionizing radiation, showed that the induction of the *PIG-3* gene took longer compared to the *p21* and *mdm2* genes (23). This may be due to the addition of other factors forming a complex with p53 which could be identified using the streptavidin magnetic bead assay.

Depending on the question being asked about the binding process, one or the other assay would provide the appropriate experimental approach. The SPA would be best if information on either the on or off rate of binding were of interest. It is also the one to use with multiple samples that requires relatively little user work for the number of samples processed. If the question is what other proteins interact with the transcription factor bound to a DNA sequence, then the streptavidin magnetic bead approach may be better. On the other hand, if the questions are related primarily to the presence of a protein on the chromatin in the living cell, then ChIP is obviously the method of choice. In any case, no one assay can provide the information required for all questions that could be asked about the complex regulation of genes in living cells. Thus, it makes sense to continue to explore alternative methods for this analysis. We have presented some evidence that three assays provide complementary information on the p53 DNA binding in extracts from human cells. Any of these assays could be modified to measure other DNA binding proteins, simply by changing DNA sequences and antibodies, thus providing additional information on the role of specific DNA binding proteins to the regulation of gene expression.

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Figure 1. EMSA analysis of DNA binding to p53 responsive sequences

(A) The blots of the gel shift experiment done using the p53 protein (100 pg) from ARO, NPA, WRO; H₂O₂ treated (MCF-7H) and untreated MCF-7 cells (MCF-7) with 20 pmoles *cyclin G* wild type (W) or mutant biotin labeled sequences (M) or without protein (Free DNA). Competition was done with 10X unlabeled *cyclin G* wild-type (W_u), *cyclin G* mutant (M_u) or *p21* sequences (p21_u) using the H₂O₂ treated (MCF-7 H) and untreated MCF-7 cell extracts (MCF-7) with biotin labeled *cyclin G* wild-type sequence. The star marks the sequence non-specific shifted band while the arrow marks the specific one. The unbound DNA was run off the gel. The figure is representative of three individual experiments performed. (B) The graph shows the intensities of the shifted bands using the cell extracts as in A with either wild-type (WT) or mutant (MT) *cyclin G* sequences calculated based on the results from 3 separate experiments with the densitometry quantified using the Quantity One software (Bio-Rad).

Figure 2. Streptavidin DNA binding assay with wild-type p53 binding to unique gene sequences Western blots of the unbound (Un) and bound (B) fractions from a binding reaction using nuclear extracts from MCF-7 cells treated or untreated with H₂O₂ (~ 100 pg of p53 protein used) and different biotinylated oligonucleotide gene sequences (20 pmole) as described in the Materials and Methods section. P is the prebound extract, while WT is the wild-type and MT is the mutant *cyclin G* sequence. The gel presented is representative of three different independent binding reactions performed. Significant differences from binding reactions using wild type *cyclin G* and *mdm2* for MCF-7 treated compared with untreated extracts were seen (*P* value < 0.05).

Figure 3. Streptavidin DNA binding assay with mutant p53 binding to unique gene sequences Western blots of the unbound (Un) and bound (B) fractions from binding reactions done using the p53 protein from ARO (120pg p53), NPA or WRO cell lines (both 100pg p53) with 4 different sequences (WT is the wild-type and MT is the mutant *cyclin G* sequence) as described above in the Material and Methods section. P is the prebound extract. The gel presented is representative of three different independent binding reactions performed. A significant difference in the binding of the ARO extract with the *cyclin G* wild type sequence compared to the *cyclin G* mutant and with *p21* compared to the *cyclin G* mutant sequence was seen (P value < 0.05). In binding reactions with the WRO extract using *p21* compared to the *cyclin G* mutant sequence was seen (P value < 0.05).

Figure 4. Competition experiment with streptavidin DNA binding assay

Western blots using p53 protein from ARO (120pg p53), NPA (100pg) and MCF-7 cells (100pg) treated with H₂O₂ adding different competitor DNA sequences. Twenty pmoles of biotin labeled *cyclin G* wild-type sequence was added (CyG WT) in some reactions with 10 times more unlabeled *cyclin G* wild-type (+Unlab Wt), *cyclin G* mutant (+Unlab Mt) or *p21* DNA (+Unlab p21). The gel is representative of three different independent binding reactions performed. A significant difference between reactions without and with wild type *cyclin G* and *p21* competitor DNA for the ARO and MCF-7 treated extracts was seen (*P* value < 0.05).

Table I

Differential binding ability of p53 in cell extracts

Source of cell extract	Specific binding counts (CPM)	Total p53 amount (pg)	Counts/pg p53
MCF-7 untreated	1427+/-44	36	40
MCF-7 H ₂ O ₂ treated	1974+/-182	36	54
ARO	1920+/-170	310	6.2
NPA	231+/-146	50	4.6
WRO	1340+/-50	25	54

Counts taken after 122 minutes (ARO, NPA, WRO) or 97 minutes (MCF-7 treated and untreated). This data is representative of 3 to 7 separate experiments.

Table II

SPA Competition for DNA binding by various sequences with cell extracts

Source of cell Extracts	Competitor DNA(% binding compared to no competitor)			
	Wild-type <i>cyclin G</i>	Mutant <i>cyclin G</i>	p21	mdm2
MCF-7 untreated	40.7+/-0.6%	49+/-11	50+/-9	47+/-2
MCF-7 H ₂ O ₂ treated	84.6+/-0.8%	99+/-9	69+/-5	77+/-2
ARO	27+/-1%	101+/-5	19+/-3	33+/-5
NPA	56+/-14%	71+/-4	53+/-9	57+/-10
WRO	23.5+/-0.4%	26.4+/-0.3	18+/-2	20+/-1

Without competitor set at 100%. Competitor was added at 20× labeled DNA. This is a representative experiment of 3 to 6 performed.