Scintillation proximity assay for DNA binding by human p53

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INTRODUCTION

DNA binding proteins are known to regulate gene expression. When that binding is altered, a disease state can result. A common method for measuring DNA binding, namely electrophoretic mobility shift assay (EMSA) is often used but it is not amenable to rapid screening of many samples. As an alternative method, we have developed a DNA binding assay for the tumor suppressor protein p53 in a 96-well microtiter plate format using scintillation proximity assay (SPA) beads. We have shown this assay to be sensitive (as little as 0.5 ng p53 can be detected), quick (assay completed in as little as 15 min), and easily quantitated using a microtiter plate scintillation counter. We also used the assay to analyze the kinetics of the DNA binding to p53. The specificity of this p53 DNA binding SPA was confirmed using competition by oligonucleotides either from the same gene or from mutated versions of this sequence. Thus, SPA is a good alternative to gel shift assays for DNA binding and may be useful for the analysis of multiple tumor cell samples or for high-throughput screens for compounds affecting DNA binding by proteins of interest.

MATERIALS AND METHODS

Preparation of Labeled DNA and p53 Protein

We prepared a double-stranded DNA probe, sequence 5'- (A)19 TGCCAAGGCTTGC CGGGCATGTGGCCT/AGGCC AGACCTGCCGGCGAACCTTG GCA-3', from the cyclin G promoter region that was known to bind p53 in a sequence-specific manner (18), and used that to develop our SPA DNA binding assay. The DNA fragment was custom labeled using (32P)TTP (GE Healthcare) and terminal transferase (kindly provided by K. Okamoto, Columbia University, New York, NY, USA) by infecting Sf9 insect cells and taking total cell extracts on different days postinfection. We quantitated the total protein in the cell extracts using bicinchoninic acid (Sigma-Aldrich, St. Louis, MO, USA) and the p53 protein specifically using a p53 enzyme-linked immunosorbent assay (ELISA) kit (Oncogene Research Products, Cambridge, MA, USA).

Gel Shift and SPA of p53 DNA Binding

We confirmed DNA binding by the p53 using EMSAs following the method of Okamoto and Beach (18). We then used those conditions to develop a DNA binding SPA. The SPA conditions were generally 0.08 μCi (0.19 pmol) 3H-labeled DNA, 100 ng anti-
from the wild-type, known to disrupt EMSA DNA binding (18), was used to test specificity and had the sequence 5'–TGCCAAGGATTTCCTGGTCAGG TCTT/AGGCCAAGCTGACCGGG AAATCCTTGCA-3'.

RESULTS AND DISCUSSION

p53 DNA Binding SPA Is Sensitive and Versatile

DNA binding of the recombinant human p53 was first confirmed using standard EMSAs (data not shown), then monitored using SPA with 3H-labeled DNA from the cyclin G promoter sequence that had been used previously to demonstrate p53 DNA binding (18). Specific binding events could easily be detected over nonspecific binding (Figure 1B). To determine the sensitivity of the p53 DNA binding SPA, we compared the detection limit of this assay format with standard EMSAs. We found consistently that 0.5 ng p53 could be detected with a SPA signal having twice the background counts (Table 1A). This is a similar sensitivity to the EMSA in our hands with this cell extract and DNA sequence (data not shown). The SPA signal could be saturated when the amount of p53 reached 20 ng using the amounts of materials indicated in the Materials and Methods section (data not shown). In addition, no p53 DNA binding in the SPA was detected using uninfected insect cell extracts (Table 1A). The specificity of the p53 DNA complex was confirmed using competition experiments (see below). The p53-DNA bound complex on the SPA beads appeared very stable, as measurements of 80% of the initial counts were detected up to 50 h after addition of the beads (data not shown). Thus, this SPA has a similar sensitivity as EMSA to measure DNA binding, but is much faster. The SPA can give data in under 30 min while the EMSA requires at least 2 h. The SPA uses much less intensive radioactive atoms compared with the 32P that is most commonly used for EMSA. Isotopes such as 125I, 35S, and 3H are generally used as the SPA beads because they have good sensitivity with these lower energy radioactive compounds. Tritium also has the advantage in that it can be conveniently used over many months because it has a much longer half-life than 32P. In addition, the configuration of the SPA using the TopCount instrument provides numerical data immediately for comparison. Bands detected on gels must first be scanned or analyzed to determine quantitative changes in DNA binding.
We also tested the effects of different nonspecific compounds. Substituting single-stranded poly(dA-dT) or poly-L-lysine for the double-stranded dA-dT had little effect on the counts obtained; however single- or double-stranded poly(dI-dC) reduced the SPA counts to 25%–35% of the original values (Table 1C and data not shown). The difference in DNA binding detected in the presence of poly(dI-dC) versus dA-dT is curious, as other DNA binding experiments published with p53 used poly(dI-dC) as the nonspecific DNA (18–22). We confirmed with EMSA that p53 DNA binding was also detected more efficiently when using dA-dT instead of dI-dC in addition to the labeled DNA fragment used for SPA (data not shown) so this difference was not something inherent with the SPA. This difference in the ability of poly(dI-dC) and poly(dA-dT) to affect specific binding may be due to the higher AT content of the binding oligonucleotide used in this assay because of the 19 adenines added at the 5′ for incorporation of the 3H-thymidine.

For these assays, we have used primarily monoclonal antibody pAb421, as it has been shown to work in supershifting p53-DNA bound complexes using EMSAs (22–24 and data not shown). This antibody recognizes amino acids 371–380 of human p53, and is known to alter the conformation of p53 to promote DNA binding by mutant p53 proteins (22–24). We used two additional monoclonal antibodies recognizing different regions of p53. When we used antibodies to other regions (antibody DO-1 recognizing amino acids 21–25 and antibody pAb246 recognizing amino acids 85–109), we noted similar or slightly stronger counts than detected with the pAb421 antibody (data not shown). The slight variations in the signal from the SPA using different anti-p53 antibodies may be related to what specific region is recognized by the antibody.

<table>
<thead>
<tr>
<th>Sample</th>
<th>p53 Protein (ng)</th>
<th>Total Protein (μg)</th>
<th>Specific SPA Counts (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected insect cell extract</td>
<td>0.25</td>
<td>0.085</td>
<td>94 ± 17</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.17</td>
<td>200 ± 36</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.84</td>
<td>1400 ± 120</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.7</td>
<td>2870 ± 100</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.4</td>
<td>9810 ± 460</td>
</tr>
<tr>
<td>Uninfected insect cell extract</td>
<td>&lt;0.33</td>
<td>8.0</td>
<td>13 ± 33</td>
</tr>
<tr>
<td></td>
<td>&lt;1.0</td>
<td>24</td>
<td>25 ± 11</td>
</tr>
</tbody>
</table>

Specific counts per minute (cpm) detected in the indicated samples after 5 h ± SEM (n = 2). Background counts with water in place of the extract gave counts of 270 ± 7 cpm, which was subtracted from the total counts to obtain the specific counts shown. SPA, scintillation proximity assay.

<table>
<thead>
<tr>
<th>B. Additional Component</th>
<th>Specific SPA Counts (cpm)</th>
<th>Control Counts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no additions)</td>
<td>11,100 ± 390</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>With poly(dI-dC)</td>
<td>3390 ± 100</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>With poly(dA-dT)</td>
<td>12,600 ± 160</td>
<td>113 ± 1</td>
</tr>
<tr>
<td>With poly-L-lysine</td>
<td>11,000 ± 380</td>
<td>99 ± 3</td>
</tr>
</tbody>
</table>

Counts detected with specific components after 3.5 h ± SEM (n = 2).

<table>
<thead>
<tr>
<th>C. Competing Oligonucleotide</th>
<th>Relative Concentration</th>
<th>Specific SPA Counts (cpm)</th>
<th>Control Counts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>—</td>
<td>2400 ± 116</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>cycG wild-type</td>
<td>3×</td>
<td>450 ± 47</td>
<td>17 ± 2</td>
</tr>
<tr>
<td></td>
<td>10×</td>
<td>309 ± 12</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>30×</td>
<td>219 ± 12</td>
<td>8.2 ± 0.5</td>
</tr>
<tr>
<td>cycG mutant</td>
<td>10×</td>
<td>1940 ± 34</td>
<td>73 ± 1</td>
</tr>
<tr>
<td></td>
<td>30×</td>
<td>1890 ± 102</td>
<td>71 ± 4</td>
</tr>
<tr>
<td></td>
<td>100×</td>
<td>1630 ± 94</td>
<td>61 ± 4</td>
</tr>
</tbody>
</table>

Specific counts per minute (cpm) detected in the indicated samples after 3 h ± SEM (n = 2). Competing unlabeled oligonucleotides were cycG wild-type, representing the same as the labeled sequence, and cyc G mutant containing a 3-base substitution in that sequence (see Materials and Methods). Concentrations of these oligonucleotides are given relative to the 0.19-pmol labeled oligonucleotide. The results in all three parts of this table are representative experiments of several performed.
antibody and the role of this region in the intact DNA binding protein. It may also be the case that binding of these antibodies to insect cell produced p53 could affect the DNA binding activity of the protein.

**Association and Dissociation of DNA Binding by p53 Can Be Measured**

One potential application for the SPA is to measure the dynamic nature of DNA binding. To test this, we first combined the buffer, protein extract, antibody, and SPA beads. After an equilibration time of 10 min, the labeled oligonucleotide (0.08 μCi) was added and samples read within 60 s. Initially, the counts from individual wells were kept as single values, as comparable wells were not counted at exactly the same time. There appeared to be a very fast association of the labeled DNA to the p53-anti-p53 antibody-protein A SPA bead complex, as the initial changes in signal detected were complete in 3 min (Figure 2A, 10-min preincubation). This was followed by a slow 10%–20% increase in the specific SPA counts detected until the maximum signal was reached in 2–6 h. Very similar counts were obtained when all the components were added at the same time (Figure 2A, 0-min preincubation). As these two configurations produced similar kinetics of binding, we hypothesize that the binding of the DNA to the p53 is the rate-limiting step in the SPA. To confirm the specificity of DNA binding detected in our SPA, we competed the signal with unlabeled mutant oligonucleotide (three base alteration, known not to compete for DNA binding by EMSA; Reference 18 and data not shown). As expected, the mutant sequence competed very poorly, not significantly reducing the SPA signal, while the wild-type sequence at lower concentrations competed very efficiently (Table 1C).

This assay was also used to measure the dissociation rate of DNA from p53. First, p53 was bound to the 3H-labeled DNA while monitoring the SPA counts for 100 min. Then, we added 10× or 100× of the unlabeled oligonucleotide and monitored the SPA counts for an additional 900 min. The counts obtained were compared with samples in which no additional DNA was added. The rate of loss of counts was substantial with the two different concentrations of excess unlabeled oligonucleotide (Figure 2B). Addition of 100× oligonucleotide resulted in a faster reduction in counts than when only 10× oligonucleotide was used.
min versus 220 min for 50% reduction in SPA counts, respectively) (Figure 2B). The experiments to show the rate of binding and dissociation of the p53-DNA complex would not generally be possible with the normal EMSA, while it was relatively easily accomplished using the SPA we developed. In some cases, mutant forms of the p53 protein may be altered in either the association or the dissociation kinetics with DNA. Those differences could easily be visualized with our approach and would not be as straightforward to detect with a typical EMSA.

Conclusion

Thus, using a baculovirus produced human p53, we have developed a DNA binding assay for this protein with SPA technology that rivals existing EMSAs. Since this assay can be performed in a microtiter plate, it is amenable to high-throughput screening and could be modified from the present 96-well format to the 384- or 1536-well formats if one uses another detection means like a charge-coupled device (CCD) camera (16,17). This is not possible for EMSAs. Other recently published assays for p53 DNA binding take an approach similar to that for ELISAs (19,20,25). Generally either biotinylated DNA is immobilized on plates coated with strepavidin, or the p53 is put on the plate through an antibody and bound biotinylated DNA is detected with strepavidin conjugated to an enzyme. These assays appear to have sensitivity similar to our assay, but require several incubation steps with washes in between, making the overall time much longer than with our SPA. One disadvantage of our assay is that it uses radioactively labeled nucleotides for the detection. But as these oligonucleotides can be labeled with the long lasting $^3$H, the experiments can be continued for a number of years. Also, to make this assay work efficiently, it requires a microtiter plate scintillation counter or luminescence reader that may not be available in the laboratory. However, we feel that the advantages for speed and versatility may outweigh those disadvantages for some researchers. In addition, this assay can have the DNA binding protein immobilized on the beads like with the ELISA-like approaches listed above. Immobilized proteins are not as accessible to the free DNA, making it somewhat more difficult to detect binding. This does not have to be the case with our method as we have generally combined the binding protein and the labeled DNA first and then immobilized the complex on the detecting SPA beads. Future applications for our SPA may be in screening human cell extracts and tissues for p53 DNA binding activity. If successful, it may become an alternative to immuno-cytocchemical methods to measure levels of p53. This type of SPA might also be incorporated into a direct screening format for compounds altering p53 DNA binding to identify drugs that could aid individuals with mutant forms of this tumor suppressor (1,2).

Using our SPA, one could screen for drugs that alter the DNA binding on- or off-rate separately. This DNA binding SPA could easily be adapted to other DNA binding proteins as long as an antibody to the DNA binding protein is available and the DNA sequence recognized has been identified. There is one report of using this assay to measure DNA binding of the transcription factor NF-κB from researchers in the Amersham Biosciences group that appeared prior to this work (26).

ACKNOWLEDGMENTS

The authors thank Koji Okamoto for baculovirus containing the human p53 gene, Nancy McGee and Gary Blizzard for helpful discussions on culturing the SF9 cells, Donna Avondet for culturing insect cells and measuring the level of p53, and Michael Blackburn for help preparing one of the figures.

COMPETING INTERESTS

STATEMENT

S.G. was given a grant between 1999 and 2000 by Amersham Biosciences (now GE Healthcare) to perform the research described in this manuscript, which included laboratory supply money and a consultancy fee. At this point, she has no scientific affiliation with the company and does not own stock in the company. At the time this research was conducted, both J.R.C. and L.H. were employed by Amersham Biosciences, which is now part of GE Healthcare.

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