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Detection of c-di-GMP-responsive DNA binding

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Abstract

Modulation of signal transduction via binding of the secondary messenger molecule cyclic di-GMP to effector proteins is a near universal regulatory schema in bacteria. In particular, direct binding of c-di-GMP to transcriptional regulators has been shown to alter gene expression of a variety of processes. Here, we illustrate a pulldown-based DNA:protein binding reaction to determine the relative importance of c-di-GMP in the binding affinity of a target protein to specific DNA sequences. Specifically, the pulldown-based assay enables DNA binding to be analyzed differing concentrations of c-di-GMP in the absence/presence of specific and non-specific competitors.

Keywords

cyclic di-GMP; transcriptional regulators; pulldown assay; immunoblotting; DNA binding

1. INTRODUCTION

Cyclic di-GMP is a secondary messenger molecule that plays a role in a wide array of regulatory processes. In the past few years, the turnover mechanisms for c-di-GMP, whereby synthesis occurs through diguanylate cyclases and hydrolysis through phosphodiesterases, have been well studied and characterized (1, 2). As attention has turned away from these mechanisms and towards uncovering downstream signal transduction pathways, it has become clear that binding of c-di-GMP to both proteins and RNA-based effectors is capable of modulating transcriptional, post-transcriptional, and post-translational processes (3). This is apparent by the recent identification of several transcriptional regulators being able to directly bind c-di-GMP, with c-di-GMP binding coinciding with differential expression of the respective target genes. In *Pseudomonas aeruginosa*, binding of c-di-GMP to the transcriptional regulator FleQ has been shown to reduce expression of genes associated with flagellar motility and relieve FleQ-mediated repression of exopolysaccharide biosynthesis genes (4). Additionally, in *P. aeruginosa*, the transcriptional regulator BrlR was found to directly bind c-di-GMP resulting in altered binding affinity for downstream DNA binding targets (5). The Clp protein in *Xanthomonas campestris* has also been shown to bind c-di-GMP and alter expression of genes linked to *Xanthomonas* virulence and motility (6, 7). As more c-di-GMP-responsive transcriptional regulators are uncovered, it will be beneficial to

have a variety of tools and procedures to verify the role of c-di-GMP in direct binding of the regulators to their DNA targets.

Here we describe a streptavidin-mediated pulldown assays to determine the role c-di-GMP in DNA binding of a protein of interest. As the assay is performed in the presence of increasing concentrations of c-di-GMP, the c-di-GMP concentration at which optimal DNA binding occurs, can be determined. Moreover, the assay can be performed in the presence of competitor DNA and other mono- and dinucleotide monophosphates, thus assisting in verifying the role of c-di-GMP in DNA binding of a particular protein. The streptavidin-mediated pulldown assay has been used by us to verify the importance of c-di-GMP in the binding of DNA by the transcriptional regulator BrlR in *P. aeruginosa* to several promoters of interest (5). The procedure itself is relatively straightforward, involving a DNA:protein binding reaction in the presence or absence of c-di-GMP, followed by a pulldown assay, SDS-PAGE, and immunoblotting. However, the method requires the use of a specific antibody be available for the protein of interest. In this procedure, we make use of a protein engineered to harbor a V5-tag and the commercially available anti-V5 antibody.

2. MATERIALS

All solutions are to be prepared in ultrapure water (purified deionized water to 18 MΩ-cm) and analytical grade reagents (unless otherwise indicated). All solutions are prepared and stored at room temperature (23°C) unless otherwise indicated.

2.1 Pulldown Assays

1. Cyclic-di-GMP (c-di-GMP). Dilute with water to a final concentration of 200 pmol/μl. Store at –80°C.
2. Streptavidin (SA) magnetic beads (Thermo Scientific, 100 μg). Store at 4°C
3. Magnetic stand suitable to hold microfuge tubes.
4. 10× Reaction buffer: 100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5. Store at –20°C.
5. EDTA: 200 mM stock solution, pH 8.0.
6. Protein: Cell-free protein extract harboring protein. (See Note 1)
7. Antibody specific to protein of interest. (See Note 1)
8. Biotinylated promoter DNA: 0.5 pmol of DNA of interest coupled to a biotin tag. (See Note 2)

¹To ensure that the protein of interest is detected in a specific manner by immunoblot analysis, it is preferable to engineer the protein of interest to harbor an antibody-specific tag. Based on our experience, a V5- or HA-tag is preferable, as anti-V5 (Life Technologies) and anti-HA (Covance) antibodies produce little to no background/cross-reactivity. These constructs are then placed into an appropriate expression vector and transformed into *E. coli*. Cells were grown to mid-exponential phase under inducing conditions, lysed via sonication, and subsequent debris removed following centrifugation at 21,000 ×g for 10 min at 4°C. Concentration of protein was determined by a Modified Lowry Assay. Concentration of protein of interest was assumed to be ~1% of total protein. Store lysates in aliquots at –20°C and avoid multiple freeze/thaw cycles.

²DNA sequence for a 5 prime biotin label was added to PCR primers used to amplify the promoter region of interest. Optimal length of resulting PCR products was between 100 – 400 nucleotides. The resulting PCR products were then cleaned up with a PCR cleanup kit and quantitated.

9. Competitor DNA: Use increasing concentrations (anywhere from 1× to 50×) of non-biotinylated promoter DNA. (See Note 3)
10. Non-specific competitors (optional): Other mono- or dinucleotide monophosphates such as cAMP or GTP can also be used in similar concentration ranges as c-di-GMP to verify specificity of binding in the presence of c-di-GMP. Store solutions at −80°C.
11. Tris-buffered saline (TBS): 0.1 M Tris-HCl, 0.9% w/v NaCl, pH 7.0. Store at 4°C
12. TBS containing 0.1% Tween-20 (TTBS). Store at 4°C
13. Thermomixer set to room temperature.

2.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 30% acrylamide/bisacrylamide (29:1). Store at 4°C.
2. Resolving gel buffer: 1.5 M Tris-Cl, pH 8.8. Weigh 90.85 g Tris-Cl and dissolve in approximately 300 ml water in a 500 ml glass bottle. Mix and adjust pH with concentrated (12 M) HCl (See Note 4). Bring up volume to 500 ml with water and store at 4°C.
3. Stacking buffer: 0.5 M Tris-HCl, pH 6.8. Weigh 6.06 g Tris-HCl and dissolved in approximately 80 ml water in a 250 ml glass bottle. Mix and adjust pH with concentrated (12 M) HCl (See Note 4) before bringing up final volume to 100 ml with water. Store at 4°C.
4. SDS: 10% solution in water. (see Note 5)
5. Ammonium persulfate: 10% solution in water. (see Note 6)
6. N,N,N',N'-tetramethylethane-1,2-diamine: Store at 4°C.
7. SDS-PAGE running buffer: 0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS.
8. 4× SDS-PAGE sample buffer: 0.048 M Tris-HCl, 8% SDS, 40% glycerine, 0.4 ml 2-Mercaptoethanol, 0.1% bromophenol blue. Aliquot and store at −20°C.

2.3 Immunoblotting

1. PVDF Membranes
2. Stacking Paper: Whatman filter paper cut to size of one gel. 10 pieces per gel.
3. Western blot transfer buffer: 100 ml SDS-PAGE running buffer, 100 ml ethanol, 800 ml water.

³Competitor DNA should be the same sequence as target DNA, just lacking the biotin label in the primer sequence. PCR, cleanup, and quantitation are performed as above. Additionally, nonspecific competitor DNA sequences can be used to verify specificity.

⁴12 M HCl can be used for initial pH adjustment to narrow large gaps between initial and target pH while lower ionic strength HCl can be used to adjust pH once a smaller gap has been obtained. This will help avoid sudden pH drops below the target pH value.

⁵SDS will precipitate out of solution if stored at 4°C.

⁶Prepare a fresh solution each time when casting a SDS-PAGE gel.

4. 95% ethanol
5. Tris-buffered saline (TBS): 0.1 M Tris-HCl, 0.9% w/v NaCl, pH 7.0. Store at 4°C
6. TBS containing 0.1% Tween-20 (TTBS). Store at 4°C.
7. Blocking solution: 1% BSA in TTBS. Store at 4°C.
8. Diluent Solution: 1% BSA in TTBS. Store at 4°C.
9. Antibody for pulldown: Anti-V5-HRP (Invitrogen). (See Note 1)
10. Clarity™ Western ECL Substrate (Bio-Rad).
11. Developer Solution: Kodak Professional developer. Prepare per manufacturer's instructions.
12. Fixer Solution: Kodak Professional fixer. Prepare per manufacturer's instructions.
13. X-ray film: CL-XPosure™ Film.
14. Three plastic containers.
15. Trans-Blot® Turbo™ Transfer system (Bio-Rad).

3. METHODS

Carry out all procedures at room temperature unless otherwise indicated.

3.1 Pulldown Assays

1. In a 1.5 ml microcentrifuge reaction, set up the binding reaction. Per 20 µl reaction, add increasing concentrations of c-di-GMP (1 – 50 pmol), 2 µl 10× reaction buffer, 0.2 µl of 200 mM EDTA, biotinylated promoter DNA (0.5 pmol) and cell extract (25 µg). (See Note 7) Bring up the reaction to a final volume of 20 µl with water.
 - a. The pulldown assay described above can be varied to include competitor DNA. To do so, set up the pulldown binding reaction (20 µl) using the optimal c-di-GMP concentration, 2 µl 10× reaction buffer, 0.2 µl of 200 mM EDTA, cell extract (25 µg), biotinylated promoter DNA (0.5 pmol), and increasing concentration of non-biotinylated competitor DNA. (e.g. 0.1 – 5 pmol). (See Note 8)
 - b. The pulldown assay described above can be varied to determine specificity of c-di-GMP binding, by include mono- and/or di-nucleotide

⁷All components should be thawed on ice prior to use. The concentration of protein lysate (or purified protein) required to obtain efficient DNA binding needs to be determined. This is best achieved by testing increasing amounts of protein. Each protein concentration will require a separate binding reaction to be set up.

⁸Competitor reactions allow for determination of specificity in the DNA binding reaction. A range of concentrations of competitor DNA should be utilized and an inverse correlation should be observed between concentration of competitor DNA and efficiency of DNA:protein binding.

monophosphates such as cAMP or GTP. These can also be used as competitors for c-di-GMP. To test for specificity, replace c-di-GMP with mono- and/or di-nucleotide monophosphates as follows: Per 20 μ l reaction, add increasing concentrations of mono- and/or di-nucleotide monophosphates (1 – 50 pmol), 2 μ l 10 $\times$ reaction buffer, 0.2 μ l of 200 mM EDTA, biotinylated promoter DNA (0.5 pmol) and cell extract (25 μ g).

2. Incubate the reaction mixture for 30 min at room temperature.
3. Binding reactions containing competitor DNA can be performed on reaction mixtures containing an optimal concentration of c-di-GMP.
4. Incubate the reaction mixture for 30 min at room temperature.
5. While waiting, prepare the SA magnetic beads by vortexing for 30 seconds.
6. Immediately after vortexing, aliquot 10 μ l of beads into a fresh 1.5 ml tube. Prepare 1 microfuge tube per pulldown reaction. (See Note 9)
7. Wash the beads twice: Add 200 μ l TTBS, vortex briefly, and collect the beads using a magnetic tube stand (~3 – 5 min) and remove the supernatant while the tube remains in the magnetic stand. Avoid pipetting up the magnetic beads. (See Note 10)
8. Combine the washed beads from step 5, the protein/biotinylated c-di-GMP reaction mixture from steps 1 – 2, and 250 μ l TTBS. Mix by pipetting.
9. Collect the beads using the magnetic stand by letting the tubes sit for ~3–5 min. Then, remove the supernatant.
10. Wash the beads by adding 1 ml TTBS, incubating for 2 min at room temperature at 1,400 rpm (thermomixer), collecting the beads using the magnetic stand (~3 – 5 min), and removing the supernatant. Repeat this step three more times.
11. Once the beads have been washed for times, beads can be stored at –20°C until further use. Otherwise, proceed to sample preparation for SDS-PAGE and immunoblotting.

3.2 Preparing 10% SDS-PAGE gel

1. In a small beaker, mix 2.5 ml 30% acrylamide/bisacrylamide, 1.88 ml resolving buffer, 75 μ l 10% SDS, and 3.5 ml water. Mix well by swirling or pipetting.
2. Add 50 μ l 10% APS and 5 μ l TEMED, then gently swirl to mix. (See Note 11)

⁹SA magnetic beads will quickly settle to the bottom of tubes. Make sure to vortex vigorously to ensure proper mixing.

¹⁰When placed in magnetic rack, SA magnetic beads should visually accumulate on the side of the tube closest to magnet. During wash steps, place pipette tip away from the beads to ensure minimal loss of beads while supernatant is removed.

¹¹SDS-PAGE gels will begin to polymerize once APS and TEMED are added to the solution. Have all pipets and material ready before adding. Once added, quickly cast gels.

3. Cast the resolving gel within cassette. Allow space for the stacking gel and gently overlay the resolving gel solution with water. Water can be added using a pipette or squirt bottle. (See Note 12)
4. Allow the gel to polymerize for 45 min. (See Note 13)
5. Once the resolving gel has polymerized, remove overlaid water from resolving gel in the casting cassette, and blot dry. (See Note 14)
6. Prepare the stacking gel by mixing 660 μ l 30% acrylamide/bisacrylamide, 1.25 ml stacking buffer, 50 μ l 10% SDS, and 3.0 ml water.
7. Add 35 μ l 10% APS and 10 μ l TEMED, then gently swirl to mix. Cast stacking gel on top of resolving gel and gently insert 10-well comb without introducing air bubbles. Let the gel sit for 60 min until fully polymerized.
8. Before loading the gel, gently remove the 10-well comb, and rinse out wells with water. Rinsing can be done using a pipette or squirt bottle.

3.3 Running SDS-PAGE gel

1. Resuspend the beads in 15 μ l of water.
2. Add 5 μ l 4 $\times$ SDS-PAGE sample buffer. Mix by vortexing.
3. Boil the sample for 10 min at 100°C. (See Note 15)
4. Remove samples and allow to cool to room temperature. Centrifuge at max speed for 1 min to collect liquid.
5. Collect the magnetic beads using the magnetic stand (~3 – 5 min).
6. Keeping the samples in the magnetic stand, use a pipette to carefully remove the entire supernatant. Avoid pipetting up the magnetic beads. Transfer the supernatant to a well of the prepared SDS-PAGE gel (section 3.2). Avoid pipetting up the magnetic beads. (See Note 16)
7. Run the SDS-PAGE gels at 100 – 150 V in SDS-PAGE running buffer for approximately 1.5 hours until the dye front has reached the bottom of the gel.

¹²Spacer plate dimensions (W $\times$ L) are 10.1 $\times$ 8.2 cm. Short plate dimensions are 10.1 $\times$ 7.3 cm. Final gel dimensions are 8.6 $\times$ 6.7 cm.

¹³Final concentration of acrylamide in resolving gel solution can be adjusted to obtain better separation of proteins depending on the size of the target protein. A 10% SDS-PAGE gel is suitable to resolve proteins having a molecular mass of 50–70 kDa proteins. A lower percentage acrylamide gel (8%) is preferable for larger molecular weight proteins (>70kDa) while a higher percentage SDS-PAGE gel (12%) may better separate proteins of lower molecular weight proteins (<50 kDa).

¹⁴Gel polymerization can be determined by gently tipping gel cassette to one side. The water should clearly separate from a solid gel mass. Excess water should then be poured off and any excess water removed with a paper towel.

¹⁵Boiling samples for 10 min in 1.5 ml tubes can result in the caps popping open, and subsequent loss of sample volume. Secure the caps with tape or a holder to prevent the cap from opening during boiling.

¹⁶A protein marker added to the SDS-PAGE gel should transfer to the membrane. If the marker contains visible dye, the prestained marker bands should be visible on the membrane following transfer. Detecting prestained marker bands on the membrane is usually a good initial indication of a successful transfer. If the ladder bands are not visible or distorted, an issue may have occurred during the transfer. Consult the manufacturer of the transfer apparatus for aid if needed.

3.4 Immunoblotting

1. Prepare three containers containing 95% ethanol, water, and blotting buffer, respectively.
2. Immediately following SDS-PAGE, turn off the power supply, and remove the gel from the unit.
3. Separate the glass plates holding the gel using a spatula.
4. Remove the stacking gel by separating it from the resolving gel with a plastic spatula and discarding it.
5. Carefully transfer the resolving gel to a plastic container containing Western blot transfer buffer and incubate for a minimum of 2 min at room temperature. Make sure that the SDS-gel is completely submerged.
6. Meanwhile, cut PVDF membrane to approximate size of the resolving gel.
7. Activate the membrane by submerging in 95% ethanol for 5 seconds, then transferring to water for 10 seconds, followed by complete submersion in Western blot transfer buffer for at least one minute.
8. While gel and membrane incubate in Western blot transfer buffer, organize stacking paper into two stacks of approximately 5 pieces each.
9. Briefly submerge one group of stacking paper in Western blot transfer buffer until wet throughout.
10. Place in transfer apparatus (Trans-Blot® Turbo™ Transfer system).
11. Gently lay PVDF membrane on top of stacking paper.
12. Place the resolving gel on top of the PVDF membrane.
13. Place the second stack of stacking paper, on top of the resolving gel.
14. Using a rolling pin, glass tube or similar device, gently roll out any air bubbles from between the layers. Start rolling from the center to the outside.
15. Place lid on transfer apparatus and run at 1.3 A and 25 V for 7 min. For two gels run together in the same transfer apparatus, use 2.5 A.
16. Prepare a fresh plastic container.
17. Following transfer, remove PVDF membrane and place the membrane in the fresh plastic container.
18. Block the membrane by adding ~10 ml blocking solution until membrane is completely submerged.
19. Incubate the membrane in the blocking solution at room temperature for 2 hours at 70 rpm.
20. Prepare 1:10,000 dilution of Anti-V5-HRP antibody in 10 ml diluent solution.
21. Following blocking step, pour off blocking solution.

22. Add antibody-containing diluent solution to the membrane.
23. Incubate at room temperature for 2 hours at 70 rpm.
24. Pour off diluent solution.
25. Wash membrane three times by adding ~10 ml TTBS, incubating for 10 min at room temperature at 70 rpm followed by pouring off of the TTBS.
26. Prepare Clarity™ Western ECL Substrate per manufacturer's instructions. Add to membrane and incubate covered from light for 3 – 5 min at room temperature.
27. Remove membrane from Western substrate, and place in plastic back. Roll out any air bubbles introduced.
28. Place the sealed membrane into a developing cassette.
29. Place X-ray on top of sealed membrane within the developing cassette and exposure X-ray for 1 – 5 minutes.
30. Briefly submerge x-ray film in developer solution and wait for bands to appear followed by quick rinse with water and complete submersion in fixer solution for a minimum of 2 minutes. Allow x-ray film to air dry.

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