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Detection of cyclic di-GMP binding proteins utilizing a biotinylated cyclic di-GMP pulldown assay

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Abstract

Cyclic di-GMP is an important regulatory messenger molecule that often directly interacts with proteins to alter function. It is therefore important to find potential c-di-GMP binding proteins and verify a direct interaction between them. Here, we describe a pulldown assay using biotinylated-c-di-GMP to capture a specific protein of interest followed by immunoblot analysis to determine relative protein abundance. This method also allows for addition of both specific and nonspecific competitors to determine specificity of c-di-GMP:protein binding. We also discuss using densitometry analysis on resulting immunoblots to calculate the dissociation constant (K_D) of the binding reaction, allowing for a determination of binding affinity.

Keywords

cyclic di-GMP; pulldown assay; Western Blot; immunoblot; biotinylated-c-di-GMP

1. INTRODUCTION

The secondary messenger molecule cyclic diguanosine monophosphate (c-di-GMP) regulates a wide variety of processes in response to both environmental and cellular signals. Modulation of c-di-GMP levels has been shown to play a vital role in the switch between the motile and sessile modes of growth, as well as regulating cell motility and antimicrobial tolerance (1–3). The near universal presence of c-di-GMP in bacteria underscores the importance of understanding its regulatory functions, in particular its downstream binding targets. Numerous approaches have been described to identify proteins capable of binding c-di-GMP, ranging from *in silico* bioinformatics approaches to affinity-binding pulldown assays (4–6). To date, many of these approaches have led to the identification of proteins involved with c-di-GMP turnover, including numerous diguanylate cyclases, involved in the synthesis of c-di-GMP, and phosphodiesterases, which play a role in its degradation (7, 8). However, identification and verification of downstream binding targets has remained challenging and hampered by predictive methods based on known motifs often failing to identify novel or previously uncharacterized c-di-GMP binding proteins. For instance, c-di-

GMP responsive transcriptional regulators include the *P. aeruginosa* flagella biosynthesis gene activator FleQ (9) and the *Mycobacterium smegmatis* LtmA that broadly regulates the expression of lipid transport and metabolism genes (10). Likewise, the *Vibrio cholerae* MshE protein (11) and the *P. aeruginosa* transcriptional regulator BrlR (12) have been demonstrated to bind c-di-GMP. While capable of c-di-GMP binding, these proteins lack regions that have a predicted secondary structure resembling those of known c-di-GMP binding regions, including the *P. aeruginosa* PelD protein, PilZ domains or the I-sites of diguanylate cyclases (4, 13–15). It is therefore important to have techniques at hand capable of easily verifying any potential binding targets.

Here, we describe a method using biotinylated c-di-GMP to identify potential target proteins. The method is based on pulldowns using streptavidin-coated magnetic beads. This approach is not designed as a broad screen to identify c-di-GMP binding proteins, as detection of c-di-GMP binding to the target protein is based on immunoblot analysis using antibodies specific to the target protein of interest. Instead, the pulldown approach makes use of an engineered/tagged candidate protein, allowing for verification of c-di-GMP binding to a protein of interest using pulldown assays and immunoblotting. In this way, potential c-di-GMP binding proteins can be easily and quickly verified. In addition, densitometry analysis using the western blots can be used to calculate binding kinetics, allowing for the comparison of binding affinities to known c-di-GMP binding proteins. Using this method, we have confirmed binding by c-di-GMP to the *P. aeruginosa* transcriptional regulator BrlR (12).

2. MATERIALS

All solutions are to be prepared using ultrapure water (18 MΩ-cm) and analytical grade reagents (unless otherwise indicated). All solutions are prepared and stored at room temperature (22°C) unless otherwise indicated.

2.1 Pulldown Assays

1. Biotinylated 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 containing protein of interest or purified protein of interest. (See Note 1)

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¹Cells were grown to mid-exponential phase under inducing conditions, lysed via sonication, and subsequent debris removed following centrifugation at 21,000 xg 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.

7. Unmarked c-di-GMP (Competitor): Prepare increasing concentrations (anywhere from $1 \times (200 \text{ pmol}) - 50 \times (10,000 \text{ pmol})$) of non-biotinylated c-di-GMP in water. Store solution at -80°C .
8. Non-specific competitors (optional): In addition to non-biotinylated c-di-GMP, other mono- or dinucleotide monophosphates such as cAMP or GTP can also be used in similar concentration ranges to verify specificity of binding. Store solution at -80°C .
9. Tris-buffered saline (TBS): 0.1 M Tris-HCl, 0.9% w/v NaCl, pH 7.0. Store at 4°C
10. TBS containing 0.1% Tween-20 (TTBS). Store at 4°C
11. 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 2). Bring up the 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 dissolve in approximately 80 ml water in a 250 ml glass bottle. Mix and adjust pH with concentrated (12 M) HCl (See Note 2) before bringing up final volume to 100 ml with water. Store at 4°C .
4. SDS: 10% solution in water. (see Note 3)
5. Ammonium persulfate: 10% solution in water. (see Note 4)
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 $\times$ 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.
4. 95% ethanol

²12 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.

³SDS will precipitate out of solution if stored at 4°C .

⁴Make this solution fresh each time SDS-PAGE gels are cast.

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)
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).

2.4 Immunoblot analysis

1. Scanner
2. ImageJ or other image analysis software for densitometry determinations

3. METHODS

Carry out all procedures at room temperature unless otherwise indicated.

3.1 Pulldown Assays

The assay is based on protein-ligand interactions, with immobilized biotinylated c-di-GMP being used to capture a protein of interest. The resulting bound protein is then detected by immunoblot analysis. In general, the protein concentration is increased while the concentration of c-di-GMP is being kept constant. A strategy for the set-up for this type of experiment and expected outcome is shown in Fig. 1A. Alternatively, the assay can be carried out at a fixed protein concentration but increasing c-di-GMP concentrations (Fig. 1B).

1. In a 1.5 ml microcentrifuge reaction, set up the protein/biotinylated c-di-GMP reaction. Per 20 µl reaction, add 1 µl biotinylated c-di-GMP (200 pmol), 2 µl 10X reaction buffer, 0.2 µl of 200 mM EDTA, and increasing concentrations of cell extract (1–10 µg). Bring up the reaction to a final volume of 20 µl with water.
2. For pulldown assays in the presence of competitors, see Method 3.2. To determine the dissociation constant K_D , refer to pulldown assays indicated in Method 3.3. Incubate the reaction mixture for 30 min at room temperature.
3. While waiting, prepare the SA magnetic beads by vortexing for 30 seconds.

4. Immediately after vortexing, aliquot 10 μ l of beads into a fresh 1.5 ml microcentrifuge tube. Prepare 1 microfuge tube per pulldown reaction. (See Note 5)
5. Wash the beads twice: Add 200 μ l TTBS, vortex briefly, and collect the beads using a magnetic tube stand ($\sim$ 3 – 5 min) and remove the supernatant while the tube remains in the magnetic stand. Avoid pipetting up the magnetic beads. (See Note 6)
6. 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.
7. Incubate for 1 hour at room temperature at 1,400 rpm using a thermomixer.
8. Collect the beads using the magnetic stand by letting the tubes sit for $\sim$ 3 – 5 min. Then, remove the supernatant.
9. 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 ($\sim$ 3 – 5 min), and removing the supernatant. Repeat this step three more times.
10. After the four washes, the beads can be stored at -20°C until further use. Otherwise, proceed to sample preparation for SDS-PAGE and immunoblotting.

3.2 Pulldown assays using competitors

Two kind of competitors can be used, specific competitors and non-specific competitors. For c-di-GMP binding proteins, unlabeled c-di-GMP can be used as a specific competitor to outcompete biotinylated c-di-GMP. A general strategy for the set-up for this type of experiment and expected outcome is shown in Fig. 1C. Non-specific competitors are those that do not compete with c-di-GMP binding. These may include cAMP or GTP. A general strategy for the set-up for this type of experiment and expected outcome is shown in Fig. 1D.

1. Set up the protein/biotinylated c-di-GMP reaction as described in step 3.1 using a single protein concentration but increasing concentrations of competitor.
2. Per 20 μ l reaction, add 1 μ l biotinylated c-di-GMP (200 pmol), protein extract (e.g. 5 μ g), 2 μ l 10 $\times$ reaction buffer, 0.2 μ l of 200 mM EDTA, and increasing concentrations of competitor (e.g. 0.4 – 10 $\times$ relative to biotinylated c-di-GMP, 50 – 2000 pmol). Bring up the reaction to a final volume of 20 μ l with water. (See Note 7)
3. Continue as outlined in 3.1, steps 2 – 11.

3.3 Pulldown assays for K_D determination

The dissociation constant K_D is commonly used to describe the affinity between a ligand (such as a c-di-GMP) and a protein, and more specifically, how tightly a ligand binds to a

⁵SA magnetic beads will quickly settle to the bottom of tubes. Make sure to vortex vigorously to ensure proper mixing.

⁶When placed in magnetic tube holder, 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.

⁷This reaction can also be set up using a fixed protein concentration and varying concentration of biotinylated c-di-GMP.

particular protein. Experimentally, assays aimed at determining K_D for c-di-GMP binding generally make use of increasing amounts ligand (c-di-GMP) that is titrated against a fixed amount of protein to determine the concentration at which an equilibrium in protein-ligand binding is reached. To do so, set up the protein/biotinylated c-di-GMP reaction as described in step 3.1 using a single protein concentration but increasing concentrations of biotinylated c-di-GMP.

1. A general strategy for the set-up of this experiment is shown in Fig. 1B.
2. Per 20 μ l reaction, add protein extract (e.g. 5 μ g), 2 μ l 10 $\times$ reaction buffer, 0.2 μ l of 200 mM EDTA, and increasing concentrations of biotinylated c-di-GMP (e.g. 50 – 1000 pmol). Bring up the reaction to a final volume of 20 μ l with water. (See Note 7)
3. Continue as outlined in 3.1, steps 2 – 11.

3.4 Preparing a 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 8)
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 9)
4. Allow the gel to polymerize for 45 min. (See Note 10)
5. Once the resolving gel has polymerized, remove overlaid water from resolving gel in the casting cassette, and blot dry. (See Note 11)
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.

3.5 Running SDS-PAGE gel

1. Resuspend the beads in 15 μ l of water.

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⁸SDS-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.

⁹Spacer 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.

¹⁰Final concentration of acrylamide in resolving gel solution can be adjusted to obtain better separation of proteins depending on size of target protein. A lower percentage acrylamide gel (8%) can be used for larger molecular weight proteins while a higher percentage gel (12%) may better separate out lower molecular weight proteins.

¹¹Gel 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.

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 12)
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.
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.

3.6 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.

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¹²Boiling 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.

14. Use a rolling pin, glass tube or similar device to 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 PFDV membrane and place the membrane in the fresh plastic container. (See Note 13)
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.

3.7 Densitometry of protein bands present on immunoblots

1. Scan the X-ray film. Save the image, preferably as a TIFF.
2. Use ImageJ software (or similar analysis tools) to measure the intensity of individual protein bands. Please note that the band intensity is a measure of the

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¹³A protein marker added to the SDS-PAGE gel should transfer to the membrane. If the marker contains visible dye, this should be visible on the membrane following transfer and can be 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 transfer apparatus for aid if needed.

fraction of protein bound to c-di-GMP. An example of a scanned blot is given in Fig. 2A. (See Note 14)

3. In addition to determining the intensity of bands, measure the intensity of the background of your scan. This value will be used to normalize your intensity measurements.
4. Subtract background intensity from band intensities.

3.8. Calculating the Dissociation constant K_D

The formula for determining K_D is as follows: $K_D = ([\text{ligand}] * [\text{protein}]) / [\text{protein-ligand complex}]$, with units for [ligand] and [protein] being concentrations (M, mM, mM, etc.). The units for K_D are concentrations (M, mM, mM, etc.). To resolve the equation, the concentrations of ligand, protein, and protein-ligand complex have to be known. However, considering that half-maximal binding of protein occurs when the free ligand concentration is equal to K_D , it is possible to estimate the K_D by inspection of the binding curve. The instructions on how to determine half-maximal binding are given below.

1. Determine the intensity of individual bands obtained from procedure 3.3.
2. Determine the concentration of c-di-GMP present in the pulldown assay. Convert pmol c-di-GMP used per pulldown assay (20 μ l reaction volume) to nmol/L (nM). For example, if you used a total of 200 pmol per 20 μ l reaction, first determine how many pmol are present in 1 L. To do so, use the following formula: $(200 \text{ pmol} * 50) = 1,000 \text{ pmol/L}$.
3. Convert pmol/L to nM. $1,000 \text{ pmol/L} = 1 \text{ nM}$. (See Note 15)
4. Graph data. There are several ways to plot equilibrium data of this kind. The easiest way is to plot the c-di-GMP concentration versus band intensity (obtained in step 3.7, band intensity is a measure of bound protein) using excel. This will generate a simple binding curve in which the fraction of bound protein is plotted vs. the concentration of c-di-GMP. If the reaction is a bimolecular equilibrium reaction, the binding curve will be similar to the hyperbolic binding curve shown in Fig. 2B.
5. From the graph, determine the band intensity at which an equilibrium in protein-ligand binding (maximal binding) has been reached (Fig. 2B, red dashed line).
6. From the graph, determine the band intensity at which half-maximal binding occurred (Fig. 2B, green dashed line). For instance, if maximal binding was noted at a band intensity of 16,000, half-maximal binding will be at a band intensity of 8,000 (Fig. 2B, green arrow).

¹⁴ImageJ is a free software and can be easily installed. Several websites are available containing step-by-step instructions on how to use ImageJ for densitometry. Two useful links are:

- a. <http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/>.
- b. <http://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf>

¹⁵There are several programs available online to calculate the conversion from pmol/L to nmol/L.

7. Next, determine the concentration of c-di-GMP at which half-maximal binding of the protein is apparent (Fig. 2B, orange arrow). In the given example, the concentration is 7 μ M. Thus, the K_D is 7 μ M.

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Figure 1. Schematic overview of pulldown assays

(A) Pulldown using biotinylated c-di-GMP (400 pmol) immobilized to streptavidin magnetic beads demonstrating that BrIR binds c-di-GMP. Increasing concentrations of V5-tagged BrIR were used (0, 1.25, 2.5, 5, and 10 μ g). (B) Pulldown using increasing concentrations of c-di-GMP (0, 5, 10, 25, 50 pmol) and a constant concentration of BrIR (5 μ g). (C) Pulldowns using a constant concentration of biotinylated-c-di-GMP (400 pmol) and a constant concentration of BrIR (5 μ g). An increasing amount of unlabeled competitor c-di-GMP was added (0, 400, 800, 2,000, and 4,000 pmol). (D) Pulldowns using a constant concentration of biotinylated-c-di-GMP (400 pmol) and a constant concentration of BrIR (5 μ g). An

increasing amount of unlabeled non-specific competitor (GTP) was added (0, 400, 800, 2,000, and 4,000 pmol). All figures adapted from (12).

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Figure 2. Overview of K_d determination

(A) Image of immunoblot showing increased presence of protein of interest following pulldown assays in the presence of increasing concentrations of c-di-GMP. (B) Graph showing the intensity of bound protein relative to c-di-GMP concentrations used in pulldown assays. Band intensities were obtained by analyzing the immunoblot shown in (A) by ImageJ. Maximal and half-maximal binding of protein to c-di-GMP is indicated by arrows. The K_d can be determined at the c-di-GMP concentration at which half-maximal binding occurs.