Understanding the binding interface of a structurally disordered protein

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Introduction

Chromosome segregation in Caulobacter

Bacteria multiply through binary fission, meaning that one parent cell divides into two daughter cells. Cell division is not limited to the physical division of the cell itself. It is important for each new cell to inherit a full copy of the bacterial chromosome. Thus, the process of cell division must also involve chromosome replication and subsequent segregation of each copy of the chromosome into daughter cells.. For rod-shaped bacteria such as C. crescentus (1), cell poles are the sites of assembly for regulatory proteins that control the time of DNA replication and chromosome segregation. (7)(6). Although bacteria are generally perceived as being simple, such complex anatomical organization suggests that even “simple” organisms require a high level of organization in order to divide efficiently (7). Polar structures are formed through the build up of “individual protein components" (7). This build up is made more complex by the fact that the structure and function of these poles changes during the progress of the cell cycle (5). This change in function is associated with the initiation of chromosome replication and the subsequent need for chromosome segregation (5). In *C. crescentus* specific proteins involved in the tethering of the chromosomes to their respective poles are critical in helping regulate this stage of the cell cycle (6). These proteins are ParA, ParB, and PopZ. The movement of the centromere during replication across the cell to anchor to the opposing pole is accomplished through these proteins and the complexes they make upon binding to one another (1).

Role of PopZ and ParB in chromosome segregation

As previously stated, PopZ and ParB serve an important purpose in segregating the chromosome copies in the pre-divisional cell. ParB works by binding to parS sites located near

the origin on the chromosome (1,2,5). This combination of protein and DNA creates what is known as the centromere(1). The protein PopZ then binds directly to the ParB protein while it is still bound to these parS sites (2,5). PopZ then anchors itself to the cell pole and tethers the centromere and chromosome along with it (5). This complex on both poles of the cell ensures that the chromosome copies in the dividing cell stay separated and are allocated into their respective daughter cells.

Role of PopZ and ParA in chromosome segregation

The protein ParA works to assist PopZ in chromosome segregation (4). After the chromosome has been fully copied PopZ then binds to the centromere to create a complex. Once this complex is created PopZ then binds to ParA which helps to taxi the chromosome copy to the other side of the cell so it can bind to the opposite pole (4). PopZ follows a gradient of ParA proteins plus ATP. As PopZ binds to the ParA proteins along this gradient towards the opposite cell pole it moves the whole PopZ-chromosome complex so it can anchor to the other side (4). This partnership is integral in the correct separation of chromosomes before cell division (4).

Comparison between Popz and other intrinsically disordered hub proteins

The specific protein PopZ is common in alphaproteobacteria but many aspects of this protein and its function are unclear. There seem to be orthologs similar to PopZ in other bacterial strains. In *M. gryphiswaldense* the knockout of PopZ ortholog, PopZ*Mgr*, results in the same growth and cell division defects as PopZ in Caulobacter (3). However, a PopZ ortholog cannot fully function when expressed in a different strain (3). Even proteins that are not direct orthologs of PopZ but have the same aspects and functions have been found. An example of this is a hub protein in gram-positive bacteria called DivIVA. DivIVA can self-assemble at the poles into a scaffold to interact with around 5 of its binding partners and participates in the tethering needed

to separate chromosomes (7). Another example is the transmembrane protein, HubP, which has a similar job in recruiting proteins including ParA. These proteins all share a common function, they bind to either ParA or ParB to relocate the centromere to the cell pole (7). Even though they perform the same task DivIVA, HubP, and PopZ are not coded by the same sequence. Their similarities lie in their structures, the C-terminal of PopZ and DivIVA both allow for the assembly of scaffolds to recruit proteins (7). The middle portions of PopZ and HubP are thought to both be intrinsically disordered regions.

Do intrinsically disordered hub proteins (PopZ) configure their structure to bind with different partners? And if so, which amino acids do both binding partners use in an intrinsically disordered protein to protein interactions? The goal for this project is to find the amino acids used by hub protein, PopZ, and binding partners, ParA and ParB. Using this information to gain a better understanding of how these protein-protein interactions occur between an intrinsically disordered protein and a binding partner.

Using a genetic screen was important because the binding sequence of PopZ exists within the first 70 amino acids. Random mutagenesis in this region should, hypothetically, produce a non-localizing PopZ mutant. Sequence comparison between the wild-type and mutant could pinpoint an essential amino acid for interaction. *E.coli* was chosen for the screen because it can easily accept genetic material during a transformation. It grows rapidly and expresses high levels of protein. The samples were grown in microwell plates so many could be screened, making the screen high-throughput.

Methods

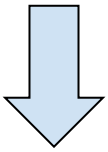
The purpose of the experiment is to find the binding region sequence in PopZ used for localization. So the first step is to construct the methods of the experiment. The main protein being studied using this screen is PopZ from Caulobacter. The binding partners being used are ParB and ParA also from Caulobacter. Plasmids that encode for PopZ and one of its binding partners can be inserted into an E. coli cell to be induced and expressed. Using a library of randomly mutated plasmids containing the sequence for PopZ and either ParB or ParA the binding region sequence can be narrowed down. Both proteins expressed in the E. coli cell will be tagged with a fluorescence so their location in the cell can be viewed underneath the microscope. Through the use of this tagging the randomly mutated plasmids can be transformed into e. coli, induced, and expressed. The plasmids that do not show binding under the microscope can be sequenced and compared to the original sequence to find the binding regions in PopZ.

To process the thousands of randomly mutated plasmids a 96-well plate assay will be used to grow and induce the E. Coli containing the plasmids. The first part of the experiment was set up to determine the optimal amounts, concentrations, and times for growth and induction in a 96-well plate. An array from 0.2% to 2% arabinose, for the induction of ParB, and 1Mm to 10Mm IPTG for the induction of PopZ.

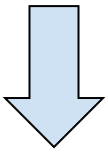
The screen consisted of the transformation of E. coli cells with the randomly mutated plasmid libraries of PopZ and ParB. After transformation the E. Coli cells are plated onto antibiotic plates that match the antibiotic resistant markers on the plasmids. The colonies from these plates are then picked off with toothpicks and grown and induced separately in the 96-wells on the well plate. After inducement the colonies in the well plate are viewed under the microscope to indicate by fluorescence whether the proteins are binding. If the proteins are shown not to bind in a well then that plasmid is sent to be sequenced for comparison.

Brief Summary of Screening Procedure

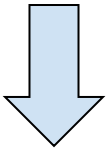
Transformation: PopZ and binding partner plasmids from the mutated library are transformed into E coli cells and plated



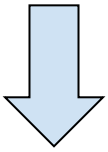
Plated cells are grown up in individual wells



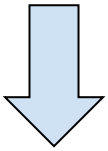
Inducement: the individual wells are induced with IPTG and arabinose to start the production of PopZ and binding partner proteins



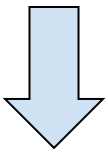
Induced strains are spotted on a microscope gel



Viewing: Fluorescence is viewed at the 40x objective on the microscope and used to determine whether the mutant proteins bind



Plasmid purification from non-localizing strains



Sequencing: plasmids purified from E coli are sent off to be sequenced for comparison

Results and Discussion

The separate screen to test for optimal growth time and inducement concentrations, amounts, and times was set up first. This revealed the ideal routine for growth and inducement to start with 100 microliters of LB in each well with the addition of antibiotics. With the colonies tooth picked off the transformation plates and individually mixed into the LB in each well. The optimal growth time for this 96-well plate was found to be overnight in a 30 degree incubator. After the growth period a second induction 96-well plate is set up. The ideals of this were found to be 50 micro liters of LB per well plus antibiotics. Along with 6 microliters of 2% arabinose and 14-16 microliters of 1MM IPTG per well. Optimal induction time was found to be 4-6 hours. These optimal induction times and amounts were found to produce the best microscope images for the next part of the experiment. However, a large gel to view all wells under the microscope was not compatible with the microscope. So the viewing method will consist of the construction of many regular sized microscope slide gels.

This first part of the overall experiment served the purpose of testing and constructing the specifics to serve as the methods for the second part of the experiment. This was necessary because a 96-well plate assay has never been done in this lab before so all the conditions and amounts needed to be perfected before moving on to the next part of the experiment. Now that the methods for the second part of the experiment have been established by these first screens,

the next experiments can be started. These next experiments include performing the screen and perfecting the transformation process with the mutated libraries.

The methods evolved throughout the screen, towards the end the 96 well plate was only used for the growth and organization/labelling of strains. The induction was done in tubes instead of in another well plate. The tubes were incubated for 4-6 hours and 0.5 microliters of 1 MM IPTG and 2.5 microliters of 2% arabinose were used. However, the transformation, growth, and microscope procedures remained the same. This proved to be a faster, more streamlined procedure. The strains induced in the microwell plate created too much variability in terms of the quality of the microscope pictures and fluorescence. The fluorescence from strains grown in tubes had better quality and less variability. To create this optimized induction procedure some grown conditions were tested including: incubation at 30 degrees versus 37 and time, 2,4,6, or 8 hours. Microscope pictures were taken every 2 hours of tubes induced and then grown in the 30 and 37 degree incubators. The pictures with the best quality were in the 30 degree incubator from 4-6 hours.

Overall, 373 colonies were screened. These strains all consisted of a PopZ library plasmid and a ParB or ParA library plasmid transformed into an E coli cell. Out of the 373 overall colonies, 297 had a PopZ library plasmid and a ParB library plasmid and 76 had a PopZ library plasmid and a ParA library plasmid. From the PopZ+ParB colonies, 19 were confirmed as non-localizing mutants, so 6.4 percent. From the PopZ+ParA colonies, 12 were confirmed as non-localizing mutants, so 15.8 percent. All mutants showed delocalized PopZ bound to ParB or ParA. None of the mutants displayed localized PopZ with delocalized ParA or ParB. Saturation would occur when the entirety of the libraries is screened for non-binding mutants, along with further sequencing of the confirmed mutants.

References

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