5-2007

Analysis of the interaction between the Cak1 kinase and the Cin5 transcription factor in budding yeast by bimolecular fluorescence complementation

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Abstract

Bimolecular fluorescence complementation (BiFC) will be applied to investigate the nature of the Cin5~Cak1 interaction *in vivo*. The method is based on the ability of interacting proteins to bring together their respective non-fluorescent fusion partners, the amino- and carboxyl- fragments of a fluorescent protein such as YFP ("YN" or "YC"). The interaction then results in regeneration of the fluorescent complex. We constructed plasmids to express fusions of the YN and YC domains to Cin5, under the control of the inducible promoter GAL1. In order to have an internal control for the fluorescent signal and facilitate the recognition of subcellular structures, strains expressing Nic96-YFP (nuclear membrane) or Bni4-YFP (bud neck) were also constructed.

Introduction

Cin5, also known as Yap4, is a bZIP family transcription factor which has mainly been studied for its functions in stress response (1). However, Cin5 was originally identified in a genetic screen for mutants that exhibit chromosome loss and sensitivity to mitotic inhibitors (CIN = **c**hromosome **in**stability), and this role remains uncharacterized (2). Cdc28 is the primary cyclin-dependent kinase (CDK) governing the yeast cell cycle. Random mutagenesis at the carboxy-terminus of Cdc28 produced mutants (*cdc28*^{CST}) which were temperature sensitive and deficient in maintaining **c**hromosome **st**ability (3). This loss of stability is associated with unregulated mitosis.

Previous studies have shown that Cin5 interacts with such a $Cdc28^{CST}$ mutant protein (Kitazono, unpublished results). Also, Cin5 has been found to moderately interact with wild type Cdc28, and very strongly with the Cdc28-activating kinase Cak1. On the other hand, it has been previously shown that high copy numbers of Cak1 suppress the temperature sensitivity and chromosome instability of the *cdc28*^{CST} mutants. The suppression was found to be independent of the kinase activity of Cak1, because both catalytically active and inactive Cak1 similarly suppressed the temperature sensitivity of *cdc28*^{CST} (3). These results suggest that Cin5 has a role in mitosis and the possibility that Cak1 regulates Cin5 function, stability and/or localization.

Using fusion or hybrid proteins as reporters of protein-protein interactions has been a standard technique in yeast studies for nearly 20 years. Fields and Song showed that splitting the inducible Gal4 transcription factor into the N and C termini and fusing the fragments onto proteins of interest could be used to report protein interactions that bring together the DNA-binding and activation domains of Gal4 (4). The development of this reporting method was seminal in the study of protein-protein interactions.

Methods of testing protein-protein interactions have become more sophisticated in recent years. In 2000, a powerful technique known as bimolecular fluorescence complementation (BiFC) was developed. Fluorescent proteins, such as YFP, are split into amino- and carboxyl- fragments ("YN" or "YC"), which are non-fluorescent, and these non-fluorescent fragments are fused to proteins of interest. If the two proteins that have

the YN and YC fragments interact, there is regeneration of the fluorescent complex. Even though yeasts were one of the first organisms used to study protein-protein interactions, BiFC was only recently introduced in yeast studies (2005), after the technique had been utilized with much more complex cells, such as mammals.

In order to determine the nature of the Cin5~Cak1 interactions *in vivo,* BiFC will be utilized. We aimed at using this system to determine the subcellular localization of the Cin5~Cak1 interactions. In order to do this, plasmids were constructed to express fusions of the YN and YC domains to Cin5, under the control of the inducible promoter *GAL1*. Cak1constructs were prepared separately. In order maintain an internal control for the fluorescent signal and facilitate the recognition of subcellular structures, the BiFC tests will be performed in strains expressing the localization markers Nic96-YFP and Bni4-YFP. Nic96 is a nuclear pore protein, and fusion to YFP allows for visualization of the nuclear membrane. Bni4 is a budneck protein.

Materials and Methods

Part I. BNI4 and NIC96

Plasmids, Strains, and General Yeast Methods

All experiments were performed in *MATa* haploid or *MATa/MATalpha* diploid cells (strains AKY3960 and AKY3963, respectively). The pDH6 plasmid was used as template to generate the YFP:kanMX6 cassettes to construct the *BNI4*-YFP and *NIC96*-YFP strains.

*Construction of BNI4-*YFP *and NIC96-*YFP

Primers were designed using the yeast resource center and GenBank and were purchased from Invitrogen. The PCR reactions include 1 uL of each forward and reverse primers (20 uM), 2 uL of plasmid DNA template (~100 ng), 1 uL taq polymerase enzyme (New England BioLabs), 5 uL 10x thermal buffer, 0.5 uL 25 mM dNTPs and 37 uL water. The PCR was run with a 3 minute initial denaturation step at 95 °C and twenty-five cycles of 55 °C for 30 seconds, 72 °C for a 3 minute and 30 seconds extension, 95 °C for 30 seconds and a final step of two-minute 72 °C.

AKY3960 and AKY3963 strains were transformed with 15 uL of each of the PCR products using standard Lithium Acetate techniques. Products were placed in the shaker and plated with 1 mL YPD on G418 plates to select for presence of the kanamycin-resistant gene. Large resistant colonies were then plated on fresh plates containing G418 to confirm phenotype. Genomic DNA was isolated from resistant colonies and used as templates for PCR tests to confirm correct integration of the YFP-kanMX6 cassettes.

Part II. Construction of plasmids harboring GalS>YC-CIN5 and GalS>YN-CIN5

A series of PCR steps were run to generate each of the DNA fragments (*GALS*, YC or YN and *CIN5*) and "fused" the respective pieces through their overlapping flanking regions. The final products (*GalS*>YC-*CIN5* and *GalS*>YN-*CIN5)* carry a *Sal*I and *BamH*I recognition site at the respective 5'- and 3'- ends.

PCR for GALS DNA fragment was run with 37 uL water, 5 uL 10x thermal buffer, 2.5 uL 100uM Magnesium Sulfate, 0.5 uL 25 uL 25 mM dNTPs, 1 uL Vent DNA

polymerase (New England BioLabs), 2uL of the Pringle's plasmid #16 for template, and 1uL each forward and reverse primers SA1GALsFW and GALsYCRV/GALsYNRV.

PCR for YC and YN DNA fragments were run using 37 uL water, 5 uL 10x thermal buffer, 2.5 uL 25 uM Magnesium sulfate, 0.5 uL dNTPs, 1 uL vent enzyme, 2 uL of the *pDH6* plasmid as template, and 1 uL each of the forward and reverse primers GALsCCFW/GALsYNFW and LINK CIN5RV.

PCR for CIN5 element was run using 38.9 uL water, 5 uL 10x thermal buffer, 0.5 uL 25 mM dNTPs, 0.2 uL vent and 0.4 uL taq polymerase enzymes, 3 uL of the isolated genomic DNA from part I as template, and 1uL each of the forward and reverse primers LINKCIN5FW and BamH1RV.

A gel was run to detect the presence of DNA fragments of the desired lengths for all three pieces of the construct.

Purification of DNA fragments

After running gels of the PCR products with all fragments comprising the construct, DNA fragments were excised from the gel. The fragments were melted at 60 °C in 600 uL of saturated sodium iodide solution, 25 uL celite suspension were added, and the mixes were incubated at room temperature for 15 min. After washing repeatedly, the celite resin was collected by centrifugation, and the supernatant containing washing buffer was discarded. 35 uL TE was added to the pellet, and the mixture was vortexed.

The solution was then incubated at 37°C for 5 minutes. The solution was centrifuged, and the supernatant was collected. Gels were run to confirm the presence of purified DNA.

Fusion-PCR of DNA fragments

PCRs were run to fuse the pieces GalS with YC/ YN and YC/ YN with Cin5. For the fusion of GalS with YC, 6 uL each of the purification product templates of GalS and YC, 5 uL 10x thermal buffer and 27.4 uL water were denatured in the PCR at 95 °C for five minutes and then immediately cooled on ice where 0.5 uL dNTPS, 0.2 uL of vent and 0.4 uL of taq enzymes, and 1 uL each of the forward and reverse primers Sal1GalSFW and LinkCin5RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C and extensions at 72 °C.

For the fusion of the YC fragment with the Cin5 fragment, 10 uL of the YC purification product template of YC and 2 uL of the Cin5 template, 5 uL 10x thermal buffer and 27.4 uL water were denatured at 95 °C for five minutes and then immediately cooled on ice. After cooling, 0.5 uL dNTPS, 0.2 uL of vent and 0.4 uL of taq enzymes, and 1 uL each of the forward and reverse primers GalSCCFW and BamH1RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C for three minutes, an elongation temperature of 72 °C for two minutes, and one minute at 95 °C.

For the fusion of GalS with YN, 5 uL of the purification product of GalS and 1uL of the purification product YN were used as templates, 5 uL 10x thermal buffer and 27.4 uL water were denatured at 95 °C for five minutes and then immediately cooled on ice where

0.5 uL dNTPS, 0.2 uL of vent and 0.4 uL of taq enzymes, and 1 uL each of the forward and reverse primers Sal1GalSFW and YNLinkCin5RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C for 3 minutes and an elongation temperature of 72 °C of 2 minutes.

For the fusion of the YN fragment with the Cin5 fragment, 10 uL of the YN purification product template of YN and 1 uL of the Cin5 template, 5 uL 10x thermal buffer and 27.4 uL water were denatured in the PCR at 95 °C for five minutes and then immediately cooled on ice. After cooling, 0.5 uL dNTPS, 0.2 uL of vent and 0.4 uL of taq enzymes, and 1 uL each of the forward and reverse primers GalSYNFW and BamH1RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C for three minutes, an elongation temperature of 72 °C for two minutes, and one minute at 95 °C.

A gel was run for each of the PCRs to detect DNA fragments of the desired lengths. Successful products for each of the fusions were then purified following the procedure outlined above.

PCR Fusion of Construct

The GalS fragment was fused with the YCCin5 fragment by the fusion PCR method. 3 uL of the GalS purification product template and 3 uL of the YCCin5 template, 5 uL 10x thermal buffer and 33.4 uL water were denatured in the PCR at 95 °C for five minutes and then immediately cooled on ice. After cooling, 0.5 uL dNTPS, 0.2 uL of vent and 0.4

uL of taq enzymes, and 1 uL each of the forward and reverse primers Sal1GalSFW and BamH1RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C and an elongation temperature of 72 °C.

For the fusion of GalSYNCin5, several combinations of prior PCR products were run for the best amount of DNA fusion products. A fusion of the GalSYN fragment with the Cin5 fragment was attempted. 5 uL of the GalSYN purification product template and 5 uL of the purified Cin5 template, 5 uL 10x thermal buffer and 33.4 uL water were denatured at 95 °C for five minutes and then immediately cooled on ice. After cooling, 0.5 uL dNTPS, 0.2 uL of vent and 0.4 uL of taq enzymes, and 1 uL each of the forward and reverse primers Sal1GalSFW and BamH1RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C and an elongation temperature of 72 °C. An additional PCR was run with the same specifications with the exception of an annealing temperature of 50 °C.

A fusion of the GalS fragment with the YNCin5 PCR product was also attempted. 6 uL of the GalS purification product template and 2 uL of the purified YNCin5 template, 5 uL 10x thermal buffer and 33.4 uL water were denatured in the PCR at 95 °C for five minutes and then immediately cooled on ice. After cooling, 0.5 uL dNTPS, 0.2 uL of vent and 0.4 uL of taq enzymes, and 1 uL each of the forward and reverse primers Sal1GalSFW and BamH1RV were added. The PCR was run for thirty-five cycles with an annealing temperature of 55 °C and an elongation temperature of 72 °C. Similar to the

previous PCR attempt, an additional PCR was run with the same specifications with the exception of an annealing temperature of 50 °C.

Gels were run to test for DNA constructs of the desired lengths for each of the fusion PCRs.

Ethanol Wash of Fusion Product GalSYCCin5

To ~150 ul of PCR product, 50uL of 7.5M Ammonium Acetate and 400uL of 100% ethanol were added. The solution was spun at 14,000 rpm for a total of 10 minutes. The supernatant was discarded and the pellet was washed three times using 70% ethyl alcohol (W/W). The solution was spun again at 14,000 rpm for three minutes, and the supernatant was discarded. The pellets were air-dried until all ethanol had evaporated (15 minutes). The construct was suspended in 50uL of TE solution, and a gel was run to determine that an acceptable amount of product was present.

Digestion of Vectors and DNA

YC products were cloned into the centromeric vector *pRS316* (allows selection in media lacking uracil) and YN products were cloned into the centromeric vector *pRS313* (selection in media lacking histidine). To digest the vectors, 10 uL of vector (~3 ug) was combined with 5uL 10x D buffer, 1uL each of the enzymes *Sal*I and *BamH*I, 0.5uL 10mg/uL bovine serum albumin, and 32.5uL water and was incubated in a 37 °C water bath for 2.5 hours.

To digest the GalSYCCin5 construct, 20uL of insert (precipitated PCR products), 5ul of 10x D buffer, 1uL *SalI*, 1uL *BamHI*, 0.5uL 10mg/mL bovine serum albumin, and 22.5uL water were combined and incubated for three hours in a 37 °C water bath. The insert was then placed in a 65 °C heating block for twenty minutes in order to deactivate the restriction enzymes.

Phenol Treatment of Vector and Ethanol Precipitation

For each of the two vectors *pRS313* and *pRS316*, to the 50uL of digested plasmid, 200uL TE and 100uL saturated phenol was added. The solution was vortexed for one minute and spun at 14,000 rpm for five minutes. The supernatant was then kept and 75uL of 7.5M Ammonium Acetate and 600uL cold 100% ethyl alcohol were added. The solution was placed on dry ice for ten minutes, and the solution was centrifuged and repeatedly rinsed with 90% ethyl alcohol.

Phosphatase Treatment

The treated vector was then combined with 5uL 10x phosphatase buffer, 1uL Antarctic phosphatase (New England BioLabs), and water to 50 uL. The solution was then incubated 37 °C for thirty minutes and placed in a 65 °C heating block to end the phosphatase activity.

Results and Discussion

Part I: Bni4 and Nic96

Products containing the *BNI4*-YFP:kanMX6 and *NIC96*-YFP:kanMX6 cassettes were successfully obtained (see Figure 1) and used to transformed haploid and diploid yeast cells. Visualization of subcellular localization was possible through the fluorescence microscopy (see Figure 2). However, the amounts of fluorescence present in the budneck due to Bni4 localization was quite weak although visible, whereas the fluorescence present in the nuclear pore caused by Nic96 was quite strong. This led to the decisions to construct diploid strains homozygous for *BNI4*-YFP and heterozygous for *NIC96*-YFP. To do this, it was first necessary to obtain strains of the opposite mating type. The obtained strains were then backcrossed and sporulated. The obtained haploids were then genotyped to determine presence of the kanMX6 cassette (resistance to G418) and mating type (formation of morphologically distinct zygotes when mixed with haploids of opposite mating type) (see figures 3 and 4).

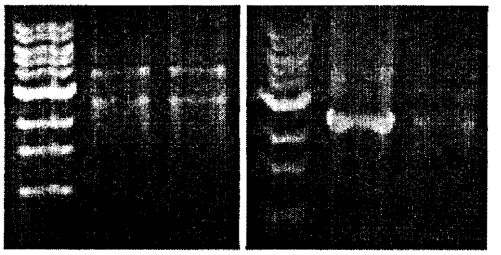

Figure 1. PCR products harboring the cassettes for tagging Bni4 and Nic96 (M = size marker)

Figure 2. Analysis by confocal microscopy confirms localization of Nic96 to the nuclear membrane.

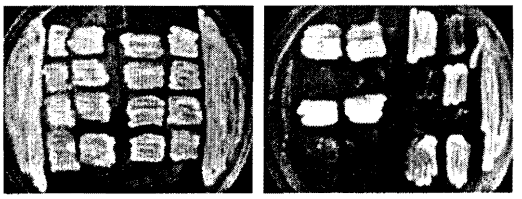

Figure 3. Comparison of Bni4 in YPD with KanMX6 Resistant Bni4

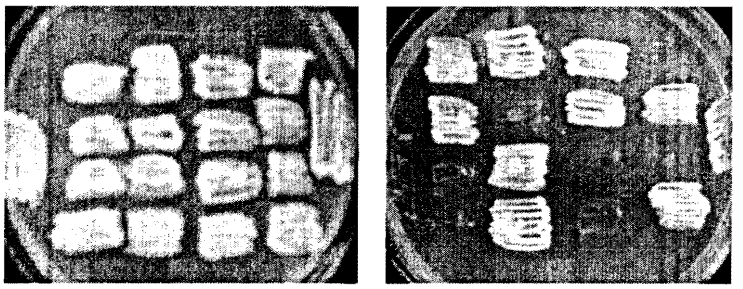

Figure 4. Comparison of Nic96 in YPD with KanMX6

Part II: Construction of BiFC Cin5 plasmids

Cloning and Purification of DNA Fragments

All parts of the Cin5 insert were successfully amplified by the PCR techniques listed above (see figure 5). The GalS promoter DNA fragment was successfully seen at the 500bp position on the gel run. The YC DNA fragment appeared at 200bp as expected, and the YN appeared at 400 bp as expected. Additionally, the Cin5 DNA fragment appeared around 1.4 kb on each gel.

Figure 5. DNA fragments prepared for fusion. Lanes 1 and 8 are 1kbp ladders. Lanes 2, 3, 9, and 10 are GalS fragments. Lanes 4 and 5 are YC fragments, and lanes 11 and 12 are YN fragments. Lanes 6, 7, 13, and 14 are Cin5 fragments.

The purification was also successful for all parts of the GalSYNCin5 construct. However, purification of YN had to be attempted several times in order to obtain the purified DNA. This was most likely due to the celite not being fully suspended. If this were the case, there would have been sufficiently low quantities of celite and the charged phosphate backbone of DNA would be unable adhere, resulting in loss of DNA.

Additionally, there were some unusual characteristics of the purification of the Cin5 fragment. After purification the gel lane for the Cin5 fragment still maintained two bands (Results not shown). This evidently did not effect fusion to YN because the YN fragment was successfully fused to the Cin5 fragment. However, it wase noted that the extra band persisted even after the fusion of Cin5 with YN (Results not shown).

Fusion PCR

The fusion of the YC and Cin5 DNA fragments was successful, and a band appeared at the expected position on the gel. The fusion of GalS with YC was never successful; that is why in the final fusion the GalS DNA fragment is fused with the YCCin5 PCR product. The complete fusion of the GalSYCCin5 insert was successful after several PCR attempts at different conditions (see figures 6 and 7). Those listed above in the materials and methods yielded the best amount of product. However, in all gels run for the fusion, there was some smearing of DNA in the gel (see figure 6). However, because of the brightness of the band at the expected position, the fusions with smearing were considered successful and sufficient for transformation.

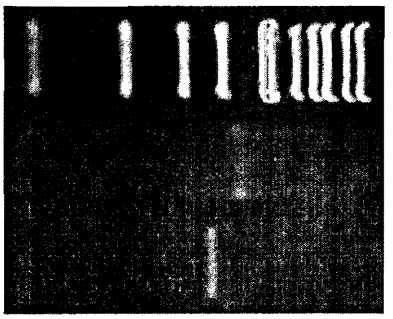

Figure 6. Fusion of GalSYC (1.4kbp) and GalSYCCin5

In the construction of the GalSYNCin5 insert, a complete fusion yielding a sufficient amount of DNA has not been made. However, the GalS promoter has been successfully fused with YN and YN and Cin5 have also

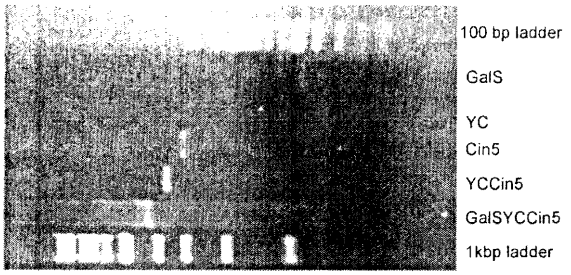

Figure 7. Complete fusion of GalSYC Cin5 Construct.

been fused (Results not shown). Additionally, a PCR to fuse the complete insert has been attempted, and some DNA of the expected length was extracted. Current experiments are being undergone to complete the fusion of the insert.

Cloning of GalSYCCin5 Insert into pRS316 Plasmid

The digestion of the vectors pRS313 and pRS316 was successful and yielded a large amount of DNA product (see figure 8). Digested vectors showed banding higher up and appeared slightly larger than their undigested counterparts.

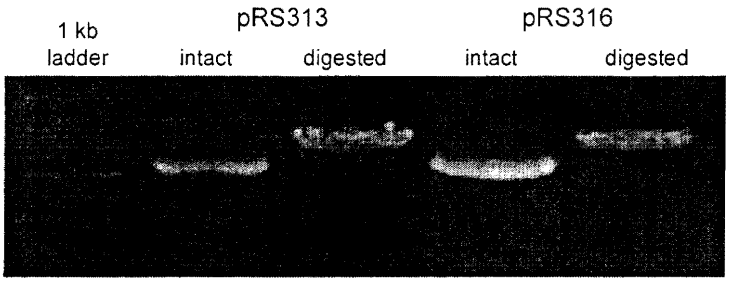

Figure 8. Intact and digested pRS313 and pRS316 vectors.

Digestion of the insert was also successful and a band was seen at the expected length (see figure 8). However, some DNA did not travel but remained the well where it was inserted. This is probably due to some ethanol still being present.

Phosphatase Treatment, Ligation, and Transformation

Despite the unusual gel for the digestion of the GalSYCCin5 insert, phosphatase treatment, ligation, and transformation were continued, and the products of these

processes were plated (see figure 9). As is noted on the figure, blue colonies indicate presence of intact vector and white colonies suggest probable presence of insert. The results are unusual in that a very high number of blue colonies are present, and a small number of white colonies appear. Because the results differ from the expected, the evidently transformed colonies are being viewed as false positives, and current experimentation is being made to ensure that a full transformation has occurred before the construct is used in further experimentation.

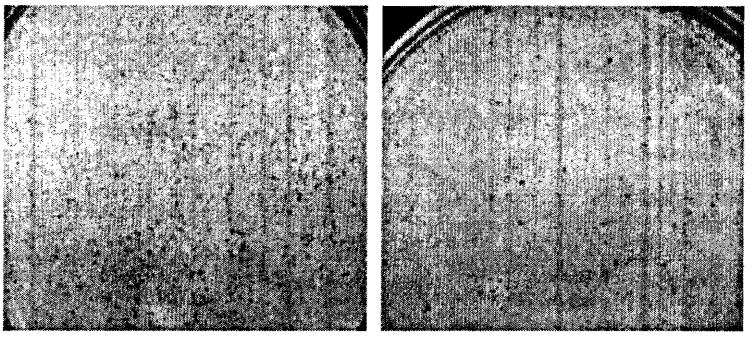

Figure 9. Transformation of GalSYCCIN5 construct. Blue Colonies indicate an intact vector, whereas white colonies suggest insertion of the construct.

While in some aspects the experiment involving the construction and transformation of GalSYCCin5 and GalSYNCin5 had unexpected results, the overall experiment was successful in streamlining the procedure used to develop and transform the inserts. Following completion of this phase of the overall experiment, the construct will be used to in the BiFC to further understanding regarding the interaction of Cin5 and Cak1.

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Acknowledgments

We would like to thank the University of Tennessee for funding our research. A special thanks goes to Ana Kitazono for her invaluable guidance through the whole process, and special thanks also go to Su-Hwa Kim who also guided us and who is working on the sister project of our own, the development of Cak1-YFP construct.

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