Functional Dissection of the Central Domain of c-Myc through Characterization of Novel Interacting Proteins

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KEYWORDS. c-Myc, yeast two-hybrid assay, cancer

BRIEF. Identifying protein-to-protein interactions within the central domain of c-Myc.

ABSTRACT. c-Myc is an oncoprotein transcription factor that controls the expression of genes necessary for cell growth and hyperproliferation. Overexpression of c-Myc, or expression at inappropriate times, triggers cells to grow irrepressibly—resulting in tumorigenesis. Generally, c-Myc can be partitioned into three fundamental domains: an amino-terminal transcriptional-activation domain, a carboxy-terminal DNA-binding domain, and a central domain of unknown function. Recent evidence reveals that the central domain of Myc plays an important role in regulating gene expression, controlling Myc by proteolysis, and in oncogenic transformation, but the underlying molecular mechanisms are unknown. To address this deficit, a two-hybrid assay was used to identify a number of novel proteins that specifically interact with the central domain of c-Myc. A quantitative two-hybrid analysis was performed, with the objective of prioritizing molecules that interact most robustly with this central domain. This research identified three proteins that bind to the central portion of Myc with apparent high affinity and specificity. Interestingly, these proteins have established links to gene expression, cell cycle progression, DNA repair, and apoptosis, suggesting that they may be involved in known functions of the c-Myc central domain. Future studies will expose the connection between these c-Myc interactors and their function in mammalian cells.

INTRODUCTION.

Contrary to common belief, cancer is not just one disease; rather it is a set of different diseases each triggered by a variety of causative agents [12]. Most normal cells undergo a programmed cell death, known as apoptosis. However, cancerous cells lose their ability to respond to signals that trigger apoptosis, and therefore grow uncontrollably. Genes that possess the potential of causing cancer, oncogenes, can alter the cell's ability to undertake apoptosis, forcing them to grow and multiply hysterically. One example is c-Myc (cellular myelocytomatosis), a regulator gene that codes for a transcription factor that controls the expression of genes necessary for cell growth as well as hyperproliferation [1].

Importantly, c-Myc is a human oncoprotein that contributes to the development of an assortment of human cancers such as prostate cancer, lymphoma, breast cancer, and leukemia [2–5]. This variation in c-Myc expression has contributed to approximately one-third of all cancer fatalities each year [6].

This oncoprotein contains three fundamental regions that simultaneously work together to make it activate. The two well researched regions are the amino terminal which contains the transcription activation domain (TAD) and a carboxyl-terminal involved in DNA binding. However, the central portion of c-Myc has been poorly studied; therefore, it stands as a region of unknown function.

Numerous proteins have been identified to interact with the c-Myc oncoprotein, with the exception of its central domain. It was previously hypothesized that this central portion of c-Myc does not contribute to its function, but recent research has indicated that this region is involved in an abundance of activities that contribute to the transformation of normal cells into cancerous ones. Thus, the central portion of c-Myc is required for oncogenic transformation, both in cell culture and mouse systems, but the underlying molecular mechanisms are still unknown [8].

Following on that previous research, the purpose of this study was to address the anonymities in the central region using a two-hybrid screen and elucidate the molecular mechanisms through which this region functions. The two-hyrbrid screen would identify protein-protein interactions with the central region of c-Myc. Yeast cells have also shown to mimic functions that would be hypothesized in mammalian cells. After the proteins that robustly bind and interact within this region of c-Myc are identified, the connection of these c-Myc interactors to their function in mammalian cells will be discovered.

This would be important because it would establish links between the oncogenic capacities of c-Myc and its ability to alter apoptotic response in cells. Ultimately, research would be capable of selectively inducing certain functions in cancer cells, such as apoptosis, by interfering with the region that is responsible for that function. Therefore, establishing the functions of the central region of c-Myc stands as a crucial step in understanding some of these underlying molecular mechanisms.

MATERIALS AND METHODS.

Yeast Two-Hybrid Assay Overview.

The yeast two hybrids simultaneously introduce bait and prey plasmids to test for physical binding between target proteins. In the yeast strain DY6877, lacZ gene expression is controlled by a promoter that contains LexA-DNA binding sites; therefore, if interaction between the bait (Myc), and the prey (potential binding partners) occurs, lacZ will become transcriptionally activated leading to the production of the lacZ gene product, β-galactosidase.

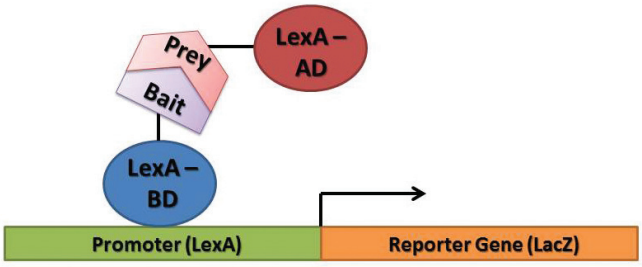

Figure 1. Yeas t Two-Hybrid System. Overview of the assay, checks for interactions between two proteins (Bait and Prey). If the bait and prey proteins interact, transcription of the reporter gene occurs. However, in the case that they do not, transcription will not be activated.

Plasmid Preparation.

To isolate and purify the plasmid DNA from the total of twelve pP6 plasmid and pB27 plasmid bacterial clones, a mini-preparation was performed using the Wizard ® Plus SV Minipreps Wizard ® Plus SV Minipreps DNA Purification System to obtain routine DNA yields [9]. Higher DNA yields were obtained through the performance of midi-preparations using the Wizard ® Plus SV Midipreps Wizard ® Plus SV Midipreps DNA Purification System. The pB27-Empty and pB27-Myc plasmids, (151-319 aa) as well as the pP6 clones (later transformed into the yeast strains with LexA binding domains) were created by the Hybrigenics Company as part of the Yeast Two-Hybrid System Service.

Mutant Identification and Validation.

A restriction enzyme digestion confirmed the identity of each of the DNA plasmids before they were used for further analysis. A double digestion was performed on the pB27 and pP6 plasmids due to the high number of samples.

The restriction enzymes PstI and MluI were used for the pB27 plasmids. The enzymes XhoI and MluI were used for eight of the pP6 plasmids, while the pP6-POU2F1 required the enzymes KpnI and NcoI in order to visualize the proper plasmid length compared to the known length of the plasmid. After one hour incubation in a 37°C water bath, gel electrophoresis with a 1.5% agarose gel was performed to visualize the DNA bands for analysis (*Supplement 1).*

Transformation.

The yeast strain DY6877 with LexA-LacZ operators was grown in YPAD (Yeast Extract-Peptone-Adenine-Dextrose) growth medium. Next, 5µg of the isolated plasmid DNA of the pB27 plasmids and the pP6 plasmids were introduced into the mutant yeast strain DY6877. The pB27 Myc and Empty served as the bait plasmids, and the pP6 clones served as the prey plasmids for the Yeast-Two Hybrid System. The transformations that contained both the pB27 and pP6 plasmid grew on CSM trypsin and leucine dropout plates in order to inhibit the growth of yeast cells without the plasmid so that the ones with the plasmid will proliferate and be identified by naked eye.

Table 1. Transformation. This table displays the combinations of bait and prey plasmids that were introduced into the mutant yeast strain DY6877.

Experimental	Control
pB27-Myc/pP6-Empty	pB27-Empty/pP6-Empty
pB27-Myc/pP6-MLL5	pB27- Empty /pP6-MLL5
pB27-Myc/pP6-MBD4	pB27- Empty /pP6-MBD4
pB27-Myc/pP6-SFRS15	pB27- Empty /pP6-SFRS15
pB27-Myc/pP6-POU2F1	pB27- Empty /pP6-POU2F1
pB27-Myc/pP6-WSB2	pB27- Empty /pP6-WSB2
pB27-Myc/pP6-SMARCC1	pB27- Empty /pP6-SMARCC1
pB27-Myc/pP6-ZNF93	pB27- Empty /pP6-ZNF93
pB27-Myc/pP6-RARγ	pB27- Empty /pP6-RARγ

β-galactosidase Assay.

This assay indirectly reflects the level of interaction between the bait (Myc) and prey plasmids, the potential partner proteins, in the yeast two-hybrid system by determining the amount of β-galactosidase enzyme activity present in yeast cells that contain the pB27 bait and pP6 prey plasmids. The level of β-galactosidase activity in the cells is determined by measuring the appearance of yellow color, with respect to time, due to the hydrolysis of o-nitrophenyl-D galactoside (ONPG) by β-galactosidase. The synthetic compound ONPG is recognized as a substrate and cleaved into galactose and o-nitrophenol which contains the yellow color. The absorbance levels at 420 nm were determined using a spectrometer in order to assess the intensity of the yellow color produced after the assay. Mathematical analysis of the optical density and absorbance levels established the β-galactosidase activity. In order to calculate the β-Galactosidase activity, the absorbance levels, the time it took for the yellow color to form due to the hydrolysis of ONPG, the volume of the reaction, and the optical density were utilized in the equation displayed.

Equation 1 $$\frac{1{,}000 \times A420}{time \times Volume \times OD600} = \textbf{β-galactosidase Activity}$$

RESULTS.

Transformation.

Before the β-Galactosidase Assay was performed, the bait (pB27 plasmid) and the prey (pP6 plasmids) were introduced into the mutant yeast strains. Numerous colonies were apparent on all of the transformation plates. Colonies were taken from each of the transformation plates and restreaked onto new ones in order to isolate genetically pure colonies. The colonies from the restreaked transformations grew successfully as well.

Yeast Two-Hybrid Assay.

The β-Galactosidase Assay measured the absorbance levels at 420 nm emitted by the interaction between each bait and prey plasmid. Typically, for a significant amount of β-Galactosidase activity to be apparent, the absorbance levels should range from 0.4-1.2. However, in some cases, the reaction time was unexpectedly low, which also produced a high value of β-Galactosidase activity. The β-galactosidase assay also confirmed that there was activity present through the display of absorbance after the assay. The absorbance levels of the pB27 bait plasmids were all significantly different ($p<0.05$) from the control pB27 empty vector plasmids for each of the potential partner proteins, except for the pP6 empty control ($p>0.05$) (figure 2). Two proteins, MBD4 and SFRS15 fall within the intended range of absorbance in order to further determine the β-galactosidase activity. However, the absorbance levels only display the intensity of the yellow color produced by the hydrolysis of ONPG. Time, volume, and initial optical density are taken into account when determining the levels of interaction.

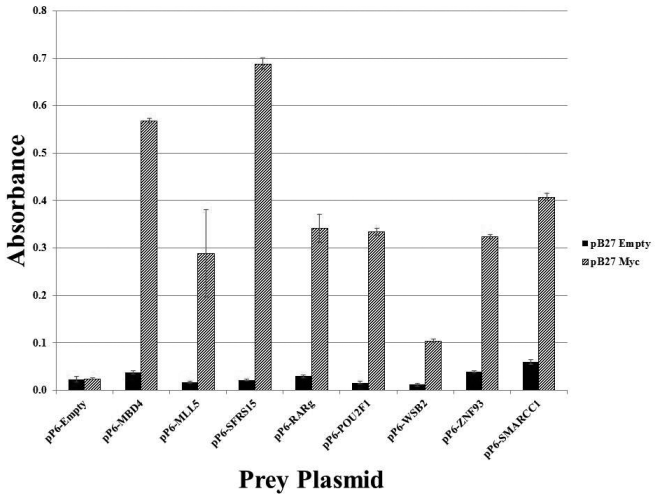

Figure 2. β-Galactosidase Assay Absorbance. The absorbance levels emitted by the different proteins after the assay. This helps predict the proteins that potentially robustly reacted with c-Myc before performing the computation of β-Galactosidase activity.

Further mathematical analysis computed the β-Galactosidase activity that was emitted by the interaction between the bait and prey plasmids. In order to determine if there is an interaction between the central region of c-Myc and the potential partner proteins, the β-Galactosidase activity of the pB27 Myc plasmid has to be significantly greater than the activity of the pB27 empty plasmid. Three proteins fit these criteria. The β-Galactosidase activity of the bait pB27 Myc plasmid was significantly different ($p<0.05$) from the activity of the pB27 empty plasmid for these three prey potential partner proteins, which were MBD4, SFRS15, and POU2F1 (figure 3). This indicates that there is an apparent interaction between the c-Myc plasmid and the proteins MBD4, SFRS15, and POU2F1.

DISCUSSION.

The purpose of this project was to further understand some of the unknown functions of the central region of c-Myc, which spans amino acid residues from 151-319. The yeast two-hybrid system identified three proteins that bind to this region with high affinity and specificity. The proteins SFRS15, MBD4, and POU2F1 displayed relatively high levels of interaction within this central domain of c-Myc, and interestingly, they have established links to gene expression, DNA repair, apoptosis, and cell cycle progression. The identity of these proteins, and their known roles, suggests that at least some of them will be relevant to c-Myc function in cancer. Prior research showed that the central region of c-Myc contains a highly-conserved region, known as "Myc Box III", which is a negative regulator of programmed cell death [8]. This conserved region has been shown to cause oncogenic transformation, both in cell culture and mouse system [8]. Consequently, this means that the central region of c-Myc is capable of transforming a normal cell into a cancerous one. However, previously, no studies addressed the proteins that could bind in this region as well as other anonymities.

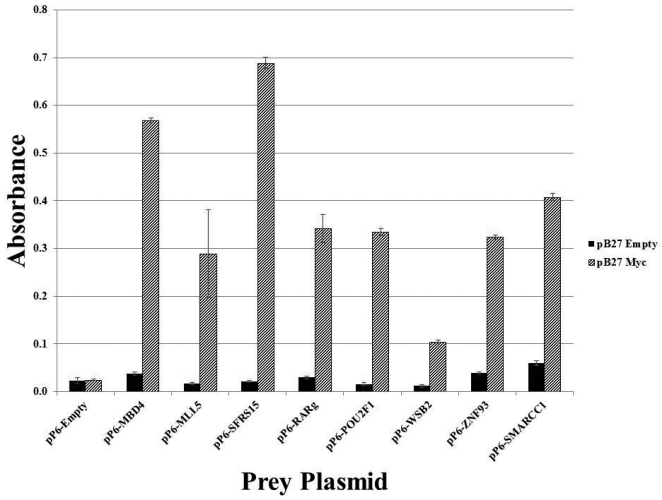

Figure 3. Interaction Levels between c-Myc and Potential Partner Proteins. β-Galactosidase Activity. Schematic overview of the proteins which displayed significant levels of interaction within the central domain of c-Myc. These interactions have incorporated the factor of time, volume, as well as optical density in order to display the interaction levels between the c-Myc protein and its potential binding partner.

The proteins identified importantly contain functions that relate to the potential formation of cancer. Identifying proteins such as MBD4 that are linked to apoptosis and interact within the central region of c-Myc could further explain the functions of this region of the protein [9]. The protein MBD4 is known to specifically bind to methylated DNA and contributes to functions of DNA repair. When cells begin to lose their ability to repair damage to their DNA, cancer often begins to form [10]. Since MBD4 has been identified to bind in this central region, further research in mouse systems could identify whether the function of MBD4 in DNA repair plays a vital role in the function that is exhibited by this central region of c-Myc. Furthermore, SFRS15 has been identified as an RNA binding protein that has been physically and functionally linked to transcription while POU2F1 is demonstrated to function as a transcription factor [11], [12].

The three proteins that have been identified to interact within the central region of c-Myc should be further studied in order to determine if these interactions hold true within mouse systems. Identifying and validating the proteins that bind within this central region of c-Myc in mouse systems will allow us to generate hypotheses about the molecular function of this region of the protein in humans. Since the functions of c-Myc have been prominently identified to feature in numerous human cancers, understanding its function will help explain its role and overexpression in numerous strains of cancer.

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