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Mapping the Interaction Between Two *Toxoplasma gondii* Cell Cycle Proteins.

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Mapping the Interaction Between Two *Toxoplasma gondii* Cell Cycle Proteins.

by

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Abstract

Toxoplasma gondii belongs to the phylum the Apicomplexa, a phylum that mainly consists of difficult to study intracellular protozoan parasites. The zoonosis caused by *T. gondii* is called toxoplasmosis. Even though most healthy individuals infected will be asymptomatic, a *T. gondii* infection can be lethal for individuals with a compromised immune system. Non-specific drugs exist to treat toxoplasmosis, but these are typically not able to eliminate *T. gondii* completely from the infected host and infection reoccurs. The proteins involved in *T. gondii*'s unique cell cycle are considered prime targets for the development of new anti-parasitic drugs. However, in order to target vital and unique *T. gondii* proteins these must first be identified and their interactions characterized. One such potential protein would be the putative *T. gondii* cell cycle protein TgCyc2. TgCyc2 has previously been found to interact with TgIMC1 and TgCDK1 in a yeast two-hybrid screen. It is hypothesized that TgCyc2 targets TgCDK1 to a specific suborganellar location by binding to the inner membrane protein TgIMC1. Here an initial attempt to elucidate this mechanism via a yeast two-hybrid test is described.

Acknowledgments

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Chapter 1: Introduction

The Apicomplexa and *Toxoplasma gondii*

Over 6000 protozoan species belong to the phylum called the Apicomplexa and a majority of them are described as intracellular parasites (Morrison, 2009). Most of the Apicomplexa parasites share similar features including a subpellicular network (SPN), rhoptries, micronemes, apical polar rings, the apical complex, and an inner membrane complex (IMC) (Morrison, 2009; Morrissette & Sibley, 2002).

One prevalent and commonly studied Apicomplexa is *Toxoplasma gondii*. *T. gondii* has a worldwide distribution and high prevalence in the human population (Tenter, Heckeroth, & Weiss, 2000). This parasite causes the disease toxoplasmosis. However, the majority of healthy individuals will either be asymptomatic or only have minor flu symptoms when they become infected. The situation is worse for vulnerable populations exposed to *T. gondii*. The immunocompromised can suffer reoccurring infections because current drugs are unable to clear the infection and the infection can lead to life threatening encephalitis (Tenter et al., 2000).

A second vulnerable population is pregnant women or more specifically their unborn children. If a mother becomes infected for the first time *T. gondii* can be passed from the mother to the unborn child, which can cause birth defects or in severe cases death (Tenter et al., 2000). Additionally, *T. gondii* can cause rare events of ocular disease in healthy individuals and studies are investigating the association between *T. gondii* infections and mental health disorders (Wilking, Thamm, Stark, Aebischer, & Seeber, 2016).

Other clinically and agriculturally important Apicomplexans include the *Plasmodium* species responsible for malaria, the *Eimeria* species, which infect poultry and cattle, and *Theileria* species, which are tick borne cattle pathogens (Morrissette & Sibley, 2002). There is a

lack of effective treatment options for many of these parasites, including *T. gondii* and the *Plasmodium* species. One area of focus among researchers is to study the Apicomplexa's cell cycle process to find new drug targets (Wei, Wang, & Liu, 2013).

Unique Cell Cycle Processes

The Apicomplexa are characterized by unique cell cycle processes, which differ from the typical eukaryotic process of mitosis. Three different Apicomplexa cellular division mechanisms have been described endodyogeny, schizogony and endopolygeny (Francia & Striepen, 2014). In humans *T. gondii* exists in what is known as the tachyzoite stage. The tachyzoite proliferate via endodyogeny and a key virulence factor for *T. gondii* is proliferation (Gubbels, White, & Szatanek, 2008). *T. gondii* utilizes endodyogeny when it is inside an intermediate host i.e. in humans, but can also reproduce via schizogony, which it does in the cat intestine (Dubey, Lindsay, & Speer, 1998; Francia & Striepen, 2014).

Endodyogeny has been described as the simplest cell cycle variation within the Apicomplexa with schizogony, utilized by *Plasmodium* species among others, and endopolygeny as more complex variations (Francia & Striepen, 2014). The ability of *T. gondii* to switch between the process of endodyogeny and schizogony could be considered a key to its success and the ability to switch also indicates that there is an intricate process for controlling cell cycle progression (Francia & Striepen, 2014; Gubbels et al., 2008).

Considering that the Apicomplexa life cycles are overlapping studying the molecular mechanisms for one species in the phylum can lead to insight into several others (Ferguson et al., 2008). Endodyogeny and schizogony differ mainly on three accounts: the amount of progeny made (two for endodyogeny versus sixteen to a hundred for schizogony), if progeny

development goes via a cytoplasmic or the peripheral route, and the intact or loss of specialization of the mother cell (Gubbels et al., 2008; Morrissette & Sibley, 2002).

A comparative approach to learn about the Apicomplexa occurs relatively frequently in the literature. For instance protein homologues of MORN1 were followed in developing *T. gondii*, three *Eimeria* species, and *Plasmodium falciparum* and implicated in IMC formation for all three (Ferguson et al., 2008). *T. gondii* is often considered to be a model organism for the Apicomplexa because of its relative ease of manipulation in the laboratory setting as well as its division via endodyogeny (Kim & Weiss, 2004).

Regulation of Endodyogeny

Entry into endodyogeny by *T. gondii* is marked by duplication of Golgi and centrioles in the G1 phase (Gubbels et al., 2008). At about the same time point DNA replication begins (Hu et al., 2002). One checkpoint during endodyogeny is suggested to be present between G1 and S termed START (Gubbels et al., 2008). Another potential checkpoint could be present during chromosome replication (Gubbels et al., 2008). The G2 phase commonly found in other eukaryotic organisms is thought to be either short or not present at all during the *T. gondii* cell cycle (Gubbels et al., 2008).

A variety of different regulatory elements are thought to be control *T. gondii* endodyogeny. Cyclin dependent kinases (CDKs) and cyclins are main regulators of the typical eukaryotic cell cycle process and have also been found to have regulatory roles in the Apicomplexa (Wei et al., 2013). Other regulatory factors that have been described are never in mitosis kinase (NIMA), transcription factors, and RNA recognition motif 1 (Francia & Striepen, 2014).

The kinases of *T. gondii* can be classified into eukaryotic kinase groups, but 78 out of 108 protein kinases predicted do not appear to have orthologues in either human or yeast kinases (Wei et al., 2013). This lack of conservation of *T. gondii* kinases is an indication of how unique Apicomplexa proteins can be and that the proteins might behave in unusual manners. Further, it suggests that unique cell cycle proteins could serve as drug targets (Wei et al., 2013).

Cyclin Dependent Kinases and Cyclins

Cyclin dependent kinases are considered to be members of the family of serine/threonine kinases. A CDK becomes activated when it binds to a cyclin and carries on its function to phosphorylate its substrate. This phosphorylation event might lead to cell cycle progression or inhibition depending on the state of the cell cycle. Cyclins are recognized as proteins that fluctuate i.e. vary in concentration during the cell cycle and regulate the activity of CDKs by binding to them. Further, cyclins can be identified by the presence of a cyclin box, which is a conserved domain at the C-terminal end (Nugent, Alfa, Young, & Hyams, 1991).

Bioinformatics analysis results have estimated that the *T. gondii* genome contains approximately 20 CDKs and close relatives to the CDKs (Peixoto et al., 2010). Further, cyclins have been isolated in *T. gondii* via the yeast two-hybrid screen and in *P. falciparum* (Kvaal, Radke, Guerini, & White, 2002; Waters, Woodard, & Prigge, 2000). Many questions still remain to be answered about the Apicomplexa CDK's and cyclins such as their substrates, interaction partners, localization, and role in controlling the diverse cell cycle modes observed in these parasites.

TgCyc2 – TgIMC1 – TgCDK1

The Putative *Toxoplasma gondii* Cyclin 2. Putative *T. gondii* cyclin 2 (TgCyc2), coding sequence (TGVEG_290020), is a 288 amino acid protein. This protein has been found to interact

with TgCDK1 and TgIMC1 in a yeast two-hybrid screen (unpublished Kvaal). Bioinformatics analysis of the coding sequence has indicated the presence of a cyclin box between bases 789 – 843 (Barnes, 2016). Further, the bioinformatics software Cyclinpred predicted that TgCyc2 was a cyclin protein (Noyes, 2013). However, overexpression of TgCyc2 in yeast has been found to induce cell cycle arrest in *S. cerevisiae* (Wade-Ferrell, 2011). This could be due to the toxicity of large amounts of TgCyc2 to yeast or because TgCyc2 is a cell cycle inhibitory protein.

Relatively little beyond this is currently known about TgCyc2.

The TgIMC1 Inner Membrane Protein. On the cytoplasmic face of the inner membrane several Apicomplexa, including both *T. gondii* and *P. falciparum*, have a subpellicular network (Khater, Sinden, & Dessens, 2004; Mann & Beckers, 2001). It is primarily thought that the subpellicular network provides structural integrity (Mann & Beckers, 2001). Two integral proteins associated with the *T. gondii* subpellicular network are the two inner membrane proteins, TgIMC1 and TgIMC2 (Mann & Beckers, 2001). TgIMC1's coding sequence is (TGME49_031640) and was isolated by Mann et al (Mann & Beckers, 2001). Further, TgIMC1 homologues have been found in other Apicomplexa such as *P. falciparum* PfIMC1 and *Plasmodium berghei* PbIMC1a (Khater et al., 2004; Mann & Beckers, 2001).

The progress of subpellicular network assembly during *T. gondii* development was studied by combining TgIMC1 with the fluorescent protein YFP while performing time-lapse video microscopy (Hu et al., 2002). First the daughter cell IMC appears when the DNA content is about 1.8N, which has been suggested as one checkpoint or pause in endodyogeny (Gubbels et al., 2008; Hu et al., 2002). This appearance of the IMC includes the appearance of the TgIMC1 protein, which is present from there on during development. Later, two dome shaped IMCs with

underlying SPNs appear. This begins the process of separating organelles and the membrane starts to surround a horseshoe shaped nucleus (Dubey et al., 1998; Hu et al., 2002).

Towards the IMC formation completion and before daughter cells leaves the mother, the TgIMC1 protein is proteolytically processed, which results in removal of TgIMC's cysteine tail (Mann, Gaskins, & Beckers, 2002). This event coincides with the daughter cell's IMC gaining structural integrity i.e. this prevents the SPN from being detergent extracted, which is a marker of a mature *T. gondii* (Mann et al., 2002).

Cyclin Dependent Kinase 1. TgCDK1, coding sequence (TGVEG_218220), was isolated by Wastling et al. in 1998 and re-isolated by Khan et al. in 2002, who further characterize this as a CDK (Khan, Tang, Qin, & Kim, 2002; Wastling & Kinnaird, 1998). Several conserved CDK domains have been identified in TgCDK1 and these include the ATP binding domain, the PSAIRE region, and the catalytic KLADFGLAR region (Khan et al., 2002).

Alignments results show that TgCDK1 have higher similarities to other Apicomplexans kinases than human and yeast. For example there is a 75% homology with *P. falciparum* compare to 63% homology to the human CDC2 (Khan et al., 2002). The functionality of TgCDK1's conserved domains has been further assessed. Co-immunoprecipitation found that mammalian cyclins A, B1, D3 and E bind to TgCDK1, and kinase activity was established in COS-7 cells (Khan et al., 2002). Further, expression of a double negative mutant of TgCDK1 leads to the accumulation of *T. gondii* in S-phase (Khan et al., 2002).

Chapter 2: Hypothesis

As stated previously TgCyc2 has been found to interact with both TgCDK1 and TgIMC1 (unpublished, Kvaal). The ability of TgCyc2 to bind to both TgIMC1 and TgCDK1 has led to an overarching hypothesis on how these interactions could be relevant for *T. gondii*'s progression through the cell cycle. If TgCyc2 binds TgCDK1 during the cell cycle then TgCyc2 can target TgCDK1 to a suborganellar location by a secondary interaction to TgIMC1. TgIMC1 is present in the developing daughter membrane and could serve as a spatial regulator of the cell cycle by interacting with the TgCyc2-TgCDK1 complex. Once TgCyc2 is bound to TgIMC1, the TgCDK1 would be in position to phosphorylate its target at a specific suborganellar location.

In order to evaluate this overarching hypothesis the purpose of this project was to determine if TgCyc2 binds TgIMC1 and TgCDK1 at different functional domains or at the same or overlapping domains. This led to the following research hypotheses:

Hypothesis 1: TgCyc2 has a functional domain separate from the cyclin box area that binds the TgIMC1 protein allowing for TgCyc2 to interact with both TgIMC1 and TgCDK1 simultaneously.

Hypothesis 2: TgCyc2 binds both the TgIMC1 protein and the TgCDK1 protein in the cyclin box area, which does not allow for simultaneous interactions.

The primary research goal was to test these two hypotheses by making three successively shorter TgCyc2 fragments with the aim of leaving only the cyclin box in the last TgCyc2 fragment. These fragments would then be cloned and expressed in the yeast two-hybrid test to

test for interaction with TgIMC1. If the TgCyc2 interaction with TgIMC1 takes place in the beginning of the protein, i.e. not in the cyclin box, then at the minimum the shortest fragment expressed in the yeast two-hybrid test would not lead to a protein – protein interaction. However, if the interaction between TgCyc2 and TgIMC1 takes place in the cyclin box a protein-protein interaction would still be observed with the shortest protein fragment.

The concept that cyclins target CDKs to subcellular locations is not a novel idea. It was found that when cyclin B1 binds to CDK1 the complex is transported from the nucleus to the cytoplasm because the complexation creates a localization signal (Hagting, Karlsson, Clute, Jackman, & Pines, 1998). Another even more relevant example is the discovery that cyclin Y complexes with the inner membrane via an N-terminal motif, which brings CDK14 closer to its substrate (Lim & Kaldis, 2013).

Chapter 3: Materials and Methods

TgCyc2 fragment PCR amplification

The *Toxoplasma gondii* cyclin 2 fragments were amplified from a *T. gondii* cDNA library provided by Christopher Kvaal, Ph.D via PCR amplification. Forward primers with NdeI restriction enzyme sites were designed for PCR amplification of three successively shorter TgCyc2 *T. gondii* fragments. The reverse primer was the same in each amplification reaction and designed with a BamHI restriction enzyme site.

Forward TgCyc2 fragment primers

TgCyc 2 (B) – 748 bp long

5' ATTCCATATGGGCCGAGGTGTTTATAC 3'

TgCyc 2 (C) – 640 bp long

5' ATTCCATATGCGTTTTCAACAGGGACG 3'

TgCyc 2 (D) – 520 bp long

5' ATTCCATATGCACCCTTCTGTCCATCC 3'

Reverse TgCyc 2 fragment primer

TgCyc 2 (B – D) reverse primer

5' TCGCGGATCCTCAGGCGCTGAAGAAGGCAGTCTC 3'

The resulting PCR products were verified with agarose gel electrophoresis run alongside a 1kb molecular weight ladder. Confirmed bands were removed and gel purified using a gel purification kit.

Cloning and Bacterial Transformation

Restriction enzymes BamH1 and Nde1 were used to digest the PCR product fragments and the pGBKT7 vector. The PCR products were ligated to the pGBKT7 GAL4-DNA binding domain vector (DNA-BD). Full length TgCyc2 had previously been incorporated into the pGBKT7 vector and full length TgIMC1 into the pGADT7 GAL4-DNA activation domain vector (DNA-AD).

Each of the pGBKT7–TgCyc2 cloned fragments were mixed separately with competent *E. coli* cells and incubated on ice for bacterial transformations. The tubes were heat shocked at 42°C for 45 seconds and rested on ice for 2 minutes. SOC media was added to the bacteria and the bacteria was allowed to grow in a shaking incubator at 37°C for 45 minutes. Undiluted and a one tenth dilution was plated on ampicillin LB plates. The plates were incubated overnight at 37°C. Colonies were selected and inoculated in ampicillin LB broth. A plasmid mini prep was used to isolate plasmid DNA from the transformed *E. coli*.

Lithium Acetate Yeast Co-transformation

The yeast strain Y187 was grown on YPD plates at 30°C. An ample amount of colonies were selected and inoculated in 20 ml YPD broth and grown in a shaking incubator at 30°C overnight. The yeast from the overnight culture was transferred to a flask containing 100 ml YPD. The fresh culture was allowed to grow for 3 hours in a shaking incubator at 30°C. After 3 hours the yeast was centrifuged for 2 minutes, the supernatant was discarded and the yeast was re-suspended in 1XTE. This was pelleted and re-suspended in 1X TE/1X LiAc and divided into 1.5 ml micro centrifuge tubes. The micro centrifuge tubes were pelleted and supernatant decanted.

A transfection mix consisting of 50% PEG, 1M LiAc, carrier DNA, and 200 ng of either of these combinations of plasmids 1) pGBKT7-FragmentC – pGADT7-TgIMC1, 2) pGBKT7-FragmentD – pGADT7-TgIMC1, 3) pGBKT7-TgCyc2FullLength – pGADT7-TgIMC1, 4) PEXpGBKT7-TgCyc2FullLenght – pGADT7-TgIMC1 was applied to each of the micro centrifuge tubes containing yeast. The resulting preparation was mixed by vortex and heat shocked at 42°C for 1 hour. The transformed yeast was plated on double drop out plates, which were missing tryptophan and leucine to select for co-transformed yeast.

Chapter 4: Results

I-Tasser Structure Predictions

Each of the three fragments and the full length TgCyc2 was analyzed with the I-Tasser structure and function prediction software (Yang et al., 2015). The software's top protein database hit for the full-length prediction was a CDK2/cyclin A in complex with the inhibitor N-&-N1. Other hits in PDB were a CDK/CyclinB1 complex and CDK4/D-type complex. The GO Ontology function predictions made by I-Tasser were "protein kinase binding", "regulation of cell cycle" and "regulation of CDK" (Yang, Roy, & Zhang, 2013). The predicted functional area corresponds to the 5 alpha-helical domain predicted to be the TgCyc2 cyclin box (Figure 1).

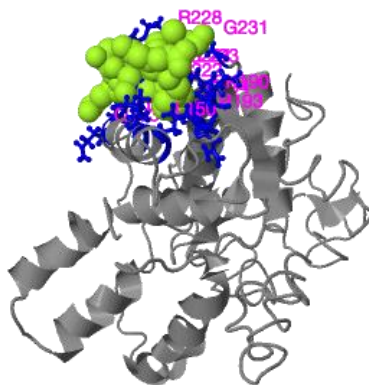


Figure 1: The I-Tasser functional prediction model generated for the full length TgCyc2. The model is shown binding a ligand in the helical domain corresponding to the cyclin box. The balls and stickballs are part of the representative ligand molecule.

The full length TgCyc2 I-Tasser structure prediction model received a C-score of -3.61 (Figure 2). As the TgCyc2 was shortened the structure predictions made by I-Tasser became more confident i.e. fragment B, C, and D received C-scores of -2.57, -0.71, and -0.11 respectively (Figure 3-5). This is consistent with the presence of the cyclin box at the C-terminal end of TgCyc2, an area, which would be easier for the software to predict considering its conserved features.

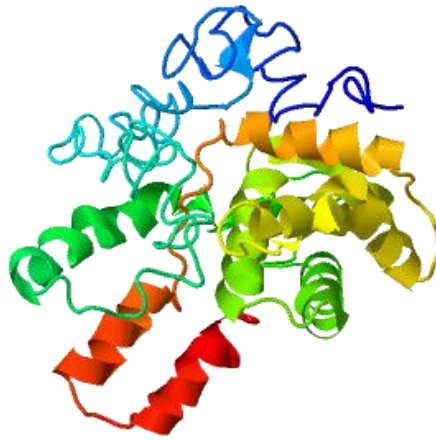


Figure 2: The I-Tasser structure prediction generated for the full-length TgCyc2 or fragment A (288 amino acids long). The predicted model received a confidence score or C-score of -3.61.



Figure 3: The I-Tasser structure prediction generated for fragment B (228 amino acids long). The predicted model received a C-score of -2.57.



Figure 4: The I-Tasser structure prediction generated for fragment C (192 amino acids long). The predicted model received a C-score of -0.71.

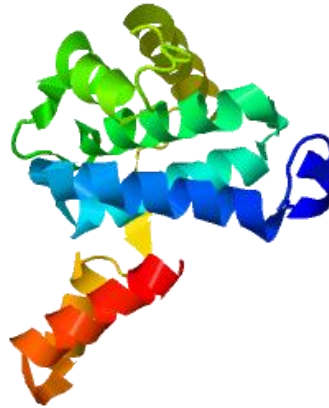


Figure 5. The I-Tasser structure prediction generated for fragment D (152 amino acids long). The predicted model received a C-score of -0.11.

Two out of Three TgCyc2 Fragments were Amplified

Two out of three TgCyc2 fragments, C and D were amplified via PCR. Bands were visualized with gel electrophoresis at approximately 640 base pairs and 520 base pairs for fragment C and D respectively (Figure 6). No band was visualized for fragment B in the gel (Figure 6). The bands indicating fragments of the expected size were cut out of the gel and gel purified for subsequent cloning experiments.

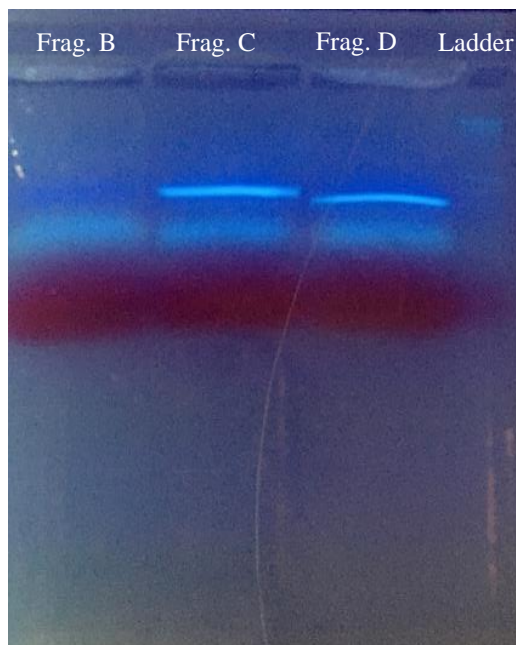


Figure 6: Two bands corresponding to fragment C at 640 base pairs and fragment D at 520 base pairs are visualized in lane 2 and 3 in the gel electrophoresis gel. This indicates that fragment C and fragment D were successfully amplified with PCR, but fragment B was not.

Amplified TgCyc2 Fragments were Cloned

After gel purifying the PCR amplified fragments, they were inserted in to plasmid pGBKT7 with BamHI and NdeI restriction enzyme digest and subsequent ligation. In order to confirm that the fragments had been successfully inserted into the plasmid a re-digest was performed on 10 μ l of pGBKT7-fragmentC and pGBKT7-fragmentD. The re-digest was visualized via gel electrophoresis and bands corresponding to the expected sizes of 640 base pairs and 520 base pairs for fragment C and D respectively (Figure 7).

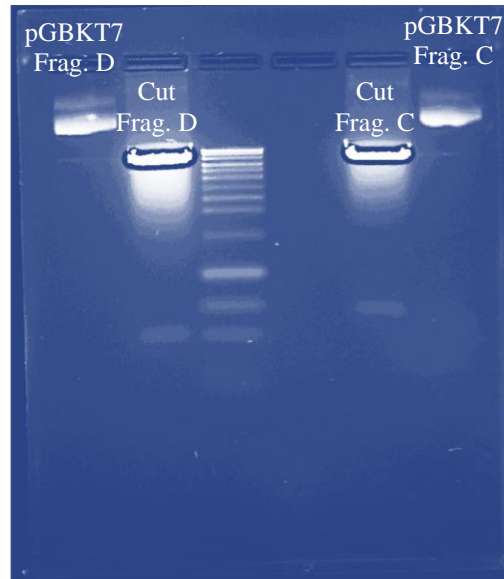


Figure 7: The pGBKT7-fragmentC and pGBKT7-fragmentD were re-digested with BamHI and NdeI, which reformed the PCR inserts at the expected sizes of 640 base pairs and 520 base pairs.

Lane one is uncut plasmid pGBKT7-fragmentD and lane two corresponds to the pGBKT7-fragmentD cut plasmid. Lane 4 is the 1kb ladder used as a marker and lane 5 is empty. Lane 6 is pGBKT7-fragmentC digested plasmid and lane 7 is pGBKT7-fragmentC uncut plasmid.

The pGBKT7-fragmentC and pGBKT7-fragmentD were transformed in *E-coli* and selected for on ampicillin plates. Selected clones that grew on the ampicillin plates were further cultured and plasmid mini-prep was performed to purify the cloned plasmids. After purification the plasmids were used to co-transform yeast.

The pGBKT7-fragmentC, pGBKT7-fragmentD, pGBKT7-TgCyc2Full-Length, and pGBKT7PEX-TgCyc2Full-Length (negative control) were co-transformed with pGADT7-TgIMC1 into the yeast strain Y187. Successful transformation was indicated by the yeasts ability to grow on double drop out plates that were lacking tryptophan and leucine.

The Yeast-Two Hybrid Test has not been Completed

Project time constrains and PCR amplification mishaps among other factors have prevented the completion of the fragment yeast-two hybrid test. Hence currently no conclusion can be drawn on where the TgCyc2-TgIMC1 protein-protein interaction will take place. However, if hypothesis number 1 is corroborated it is expected that as the fragments are shortened the TgCyc2-TgIMC1 interaction will cease. The opposite is expected if hypothesis number 2 is corroborated i.e. the last fragment containing the cyclin box will interact with TgIMC1 in the yeast two-hybrid test.

Chapter 5: Discussion

The Nature of the TgCyc2 – TgIMC1 – TgCDK1 Interaction Remains

The question still remains on the nature of the interaction between TgCyc2, TgIMC1, and TgCDK1. However, with co-transformed yeast one could easily perform the fragment protein yeast two-hybrid interaction test. Depending on the result of the yeast two-hybrid test these results could indicate the potential presence of a unique targeting mechanism performed by TgCyc2 on TgCDK1. On the other hand the results could indicate that TgCyc2 – TgIMC1 – TgCDK1 cannot form a complex since the binding of TgCyc2 to both occur at the same location.

In addition to temporal control by cyclins, the hypothesis has emerged on the role of the Apicomplexa cytoskeleton elements for spatial control over the cell cycle (Francia & Striepen, 2014). The existence of the TgCyc2 – TgIMC1 protein interaction could be support for the hypothesis that structural elements provide spatial regulation of the cell cycle. Further, the discovery that for example cyclin Y in mammals targets CDK14 to a specific inner membrane position suggest that similar targeting mechanisms could be present in other eukaryotic organisms (Lim & Kaldis, 2013). It will be interesting to see what information the yeast two-hybrid test generates with the cyclin fragments.

If TgCyc2 were found to target TgCDK1 to a suborganellar location by binding TgIMC1, then the TgCyc2 – TgIMC1 interaction would make for an attractive drug target. TgIMC1 is a unique protein to the Apicomplexan, with the closest homology to articulins in *Euglena* species (Mann et al., 2002). Therefore, a drug that inhibits the TgCyc2 – TgIMC1 interaction could lead to minimal toxicity for the host. Nonetheless, inhibiting the interaction could potentially result in cell cycle arrest for *T. gondii* by disrupting the TgCDK1 suborganellar localization.

Yeast Two-Hybrid Method and Limitations

A yeast two-hybrid screen was originally used to identify that TgCyc2 interacts with TgIMC1 and TgCDK1. This same method has previously been used to identify positive cyclin interactions for *T. gondii* (Kvaal et al., 2002). However, limitations to the yeast two-hybrid screen exist. There are concerns that the proteins will not fold properly when expressed or that foreign proteins expressed in yeast will not receive its proper post-translational modifications (Coates & Hall, 2003).

Post-translational modifications might be a concern for *T. gondii* proteins expressed in yeast. For instance *T. gondii* tubulin proteins have been found to be post-translational modified, which indicates that this is a potential issue for *T. gondii* yeast two-hybrid interactions (Xiao et al., 2010). There is also a risk that the yeast two-hybrid screen finds artificial interactions between proteins that normally would not be in proximity to each other nor expressed during the same time (Coates & Hall, 2003). However, experimental evidence exists that show TgIMC1's expression throughout the development of the *T. gondii* daughter cells (Mann & Beckers, 2001). Therefore, it would be expected that cell cycle proteins such as TgCyc2 and TgCDK1 would be expressed at the same time as TgIMC1.

Further, there are some concerns about the proposed methodology. Protein fragments that are successively shorter might not fold correctly. This might result in false negative protein-protein interaction results in a yeast two-hybrid test, which is not due to a lack of functional domain, but due to improper protein folding. Further, as the fragments become successively shorter the transcriptional binding domain might become closer to the functional domain. The presence of the binding domain could lead to steric hindrance and this could also lead to false negative result (Coates & Hall, 2003).

I-Tasser Function Predictions

The I-Tasser structure predictions showed an interesting trend where the confidence score or C-score increased, as the TgCyc2 fragment became successively shorter -3.61 to -0.11. This fits the idea that the cyclin box is present at the end of TgCyc2. I-Tasser searches the protein data bank for known structures determined via for example x-ray diffraction (Yang et al., 2015). Due to the fact that the cyclin box is a highly conserved domain among eukaryotic organisms there are plenty of mammalian cyclins to use as templates for the structure of this area.

On the other hand the area hypothesized to bind to TgIMC1 is predicted to be a coiled region. When this region is included on TgCyc2 the structure prediction is poor (-3.61) i.e. closer to the lowest score possible, which is -5. This indicates that no known related structures could be found in the protein database and supports the idea that *T. gondii*'s cell cycle proteins and Apicomplexa proteins in general are unique eukaryotic proteins. Considering the distinctiveness of Apicomplexa cell cycle proteins it is not difficult to see why these would be considered to be potential drug targets. Further, it also supports the idea that TgCyc2 might be involved in unique functions such as targeting a CDK to a suborganellar location by binding TgCyc2.

Conclusions

Using yeast two-hybrid testing to search for the area on TgCyc2, which is responsible for binding TgIMC1, was not meant to provide a conclusive answer, but lead experimentation into new directions. Determination of the submolecular interaction of proteins provides valuable information towards function. Additionally, this approach would also never fully explain the reason for TgCyc2's ability to bind both TgIMC1 and TgCyc2. Neither would it hone in on the exact functional domains present in TgCyc2. However, if encouraging results can be achieved

with the yeast two-hybrid test, which is an economical and timesaving approach, these encouraging results could warrant further studies. Supplementary testing might include techniques such as co-immunoprecipitation and indirect immunofluorescence to corroborate findings from the yeast two-hybrid test.

It will be interesting to follow the future research on the TgCyc2 – TgIMC1 – TgCDK1 interaction. It is worth mentioning here in the concluding remarks that results indicating that the novel interaction hypothesis between TgCyc2 – TgIMC1 – TgCDK1 is corroborated would also warrant cross-Apicomplexa examination for similar interactions. It will be interesting to see what the results are from the yeast two-hybrid test suggested here. However, only more experiments will be able to elucidate if these protein – protein interactions are simply spurious artifacts of the yeast two-hybrid system or if it could be the basis for developing the next *Toxoplasma gondii* drug.

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