Aggregation-resistant mutants of schizophrenia-related protein TRIOBP-1

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Bachelor's thesis

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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Preddiplomski sveučilišni studij

Biotehnologija i istraživanje lijekova

Anja Hart

OTPORNOST NA AGREGACIJU MUTANATA PROTEINA TRIOBP-1 POVEZANOG SA SCHIZOFRENIJOM

Završni rad

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Mentor: doc.dr.sc. Nicholas J.Bradshaw

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In front of the Committee:

1. Associate Prof. Antonija Jurak Begonja

2. Assistant Prof. Christian Reynolds

3. Assistant Prof. Nicholas J. Bradshaw

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Abstract

Schizophrenia is a chronic mental illness, characterized by severe symptoms such as delusions, hallucinations, disorganized speech, lack of motivation, and unpredictable behavior. The development of schizophrenia is caused by genetic and environmental factors. The complexity of these factors has made it difficult to develop new methods for the successful treatment of patients. Recent research has shown that disruption of proteostasis may also impact on the progression of schizophrenia. Disruption in proteostasis causes certain proteins to misfold and therefore aggregate if the cell fails to degrade them. Aggregation is a process in which large insoluble structures known as aggregates are formed. So far, five proteins have been identified that may aggregate in schizophrenia. Among these five is TRIO and F-actin-binding protein (TRIOBP-1), the protein of interest in this thesis. Using C-terminally truncated constructs, it was discovered that the critical region for aggregation of TRIOBP-1 is located in the central section of the protein. As a next step, different truncated variants of TRIOBP-1 were expressed in neuroblastoma cells and immunofluorescent microscopy was used as a way to visualize aggregation. In this way, the critical region for aggregation was narrowed down to a sequence of less than 10 amino acids. To expand and verify this, the next step was to use full-length constructs with a small deletion from their center. In this thesis, four such constructs were used. Unexpectedly, all four of them formed aggregates in cells. This finding indicates that either the PH domain, or an optional 59 disordered amino acids at the N-terminus have effect on TRIOBP-1's aggregation propensity which was previously thought to be very unlikely. The next step is to generate plasmids lacking the optionally translated N-terminal unstructured region, and look into the effect it may have on aggregation. By generating a TRIOBP-1 mutant with the minimal number of mutations required to prevent aggregation, we will be able to generate

model systems for studying TRIOBP-1 aggregation. This will allow us to better understand the role of TRIOBP-1 in the progression of schizophrenia.

KEY WORDS: Schizophrenia, TRIOBP-1, protein aggregation, mental illness

Sažetak

Shizofrenija je kronična mentalna bolest koju karakteriziraju teški simptomi kao što su zabluda, halucinacije, neorganiziran govor, nedostatak motivacije i nepredvidivo ponašanje. Razvoj shizofrenije uzrokovan je genetskim i okolišnim čimbenicima. Složenost ovih čimbenika otežala je razvoj novih metoda za uspješno liječenje pacijenata. Nedavna istraživanja pokazala su da poremećaj proteostaze također može utjecati na napredovanje shizofrenije. Poremećaj proteostaze uzrokuje pogrešno savijanje određenih proteina i s time stvaranje agregata ako ih stanica ne razgradi. Agregacija je proces u kojem se formiraju netopive velike strukture poznate kao agregati. Do sada je identificirano pet proteina koji mogu stvarati agregate u shizofreniji. Među tih pet je i TRIO and F-actin-binding protein (TRIOBP-1), protein od interesa u ovom radu. Koristeći C-terminalno skraćene konstrukte, otkriveno je da se kritična regija za agregaciju TRIOBP-1 nalazi u središnjoj regiji proteina. Kao sljedeći korak, različite skraćene varijante TRIOBP-1 izražene su u stanicama neuroblastoma, a agregacija je vizualizirana pomoću imunofluorescentne mikroskopije. Na taj se način kritična regija za agregaciju suzila na niz od manje od 10 aminokiselina. Da bi se ovo potvrdilo, sljedeći korak je bio upotreba konstrukata pune duljine s malim delecijama iz njihovog središta. U ovom su radu korištena 4 takva konstrukta. Neočekivano, sva 4 konstrukta su tvorila agregate u stanicama. Ovo otkriće ukazuje da ili PH domena ili 59 opcinalnih nestrukturiranih aminokiselina na N-terminalnom kraju utječu na sklonost agregacije TRIOBP-1, za koju se ranije smatralo obrnuto. Sljedeći korak je stvaranje plazmida kojima nedostaje opcionalno translatirana N-terminalna nestrukturirana regija, te razmotriti učinak koji ona može imati na agregaciju. Generiranjem TRIOBP-1 mutanta s minimalnim brojem mutacija potrebnih da se spriječi agregacija, moći ćemo generirati modelne sustave za proučavanje TRIOBP-1 agregacije. To će nam omogućiti da bolje razumijemo ulogu TRIOBP-1 u napredovanju shizofrenije.

KLJUČNE RIJEČI: Shizofrenija, TRIOBP-1, agregacija proteina, mentalne bolesti

Contents

Introduction

Chronic mental illness

Mental health is important at every stage of life and it includes the emotional, psychological and social well-being of an individual. Many factors can effectuate mental health problems, including: biological factors, such as genes or brain chemistry, life experiences, such as trauma or abuse, and family history of mental health problems[1]. Mental illnesses, also called mental health disorders, are a group of conditions that affect a person's ability to cope with the routine daily activities. The World Health Organisation (WHO) characterizes mental disorders as a combination of abnormal thoughts, perceptions, emotions, behavior, and relationships with others, and one in four people in the world will suffer from a mental disorder at some point in their lives[2,3]. There are many different mental health disorders, with different expressions of symptoms. Mental health disorders include illnesses such as major depression, schizophrenia, bipolar disorder, obsessive-compulsive disorder, and post-traumatic stress disorder, some of which can be categorized as chronic (chronic mental illnesses, CMIs) due to their lifelong recurring nature. Just like other chronic diseases, some mental health disorders are treatable, although, diagnosis of mental disorders remains almost exclusively based on standardized psychiatric evaluations, as there are currently no biological tests to determine biological causes to these disorders[4]. About 15% of schizophrenia and 30% of depression is not treatable using current drugs or other types of therapies. As mental disorders are among the leading causes of disability worldwide, further research into the molecular mechanisms and biological causes are obligatory to provide adequate treatments.

Schizophrenia

Schizophrenia is a severe and long-term mental illness, which is characterized by cognitive and behavioral changes and has a long-lasting effect on how a person feels, thinks, and acts. According to the WHO, schizophrenia affects 20 million people worldwide[5]. People with schizophrenia suffer from symptoms either continuously or intermittently throughout life and those symptoms can be classified as positive or negative. Positive symptoms are generally not seen in healthy individuals but are present in patients during a psychotic episode in schizophrenia. These include hallucinations (normally auditory or visual), delusions, disorganized thoughts and speech, and catatonia. On the other hand, negative symptoms refer to a lack of normal mental functions which include avolition, social withdrawal, lack of emotional expression and anhedonia[6]. Schizophrenia is typically diagnosed in late adolescence or early adulthood, although it is generally considered that the condition begins earlier than that, with a tendency to have a later onset in females[7]. The development of schizophrenia is caused by a combination of genetic and environmental factors[8]. Confirmation that genetics play a significant role in developing schizophrenia was provided by twin and heritability studies stating that heritability of schizophrenia is around 80%[9]. Unfortunately, there is still a lack of knowledge regarding condition's pathological mechanism and its strong genetic basis that has only partially been explained to date through conventional and genome-wide genetic association and linkage studies[10].

Protein aggregation

Proteostasis (protein homeostasis) promotes viability at the cellular and organismal levels by maintaining a functional proteome. Proteostasis

maintains this by controlling the biogenesis, folding, trafficking, and degradation of proteins present inside and outside of the cell. Proteins usually fold in the most thermodynamically favorable three-dimensional conformation[11]. Disruption in proteostasis causes certain proteins to misfold and potentially form insoluble aggregates if the cell fails to degrade them by the proteasome or autophagy[4] (Fig 1). Protein aggregation can occur depending on various factors such as changes in amino acid sequence, stress and imbalance in the expression of the protein, or conditions inside and out of the cell[12].

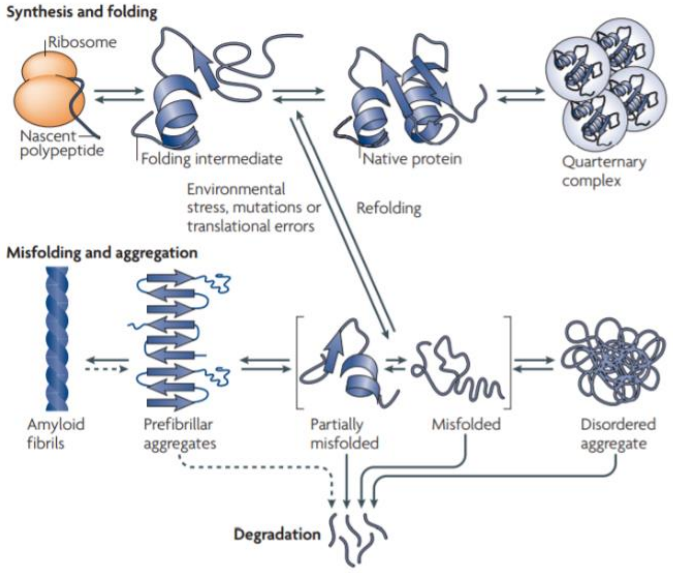

Figure 1: Overview of protein folding pathway. Proteins undergo a series of processes through different intermediates to become a fully functional, three-dimensional folded structure. If some of those processes are affected by proteotoxic stresses or imbalanced conditions inside or outside of the cell, proteins can get misfolded. Those misfolded intermediates can either be refolded to its native three-dimensional state or degraded by cellular proteolysis mechanisms. If that fails, misfolded proteins can accumulate and form insoluble aggregates. Taken from Tyedmers et al. 2010[13]

Proteins aggregating in chronic mental illness

As it was discovered that there is a link between overexpression and misfolding of proteins in a wide range of neurodegenerative disorders, a recent

approach in the field has been putting the focus on finding similar occurrences in patients with CMIs. Neurodegenerative disorders in which large plaques or aggregated protein structure were discovered include Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and frontotemporal dementia[14]. While no such large plaques or other aggregated protein structures exist in CMIs such as schizophrenia, as a non-genetic cause of those conditions, the formation of micro-aggregates triggered by extracellular stressors was discovered[4,15]. The five aggregate-forming proteins in patients with CMIs that have been best characterized are Disrupted in Schizophrenia 1 (DISC1), Neuronal PAS domain protein 3 (NPAS3), dysbindin-1, Collapsin Response Mediator Protein 1 (CRMP1) and Trio and F-actin-Binding Protein 1 (TRIOBP-1), our protein of interest in this thesis[4].

TRIO and F-actin-Binding Protein 1

Trio and F-actin-Binding Protein 1 (TRIOBP-1 or Tara), whose specific roles in the brain remain unclear, is generally known to be a critical promoter of actin polymerization, binding directly to polymerized fibers of F-actin, and it is encoded by the *TRIOBP* gene. *TRIOBP* is a complicated gene, which encodes for multiple splice variants, that among others include TRIOBP-1 and TRIOBP-4[15]. Proteins from the 3’ end of the locus, such as TRIOBP-1, are expressed in most cell types, and are involved in formation of actin fibers. Proteins from the 5’ end of the locus, such as TRIOBP-4, are mainly expressed in retina and inner ear where they are involved in function of rootlets of the ear – mutations in this part of *TRIOBP* cause deafness[16,17]. Although both TRIOBP-1 and TRIOBP-4 are vital for actin polymerization, the two share no exons between them. In contrast, TRIOBP-5 is a long isoform of more than 200kDa and it incorporates exons from both TRIOBP-1 and TRIOBP-4, but its further charaterization is lacking[4,15] (Fig 2). TRIOBP-4 is only expressed in the ears

and eyes, making it very unlikely to be connected to mental disorders[15]. Although there is a lack of knowledge in specific roles of TRIOBP-1 in the brain, aside from actin polymerization, studies have confirmed its involvement in neurite outgrowth, cell migration, proliferation and chromosome segregation during mitosis[16,18].

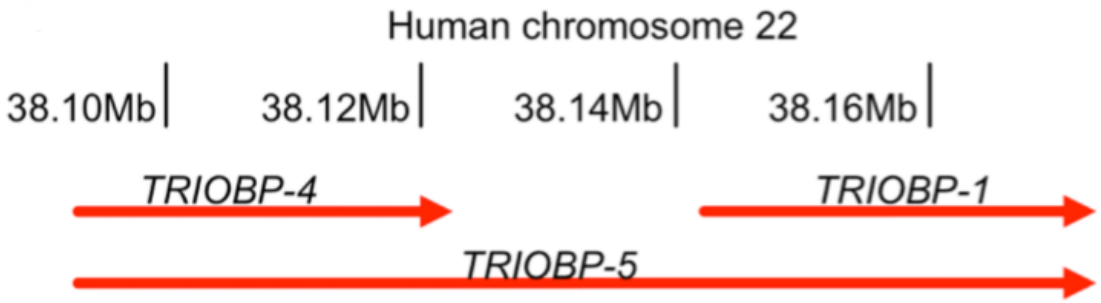

Figure 2: TRIOBP splice variants. Approximate chromosomal positions of the transcripts on human chromosome 22. Taken from Bradshaw et al. 2014 [15]

TRIOBP-1 in mental illness

A recent study on ubiquitously expressed TRIOBP-1 has discovered its propensity to form insoluble aggregates in CMIs through analyzation of post mortem tissue samples obtained from schizophrenia patients[15]. Prior to this, monoclonal antibodies that specifically recognize unique epitopes were identified. It was found that one of the derived antibodies (6H11), which shows reactivity specifically to the brain aggregomes of schizophrenia patients, recognized an epitope on CRMP1[19], however it also detected at least one additional protein. Later on, in another study, it was discovered that TRIOBP-1 is the main antibody substrate for that same monoclonal antibody. It was speculated that this occurrence could be due to a certain physiological mechanism, such as incorrect folding or post-translational modification[4,15]. In 2017, Bradshaw et al. have discovered that the aggregation propensity of

TRIOBP-1 arises from its central domain. In this central domain, a short 'linker' region of 25 amino acids was found to be essential for the formation of TRIOBP-1 aggregates[20]. Although, this region was later narrowed down to a sequence of 10 amino acids (Odorčić and Bradshaw, unpublished). Firstly, by expressing two TRIOBP-1 constructs (aa 333-652 and aa 324-652) it was discovered that the first nine amino acids, in which these two differ, are not required for the protein's aggregation propensity as both constructs aggregated. Moreover, in the same experiment, the other two TRIOBP-1 truncations (aa 341-652 and aa 343-652) showed partial aggregation in the cell[21].

TRIOBP-1 domain structure

To better understand the specific roles of TRIOBP-1 or its trigger for aggregation it is necessary to research its domain structure. Bradshaw et al. proposed that TRIOBP-1 consists of three separate domains and is 652 aa long. One of those is the N-terminal pleckstrin homology (PH) distinctly folded domain (aa 60-189) that has high β-sheet content (~50%) and, when expressed as a recombinant protein, forms a soluble, stable dimer. The other two domains are in the C-terminal half, the central domain (aa 281-555) and the C-terminal domain (aa 556-652), both forming α-helices, most likely in the form of coiled-coil domains (CC). Both the PH and central domain's function is linked to neurite growth. The C-terminal domain forms a stable monomeric protein if expressed alone. They predicted that the C-terminal half consists of six coiled-coil regions, labeled CC1-CC6, with the central domain including CC1-CC3/CC4, and the C-terminal domain including CC5-CC6. The central domain has an important role in the oligomerization of TRIOBP-1 and inhibiting the depolymerization of F-actin. In front of the PH domain is an additional 59-aa (aa 1-59) N-terminal region that is rich in both prolines and

positively charged residues. This optional 59-aa N-terminal region localizes to both the cytoplasm and nucleus when expressed in neuroblastoma cells. Inbetween the first two coiled-coil regions of the central domain, CC1 and CC2, is a 25 amino acid-long essential stretch, or a so-called ''linker'' region (aa 324-349), responsible for aggregation properties of the protein. The linker region was discovered by studying SH-SY5Y neuroblastoma cells, with different overexpressed FLAG-tagged constructs that contained certain domains of the full TRIOBP-1, under a fluorescent microscope[20].

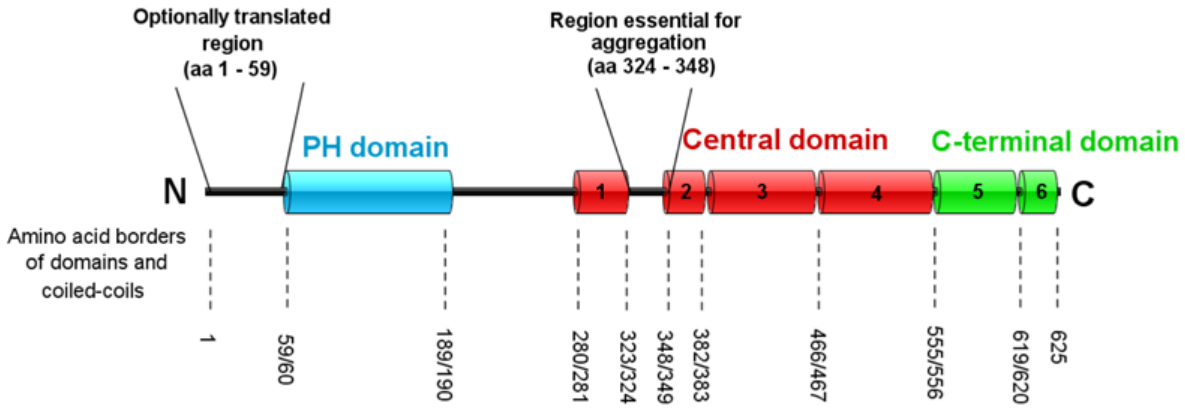

Figure 3: The domain structure of TRIOBP-1.TRIOBP-1 consists of three separate domains, each shown in a different color. The C-terminal half incorporates six individual coiled-coils, labeled CC1-CC6. Central domain consists of CC1-CC4, and C-terminal domain of CC5-CC6. Linker region (aa 324-349) essential for aggregation is shown between CC1-CC2. Adapted from Bradshaw et al. 2017[20]

Figure 4: Linker region. Sequence of the 25 amino acid aggregation prone ''linker'' region. Prolines are shown in purple and charged residues are shown either as red for positive charge or blue for negative. Taken from Bradshaw et al. 2017[20]

Aims of the thesis

As major mental illnesses can develop and progress as a result of both biological and environmental factors, it is necessary to focus more on their biological background due to the fact that there is a significant lack of knowledge and research in that part. Research on the biological background of CMIs is essential for better understanding, diagnosing, and treating mental illness. Since the discovery of mechanisms involved in forming insoluble deposits in brain samples of patients suffering from Alzheimer's disease, protein aggregation has been linked with chronic brain disorders. Later on, a more subtle misassembly and aggregation of proteins, which occur as a result of aberrant proteostasis, was seen in patients suffering from CMI. Protein aggregation has therefore been an emerging theme in the study of schizophrenia in particular. Five proteins have been identified as aggregation-prone proteins in patients with schizophrenia, these include DISC1, dysbindin-1, CRMP1, TRIOBP-1, and NPAS3[4]. The focus of this thesis was on the recently discovered TRIOBP-1 protein and its 'linker' region in the central domain which is responsible for the proteins aggregation properties[15,20].

Based on previous TRIOBP-1 research [15,20,21], in this thesis we aimed to:

1) Establish whether the shorter linker region of 10 amino acids, located between CC1 and CC2, which is responsible for the propensity of TRIOBP-1 to aggregate, discovered using C-terminal only plasmids also promotes aggregation of full-length proteins containing both N and C-terminal regions
2) Narrow down the linker region from 10 amino acids, in full-length TRIOBP-1, in order to generate a version of TRIOBP-1 with the

minimal number of deletions required to lose its aggregation propensity

Materials and methods

Materials

Antibodies

List of antibodies

Anti-Flag - Monoclonal ANTI-FLAG M2 antibody (mouse), 1 mg/ml, Sigma

GAM - Alexa Fluor 594 Goat anti-Mouse IgG, 2 mg/ml, Invitrogen by Thermo Fisher

Plasmids and vectors

Table 1: List of plasmids. Plasmids in the pdcDNA-Flag vector, with antibiotic resistance to AMP

	Origin	**Protein encoded**	**Publication**
1.	Nicholas Bradshaw and Carsten Korth, Düsseldorf	TRIOBP-1	Bradshaw et al. (2014)[15]
2.	Nicholas Bradshaw, Rijeka	TRIOBP-1 (Δ333-340)	Unpublished
3.	Nicholas Bradshaw, Rijeka	TRIOBP-1 (Δ333-343)	Unpublished
4.	Beti Zaharija and Nicholas Bradshaw, Rijeka	TRIOBP-1 (Δ341-345)	Unpublished
5.	Beti Zaharija and Nicholas Bradshaw, Rijeka	TRIOBP-1 (Δ344-345)	Unpublished

Methods

Bacterial culture, transformation, and inoculation

For bacterial transformation of NEB5α competent bacterial cells with plasmids containing the desired DNA constructs, 1 µL of plasmid construct (TRIOBP-1 (full-length), TRIOBP-1 (Δ333-340), TRIOBP-1 (Δ333-343), TRIOBP-1 (Δ341-345) or TRIOBP-1 (Δ344-345)) was added into a sterile 1.5 mL tube with 50 µL of freshly thawed NEB5α cells and incubated on ice for 30 minutes. Incubation on ice was followed by heat shock transformation, at 42°C for 30 seconds, and 5-minute recovery on ice. The suspension was then inoculated on LB agar plates that contained antibiotic ampicillin (1 µL/mL) and grown with plates upside-down overnight in the incubator at 37°C. The next day, one separate colony was picked and grown in 3 mL of LB media with antibiotic ampicillin overnight in a shaking incubator (37°C/250 rpm). When the culture in the Falcon tube had grown, it was centrifuged at 3700 rpm for 15 minutes and plasmid DNA was isolated per the protocol described below.

Plasmid DNA purification

Plasmid DNA was isolated with QIAprep Spin Miniprep Kit according to the manufacturer's protocol. The bacterial pellet in a falcon tube was resuspended in buffer P1 and lysed with buffer P2. The suspension was neutralized using buffer N3 for 5 minutes after which it was centrifuged in a table-top microcentrifuge at 13000 rpm for 10 minutes. The supernatant was then applied to the QIAprep 2.0 spin column and again centrifuged for 30-60 seconds. After every subsequent centrifugation, the supernatant was discarded. Next, QIAprep 2.0 spin column was washed with buffer PB and centrifuged again. This was followed by adding buffer PE and another centrifugation. To ensure that all residual washing buffer was removed,

QIAprep 2.0 spin column was additionally centrifuged for 1 more minute. Buffer EB was used to elute DNA from the column by shortly incubating and centrifuging for 1 minute. Samples were stored in the freezer at 4°C.

Micro-volume measurement of plasmid DNA

Samples obtained by growing bacterial culture and purifying plasmid DNA were analyzed by measuring their concentration using BioDrop µLITE spectrophotometer, with absorbance wavelength set at 260 nm. Elution buffer EB was used as a blank probe. 1 µL of the sample was analyzed. Measured concentrations were expressed in µg/mL.

Agarose gel electrophoresis

Purified samples of plasmid DNA were confirmed by agarose gel electrophoresis. Agarose gel electrophoresis is a method used for separating DNA samples based on their size. An agarose gel (agarose 0.5 g, 1x TAE buffer 50 mL, GelGreen 0.5 µL) was mixed and heated in the microwave for 1-2 minutes until agarose was dissolved completely. When the gel hardened it was placed in the electrophoresis tank filled with 1x TAE buffer. 10 µL of each sample (solution of dH_2O, DNA loading buffer, and plasmid DNA) was loaded as well as 5 µL of marker (solution of DNA Ladder and DNA Loading buffer). The electrophoresis was run at 140 V for 15-20 minutes. The gel was visualized using BioRad Chemi-Doc MP Imaging System.

Cell culture

Two cell lines were grown separately, HEK293 and SH-SY5Y. HEK293 cells are human kidney cells used for their fast growth and easy transfection, and SH-SY5Y cells are human neuroblastoma cells used because they provide a better model for aspects of neurons. Different media was used for each cell line, DMEM media for HEK293 and DMEM/F-12 for SH-SY5Y. Both cell lines were grown and adherent to the bottom surface of cell culture T25 flasks. When they reached a high confluency, they were split into a new T25 flask or 6 or 12-well plates, depending on what they were needed for, with preheated trypsin solution and corresponding medium (at 37°C). It was necessary to spray everything with 70% ethanol before use to obtain sterile conditions in the hood. Firstly, old media was pipetted out and 1 mL of trypsin solution was added to T25 flask with cell ready for splitting and left on room temperature for 10 minutes. After incubation, flasks were bashed to additionally loosen the cells. In that old flask, 4 mL of corresponding media was added. New flasks or plates were prepared with fresh media. If 12-well plates were used for SH-SY5Y cells to be looked under a microscope, glass coverslips were added in wells before adding media and cells. Cells from old flasks were then split into new one or in plates according to wanted dilution depending on cell confluency in the old flask. If HEK293 cells were intended to use for lysing and Western blotting they were split in 6-well plates. SH-SY5Y cells were split into 12-well plates with glass coverslips to use them for immunocytochemistry and microscopy. Flasks and plates were kept in the incubator (Nüve CO2 incubator) at 37°C/5% CO2.

Transfection

After splitting cells, the next step is transfection, a process where DNA plasmids of interest are put into mammalian cells. HEK293 cells were transfected with Metafectene and SH-SY5Y cells were transfected with Lipofectamine. As mentioned before, different media is used for each cell line (DMEM/HEK293, DMEM-F12/SH-SY5Y), only without fetal calf serum (FCS) or antibiotics, marked "-/-". Two solutions were prepared, DNA solution with 100 µL/300 µL (depending on a plate of 6 or 12 wells) of "-/-" media and calculated amount of plasmid (depending on measured concentrations), and Metafectene/Lipofectamine solution containing 2 µL of Metafectene or 6 µL of Lipofectamine and 100 µL/300 µL of "-/-" media. After 5-minute incubation at room temperature, solutions were mixed together and incubated once again for 20 minutes at room temperature. In the meantime, normal "+/+" (containing serum and antibiotics) media was removed from the wells, and the wells were washed with 0.5 mL/1.5 mL of corresponding "-/-" media. That media was removed and 300 µL/900 µL of fresh "-/-" media was added to the wells. Next, solution (DNA solution+ Metafectene/Lipofectamine solution) was added to the wells, and plates were left to incubate in an incubator for 6 hours at 37 $^{\circ}$C. After 6 hours, "-/-" media was removed and replaced with fresh "+/+" media and left to incubate overnight. Following transfection, HEK293 cells were lysed and cell lysates were used for Western blot, SH-SY5Y cells were instead used for immunocytochemistry and microscopy.

Cell lysis

After overnight incubation, the media was removed from HEK293 cells, and cells were washed twice with PBS. PBS was then drained from wells and cell lysis buffer was added instead and left to stand for approximately 5 minutes.

DNAseI (1 μL per mL of buffer) and protease inhibitor cocktail (at 1x final concentration) were added to the cell lysis buffer immediately before use. Using the buffer, the base of the wells was pipetted to remove all remaining cells, and then the solution was transferred to 1.5 mL Eppendorf tubes. Solutions were incubated on ice for 1 hour and vortexed periodically. Samples were then stored at -20 °C or prepared for Western blotting.

SDS-PAGE and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method used for separating proteins by their molecular weight. 8% acrylamide gels (H_2O 5.5 mL, 30% acrylamide 3.2 mL, 1.5 M Tris [pH 8.8] 3.0 mL, 10% SDS 120 μL, 10% APS 120 μL, TEMED 12 μL) with acrylamide stacking gel (H_2O 2.6 mL, 30% acrylamide 1.0 mL, 1.5 M Tris [pH 6.8] 625 μL, 10% SDS 50 μL, 10% APS 50 μL, TEMED 5 μL) were used for SDS-PAGE. Both acrylamide gels were hand casted using the Mini-PROTEAN Tetra Handcast Systems (Bio-Rad). The stacking gel, with its lower polyacrylamide concentration, is placed on top of the more concentrated running gel. It is used to create an ionic gradient that concentrates all the protein in one band. Once the protein reaches the more concentrated running gel with smaller pores, it will separate according to its molecular weight. The stacking gel was made and added 25-30min later. A well comb was added into the plates and they were left for 15-30 min until the stacking gel was set. Lysates for Western blot analysis were thawed and samples were prepared by adding Protein Loading Buffer to lysates in the same amount as Cell Lysis Buffer was added before, and 1M DTT, 10% the volume of Cell Lysis Buffer. The solution was then heated at 95°C for 5 minutes to denature the proteins. Next, samples were loaded onto the gel alongside a Prestained Protein Ladder marker used for protein size-tracking. SDS-PAGE was run for 50 minutes at 180V in SDS-PAGE Running

Buffer. Next, proteins from the obtained gel were transferred on to the polyvinylidene difluoride (PVDF) membrane (Macherey-Nagel, 0.20 µm pore) using the Trans-Blot Turbo Transfer System (Bio-Rad) which ran at 0.5 A for 30 minutes. After it was done, membranes were washed with water, stained with Ponceau S solution to see the total protein, and washed again to remove the remaining staining solution. Membranes were washed again but this time with PBS-Tween and blocked overnight at 4°C in PBS-T with 5% dry milk. This was followed by incubation with primary antibodies diluted in PBS-T solution (1:1000) at room temperature for 2-4 hours on a shaker. Next, membranes were incubated in secondary antibody diluted in PBS-T (1:2000) for 1 hour at room temperature. Following each antibody incubation, membranes were washed with PBS-T 3 times over 30 minutes. Detecting protein bands was done using Pierce ECL Western Blotting Substrate (Thermo Fisher) and chemiluminescent pictures were acquired with ChemiDoc MP Imaging System (Bio-Rad).

Immunocytochemistry and microscopy

After transfection and overnight incubation, SH-SY5Y cell line was used for immunocytochemistry and microscopy. DMEM-F12 "+/+" media was removed and transfected SH-SY5Y cells growing on glass coverslips were gently washed with 0.5 ml PBS per well. Next, they were fixed with Fixation buffer for 15 minutes, followed by permeabolization with Permeabolization buffer for an additional 10 minutes. Cells were then washed three times with PBS and blocked with 10% goat serum/PBS for at least 30 minutes. After 30 minutes, blocking media was removed and PBS containing the primary antibody and goat serum was added. Cells incubated in primary antibody, Anti-Flag, diluted in 10% goat serum/PBS for 1-3 hours. Next, cells were washed three times with PBS over 15 minutes and incubated in the dark with secondary antibody,

Goat anti-Mouse IgG, with DAPI (1:500) and Phalloidin 488 nm (fluorochrome: Acti-stain 488) diluted in 10% goat serum/PBS for 1 hour, covered with a wet paper towel for humidity. The secondary antibody was then removed and cells were washed 3 times over 15 minutes with PBS. The procedure was done entirely at room temperature. At this point, glass coverslips were attached on slides using Mounting medium and stored in -4 ℃.

Prepared coverslips were viewed on an Olympus IX83 fluorescent microscope under 60x magnification. Images were taken using Hamamatsu Orca R2 CCD camera and CellSens software and further analyzed with ImageJ program.

Results

Several studies have shown that TRIOBP-1 has a propensity to form insoluble aggregates when over-expressed in mammalian cell culture. A 'linker' region responsible for TRIOBP-1's aggregation was first discovered to be 25 amino acid long, but it was narrowed down to a sequence of 10 amino acids. The 'linker' region is located in TRIOBP-1's central domain, between the first two coiled coils. Based on the last finding, four different plasmid constructs have been generated ((Table 1, 2.-5.), (Fig. 5, 2.-5.)) to try and establish if the shorter linker region (10 aa long), discovered by Odorčić and Bradshaw (unpublished) using C-terminal only plasmids, also works for full length proteins. These constructs all contain full C and N-terminal regions but differ in sequence in the central domain of the protein, which has various deletions in the 'linker' region. In an attempt to do so, each of the plasmid constructs ((Table 1), (Fig. 5)) was expressed individually in vitro. Before researching these 5 plasmids, they were all confirmed by sequencing, including the plasmid in well number 4 (Fig. 6), which was then excluded based on the sequencing results. Cell lines HEK293 and SH-SY5Y were grown and transfected with each of the following:

1) Full-length TRIOBP-1 (aa 1-652) with an N-terminal Flag-tag encoding aggregation-prone TRIOBP-1 protein

2) Full-length TRIOBP-1 (aa 1-652) with an N-terminal Flag-tag, lacking amino acids from 333-340

3) Full-length TRIOBP-1 (aa 1-652) with an N-terminal Flag-tag, lacking amino acids from 333-343

4) Full-length TRIOBP-1 (aa 1-652) with an N-terminal Flag-tag, lacking amino acids from 341-345

5) Full-length TRIOBP-1 (aa 1-652) with an N-terminal Flag-tag, lacking amino acids from 344-345

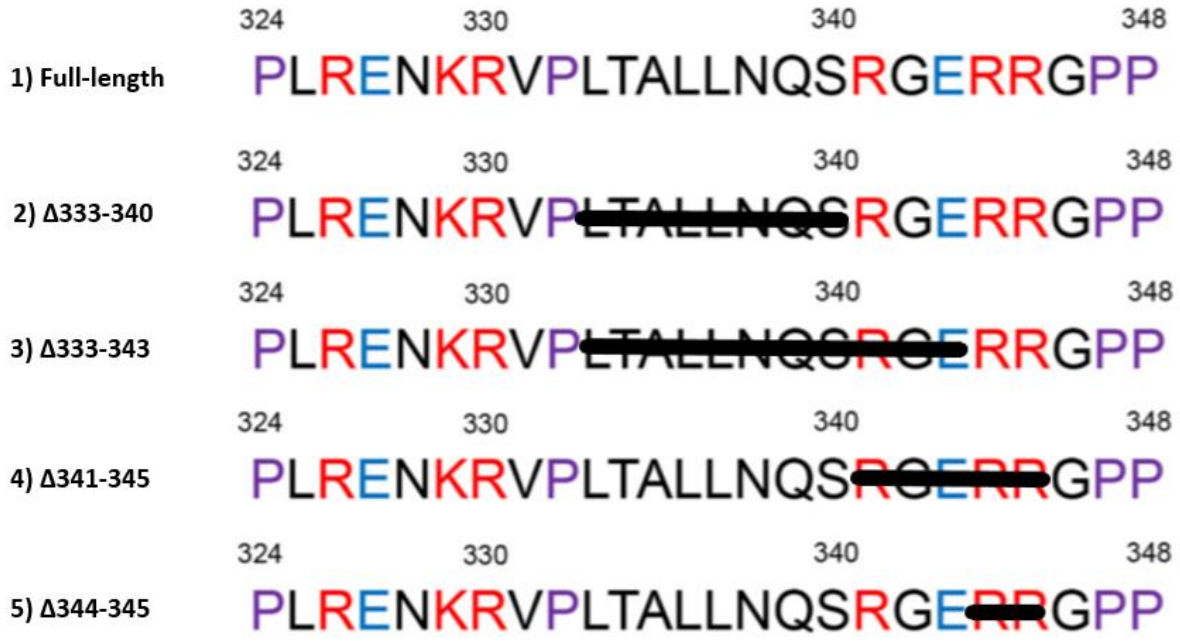

Figure 5: Linker regions of TRIOBP-1 and its constructs. Linker region of full-length TRIOBP-1 and its constructs used in this thesis with crossed over amino acids that are deleted.

TRIOBP-1 constructs expressed as proteins in the HEK293T cell line

Before investigating whether these constructs form aggregates in cell culture, we decided to confirm successful construct expression in mammalian cells. For that purpose, the human embryonic kidney (HEK293) cell line was grown and transfected with constructs respectively.

Firstly, full-length TRIOBP-1 and four plasmid constructs were grown and isolated from NEB5α competent bacterial cells. Then, each of the plasmids was added in wells containing HEK293 cells, which are used for its easy transfection and therefore it is very good for Western blot analysis. The cells were transfected using Metafectene as a transfection reagent. Following

transfection, the HEK293 cells were lysed and the samples were used for Western blot analysis. An anti-Flag antibody was used as the primary antibody to bind to the expressed Flag tag in the protein, and Goat Anti-Mouse antibody as the secondary for detection. The membranes were then visualized under ChemiDoc using the ECL visualization kit. The obtained blot (Fig. 5) represents the expression levels of the five distinct TRIOBP-1 proteins.

The two lines in the wells number 5 and 6 (Fig. 5) appeared as a result of accidentally moving the blotting paper with the membrane and the obtained gel while closing the Trans-Blot Turbo Transfer System (Bio-Rad) transfer cassette. The reason for the band number 2 being bigger than the others is probably that there was a generous amount of DNA in the lysates.

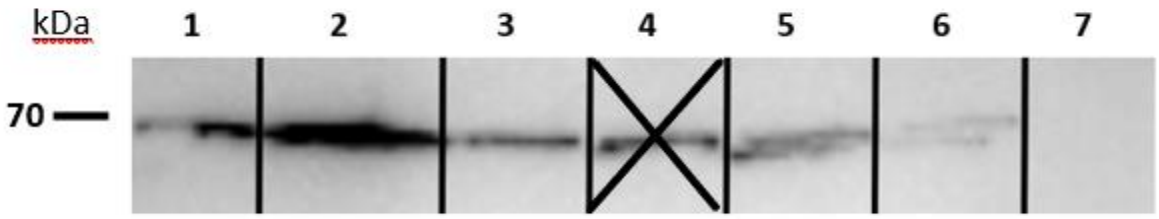

Figure 6: Distinct expression of full length TRIOBP-1 and 4 of its constructs expressed in human HEK293 cell line. Full-length TRIOBP-1 and four distinct TRIOBP-1 plasmid constructs were used for transfecting the HEK293T cell culture in a 6-well plate. Transfected cells were lysed and samples were used for Western blotting. The membrane shows expressed protein from the lysed cells, starting with the full-length TRIOBP-1 (**1**), followed by its constructs respectively (TRIOBP-1 (Δ333-340)(**2**), TRIOBP-1 (Δ333-343)(**3**), TRIOBP-1 (Δ341-345)(**5**) and TRIOBP-1 (Δ344-345)(**6**)), and ending with the untransfected mock control sample (**7**). Construct in well number **4** was exculded based on sequencing. The sizes were compared with Prestained Protein Ladder 10-180 kDa. Controls, which were mock transfected cells, were used for all western blots from which one is presented in the figure. Protein sizs in kDa is indicated at the left of the blot. The membranes containing proteins were stained with primary antibody anti-Flag and then with secondary antibody Goat Anti-Mouse. The pictures were visualized with ECL kit under the ChemiDoc. Constructs were confirmed by western blot in 2 independent western blots.

Fluorescent microscopy analysis

After confirming expression of constructs in mammalian cells, we wanted to investigate whether these proteins formed visible aggregates in cell culture and to do so, we used the SH-SY5Y human neuroblastoma cell line. The SH-SY5Y cell culture was grown on coverslips and transfected with the constructs in 12-well plates. The cells were transfected using Lipofectamine 2000 as a transfection reagent and incubated for 16 hours. After 16 hours, transfected cells were fixed, permeabilized, and stained with Monoclonal anti-FLAG M2 as a primary antibody, and with Alexa Fluor 594 Goat anti-Mouse IgG as secondary. During this second staining with the secondary antibody Goat Anti-Mouse 594 nm, Phalloidin 488 nm and DAPI were also added. Glass coverslips were attached on slides using Mounting medium before they were observed under the microscope. The whole process was repeated three times for confirmation of the results.

When viewed under a fluorescent microscope, the proteins can be seen as bright red fluorescence as a result of the secondary antibody binding to the primary anti-Flag antibody that emits red light when visualized. Only the proteins expressed from the plasmids were stained with red. Fluorescently-labeled Phalloidin was used to visualize cellular actin, and it fluoresces with a bright green color. The cells were visualized with fluorescent microscope and the CellSens software.

Images acquired from the experiments (Fig. 6) indicate that all TRIOBP-1 constructs and full-length TRIOBP-1 formed visible aggregates in the cell.

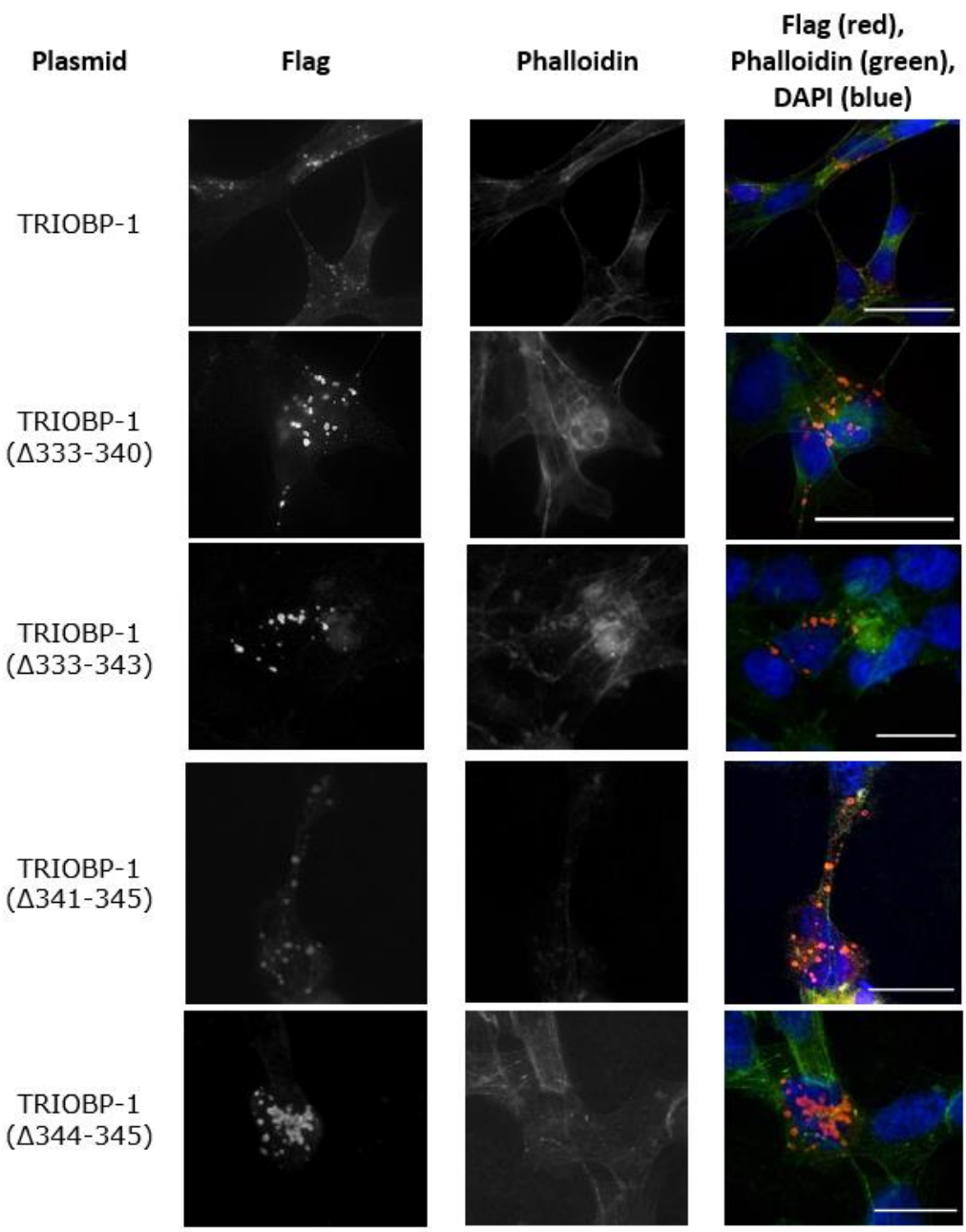

Figure 7: Fluorescent microscopy of full length TRIOBP-1 and its 4 constructs, all showing clear aggregation. The proteins were stained with primary antibody anti-Flag. The second staining contained the secondary antibody Goat Anti-Mouse 594 nm and phalloidin 488 nm. When the secondary antibody connects to the primary it gives a bright red fluorescent which the color of the proteins. Phalloidin connects to the actin and gives a bright green color. DAPI was used to stain the nucleus blue. Obtained images were captured under 60x magnification on a fluorescent microscope with the CellSens software. The scale bars represent 15 μm. Constructs were analysed by fluorescent microscopy in 3 independent experiments.

Discussion

Certain disorders, such as major depressive disorder, bipolar affective disorder and schizophrenia, can be categorized as CMIs due to the lifelong chronic or recurring nature of the disorders. These complex conditions partially overlap when it comes to their genetic influences as well as their symptomatology. The intricacy of its causes and mechanisms by which it occurs is why there is a lack of knowledge regarding the nature of these conditions. CMIs have a significant impact on a person's everyday life, by directly affecting their emotions, thoughts, and behavior[22]. As CMIs are considered one of the leading causes of disability worldwide, the growing awareness and recognition of such disorders have inspired researchers to focus on determining the biological causes that contribute to the pathology of these disorders in order to enable better diagnosis and treatment. Investigating the non-genetic factors of changes in protein homeostasis, also known as proteostasis, resulting in aggregated or misfolded proteins found in the brains of the patients, has been an emerging approach in the field. Thus far, five proteins with the potential to aggregate have been discovered[4].

This thesis aimed to investigate in more detail the aggregation propensity of TRIOBP-1, a protein implicated in schizophrenia. We set out to confirm and narrow down the TRIOBP-1 linker region positioned in between two coiled coils, CC1 and CC2, presumed responsible for the aggregation of the whole protein.

Bradshaw et al. investigated the domain structure of TRIOBP-1 by expressing different fragments of TRIOBP-1 as recombinant proteins. By doing that, they discovered that the protein consists of few distinct parts: an optionally translated region (aa 1-59), the PH domain (aa 60-189), the Central domain (aa 281-555), and the C-terminal domain (aa 556-652). It was demonstrated that the Central domain and potentially the C-terminal domain, but not the PH

domain, are capable of interacting with F-actin and protecting it against de-polymerization, concluding that the presence of the PH domain did not affect the dynamics of the actin. Moreover, in the Central domain, between two coiled coils, CC1 and CC2, they discovered and defined the linker region as 25-amino acid (aa 324-348) long stretch, which is rich with charged amino acids (Fig. 4) and is required for the aggregation propensity of TRIOBP-1[20]. Recently, the linker region was narrowed down to a sequence of 10 amino acids needed for the aggregation of the protein[21]. This was done by expressing four different TRIOBP-1 truncations (Table 2, plasmids 4, 10-12) and observing their aggregation under a microscope. The first two constructus (Table 2, plasmids 4 and 10) differed only in the first nine amino acids and they both aggregated, implicating that those nine amino acids are not required for the protein's aggregation propensity. Furthermore, the other two constructs (Table 2, plasmids 11 and 12) showed partial aggregation in the cell which, after a blinded assay of quantifying the aggregation, turned out to be non-aggregating in 64 ± 6% (aa 341-652) and in 82 ± 6% (aa 343-652) of the cells. Both proteins were described as aggregating in only 2 ± 1% of the transfected cells. Lastly, by performing a *Welch's t-test* (two-tailed distribution, two-sample, unequal variance) it was implicated that: 1. TRIOBP-1's amino acids 324-340 are crucial for aggregation, 2. two amino acids (341-342) are important in forming some aggregates, even though they do not cause the whole protein to fully aggregate, and 3. amino acids 343-349, while not crucial, still possess some ability to form aggregates.[21] The amino acid composition of the linker region implies that it is very likely that the aggregation is dependent on protein-protein interactions, with possible interaction partners being HECTD3, TRF1 and Plk1[23–25].

Our goal of confirming and narrowing down the linker region was carried out by expressing truncated proteins in the SH-SY5Y human neuroblastoma cell line. As expression patterns of truncated proteins are similar to the full-length

protein, these experiments present important evidence even if such truncated proteins would not be expressed *in vivo*.

Based on the previous research on TRIOBP-1's aggregation propensity and the linker region responsible for that (Table 2), four constructs of the TRIOBP-1 protein which contained incomplete versions of the linker region were tested, with each construct truncated by a variable number of amino acids (Fig. 5). These constructs were made and investigated depending on conclusions from previous experiments of which amino acids in the linker region may be responsible for aggregation of TRIOBP-1. The expression of proteins in mammalian cells was confirmed by Western blot analysis. All proteins were the size that was expected. After confirming expression of TRIOBP-1 and its constructs in mammalian cells, we wanted to investigate whether these expressed proteins formed visible aggregates in cell culture and to do so, we used the SH-SY5Y human neuroblastoma cell line. Full-length TRIOBP-1 showed clear aggregation, as expected, (Fig. 6) confirming its tendency to form insoluble aggregates[15]. Unexpectedly, when expressed in neuroblastoma cells, all four constructs with deletions of the linker regions also formed clear aggregates in the cells (Fig. 6) with the expression pattern similar to the full-length TRIOBP-1. This finding was surprising because previous research showed that deleting the N-terminal region and the PH domain (Table 2, plasmid 7) did not ablate TRIOBP-1's aggregation propensity as it aggregated when over-expressed in mammalian cells, but it did not aggregate when the linker region was also deleted from this construct (Table 2, plasmid 8), impicating that neither an optionally translated region nor the PH domain are involved in forming aggregates[20]. Therefore, it is surprising that deleting the linker region in full-length constructs did not have a similar effect. This led us to conclude that either the N-terminal region (aa 1-59) or the PH domain (aa 60-189) must have an effect.

An optionally translated region is a 59 amino acid long region, rich in both

prolines and positively charged residues, found at the 5' end of the TRIOBP-1 transcript. It consists of two putative Kozak sequences and methionine residues, and when expressed in neuroblastoma cells it localizes to both the cytoplasm and nucleus. According to Bradshaw at al., "TRIOBP-1 species containing this region appear to represent a minority in cells and, therefore, arise either from a less common translation event or else they represent the approximate position of a "pro" domain, which is normally cleaved off in the cell"[20]. On the other hand, the pleckstrin homology (PH) domain was suggested to have an effect on aggregation by bioinformatics analysis as oneof the two mechanisms for TRIOBP-1 aggregation through β-sheet-based aggregation. However, deleting the PH domain of TRIOBP-1 did not ablate its aggregation propensity, to be more precise, TRIOBP-1ΔPH aggregated when over-expressed in CL4 epithelial cells, and when expressed in SH-SY5Y neuroblastoma cells it aggregated in the same manner as the full length construct[15]. Nevertheless, it is technically possible that both the PH domain and the linker region are capable of inducing aggregation, and therefore deletion of only one or the other does not stop aggregation.

In line with the present research, a next step would be generating a full-length recombinant TRIOBP-1 that lacks the optionally translated region and the linker region and over-expressing it in cell culture to investigate whether the presence of both is the reason for aggregation. Based on previous research, a TRIOBP-1 construct lacking these regions would not be expected to aggregate, and if confirmed in mammalian cells, could be used for future experiments for narrowing down the linker region to a minimal number of mutations required to prevent aggregation. Also, performing a blinded assay of quantifying the aggregation on these four constructs would be one of the next steps to show the percentage of cells with aggregates, to see if there is a difference between constructs and the amino acids they are missing.

Locating and defining the exact amino acids in TRIOBP-1's aggregation domain

required for the aggregation to occur is necessary as we would like to generate good transgenic animal models of TRIOBP-1 and schizophrenia. To make it happen, we would ideally have three lines of animals: one wild type, one which expresses aggregating TRIOBP-1, and one which expresses non-aggregating TRIOBP-1. This would allow us to differentiate between effects caused by having too much TRIOBP-1 generally, and ones caused specifically by having too much aggregating TRIOBP-1. But in order to do this, we need to be able to clone in a TRIOBP-1 gene that has the minimum number of mutations necessary to prevent aggregation. In short term, the plan is to do this in Drosophila fruit flies, and in long term, either mice or rats.

CMIs have a very complex nature which makes them very hard to treat and diagnose, researchers, trying to finding new and better ways of doing so, focused on the biological background of the illnesses. A recent revelation of connection of protein aggregation and CMIs was followed by the discovery of few proteins with a tendency to form insoluble aggregates in the brains of the patients with CMIs. One of those proteins, TRIOBP-1, implicated in schizophrenia, was closely investigated and a region of the TRIOBP-1 was identified as essential for its aggregation to occur. Although the research of pathways leading to the accumulation and aggregation of this protein and many others is still lacking, it will potentially lead to novel diagnostic and potential prevention strategies for these disorders.

Table 2: List of construct used in research so far

	Plasmid	Tendendency to aggregate when over-expressed	Publication
1	Mouse full-length	Yes	[15]
2	TRIOBP-1ΔPH	Yes	[15]
3	Human full-length (652 aa)	Yes	[20,21]
4	aa 281-652 (CC1-6)	Yes	[20]
5	aa 324-652 (construct #4, minus CC1)	Yes	[20]
6	aa 349-652 (construct #5, minus linker)	No	[20]
7	aa 190-652 (full length, minus N-terminal region and PH domain)	Yes	[20]
8	aa 190– 652 Δ324–348 (construct #7, minus linker)	No	[20]
9	aa 1-382 (full length, minus CC3-6) *	No	[20]
10	aa 333-652 (construct #5, minus partial linker)	Yes	[21]
11	aa 341-652 (construct #5, minus partial linker)	Partial aggregation**	[21]
12	aa 343-652 (construct #5, minus partial linker)	Partial aggregation**	[21]

***** This construct is demonstrating that although this 25-aa linker region is essential for aggregation to occur, it is not itself sufficient to induce aggregation, requiring the presence of at least some other part of the central domain

****** In most of the transfected cells the protein expressed evenly throughout the whole cell, but in some cases aggregates were also visible in the cell at the same time

Conclusion

With CMIs being a growing health issue all around the world, novel therapeutic approaches to their pathology on a molecular level have become an emerging focus for research. Having a very complex nature, CMIs are very difficult to treat and diagnose in the early stages of the condition. As following a treatment plan specifically designed for patients with CMIs by a trained psychologist or psychiatrist is often not enough, researchers have focused on the biological background of the illnesses and have recently found that protein aggregation could be the cause of its development. A number of aggregate forming proteins in the brains of patients with CMIs have been discovered in the last decade. Recent work has found that there are at least five proteins with aggregation propensity in patients with schizophrenia.

It was discovered that one of the schizophrenia candidate proteins, TRIO and F-actin binding protein 1, loses its aggregation propensity if the 'linker' region is cut out, with the N-terminal pleckstrin homology domain having no effect to this. Constructs of TRIOBP-1, missing its PH domain and the 'linker' region, have shown to not aggregate.

In an effort to narrow down the critical region for aggregation (the 'linker' region) to a sequence of less than 10 amino acids, in this thesis, we investigated 4 plasmid constructs of full-length TRIOBP-1 with small deletions in the central domain, more precisely in the 'linker' region. Levels of expression of those constructs were studied in HEK293 as well as in neuroblastoma cell line SH-SY5Y. Despite previous findings of the PH domain, or the optional 59 disordered amino acids at the N-terminus as having no effect on the dynamics of actin, it was discovered that the presence of one of those in constructs causes them to aggregate. Thus, giving us an insight into the next part of the research and that would be to further investigate the

effect the PH domain and the optional 59-aa N-terminal region have on TRIOBP-1's aggregation propensity.

Finding the exact cause for TRIOBP-1's aggregation propensity is important because over-expressing the construct of TRIOBP-1 lacking that parts in transgenic animal models would give us an insight into the patholoy of this protein, specifically how it affects neuronal function and behavior.

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Appendix

Enzymes, stains and commercially prepared kits

Bio-Budget Technologies GmbH	QIAGEN
my-Budget DNA/RNA Stain Green	QIAprep Spin Miniprep Kit

Size markers

my-Budget 1 kb DNA Ladder (200 mg/mL) (Bio-Budget Technologies GmbH)

-The marker consists of 13 blunt-ended fragments in the range from 250 base pairs (bp) to 10 kilobase pairs (kb).

my-Budget Prestained Protein Ladder 10 kDa - 180 kDa (0.2-0.4 µg/µL) (Bio-Budget Technologies GmbH)

-The marker consists of proteins in the size range between 10 kDa - 180 kDa with an additional fragment at 1500 bp and is used for Western Blot transfers performed in Tris-Glycine buffer.

Buffers and solutions

Buffers and solutions used in this thesis were made in deionized distilled water (dH_2O) and are listed below, in alphabetical order.

30% acrylamide solution

14.6 g acrylamide

0.5 g N,N'-methylbisacrylamide

Added water up to 100 mL

Cell Permeabilizing buffer

10 mL 10x PBS

500 µL Triton X-100

Up to 1000 mL dH_2O

Cell Fixing buffer

8 g paraformaldehyde

20 mL 10x PBS

dH_2O up to 200 mL

set pH to 7.4

DNA Loading buffer

5 µL 10% SDS

80 0.25M EDTA

~ 5 mg bromophenol blue

Added water up to 50 mL

Cell Lysis buffer

5 mL 10x PBS (1x)

5 mL 10% Triton X-100 (1%)

1 mL 1M Magnesium chloride (20 mM)

Added water up to 50 mL

HEK293 cell media (+/+)

500 mL DMEM

50 mL FCS

5 mL non-essential amino acids

5 mL Penicillin/Streptomycin

LB agar

1 g tryptone

0.5 g yeast extract

0.5 g NaCl

1.5 g agar

Added water up to 100 mL

LB media

10 g tryptone

5 g yeast extract

5 g NaCl

Added water up to 1 L

PBS-Tween

50 mL 10x PBS

450 mL dH_2O

250 mL Tween 20

Phosphate buffered saline (PBS)

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO4

Added water up to 1 L

Adjusted pH to 7.4

Ponceau S stain

1 g Ponceau S

4 mL acetic acid

Added water up to 200 mL

Protein loading buffer

6.25 1M Tris [pH 6.8]

10 mL glycerol

20 mL 10% SDS

3.75 mL dH_2O

~ 5 mg bromophenol blue

SDS-PAGE running buffer

30 g Tris

144 g glycine

10 g SDS

Added water up to 1 L

SH-SY5Y Cell media (+/+)

500 mL DMEM/F-12

50 mL FCS (Fetal calf serum)

5 mL 100x Penicillin/

Streptomycin

5 mL 100x Non- Essential Amino acids

50x TAE buffer

242 g Tris

18.61 g EDTA

57.1 mL acetic acid

Added water up to 1L

TE buffer

0.5 mL 1M Tris (pH 7.4)

200 µL 0.25M EDTA

Added water up to 50 mL

Transfer buffer (1x)

5.8 g Tris

2.9 g glycine

4 mL 10% SDS

200 mL methanol

Added water up to 1 L

List of Figures and Tables

Figures

Tables

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