The involvement of the ceramide transporter in Alzheimer's disease

Investigating the binding properties of the ceramide transporter in the human brain by optimizing the split-ubiquitin Yeast Two-Hybrid system

A.M.G.S. Jansen
Maastricht University
amgs.jansen@student.maastrichtuniversity.nl

Abstract

Background: Alzheimer's disease (AD) is the most common form of dementia, characterized by aggregates of Amyloid-ß (Aβ) peptides. The ceramide transporter ($CERT_L$) exists in a complex with serum amyloid P component in the Aβ plaques of AD patients. The goal is to obtain more information about the protein binding activity of $CERT_L$ in the human brain. To be able to do so, the Yeast Two-Hybrid (Y2H) technique, that was used to confirm the results from a previously executed $CERT_L$ Y2H screening, had to be optimized. Technical problems were encountered with the plasmid purification of the yeast NMY51 using the EZNA plasmid DNA minikit I and with the transformation in competent E.coli cells. Initially it was believed that there was a problem with the EZNA plasmid DNA minikit I. In this report troubleshooting was performed to identify the problem of the plasmid purification technique that has been used. **Methods**: To examine the protein binding activity of $CERT_L$ a split-ubiquitin Y2H system was used. In this system proteins are attached to two parts of one complete ubiquitin molecule, called the split-ubiquitin. The middle region domain of $CERT_L$ (the bait) was attached to the C-terminal half of the split-ubiquitin and the transcription factor LexA-VP16. The possible interactor, a prey library containing human brain proteins was fused to the N-terminal half of the split-ubiquitin. The presence of an interaction between bait and prey will results in the re-assembly of the split-ubiquitin, which activates ubiquitin proteases. The transcription factor is released and reporter genes are activated.The results of a previously executed Y2H screening had to be confirmed by

performing another Y2H screening. The new screening had to be carried out using glycerol stocks of the yeast NMY51, containing both bait and prey, from the earlier performed Y2H screening. To optimize prey plasmid purification from the yeast using the EZNA plasmid DNA minikit I, troubleshooting was performed on the technique. Next to this, other methods to isolate the plasmid DNA, a QIAprep spin miniprep kit protocol and a phenol extraction and ethanol precipitation, were performed. The failing results were possibly due to the yeast being stored as a glycerol stock. In order to correct for this, a freshly transformed yeast sample was made. **Results:** Extracting the plasmid from glycerol stocks from the yeast NMY51 did not succeed. Plasmid purification and transformation of the prey plasmid from the fresh transformed yeast in E.coli competent cells was successful. **Conclusion:** From the results it is now believed that storing the yeast NMY51 containing the prey plasmid in glycerol stocks causes the prey plasmid to integrate into the genomic DNA of the yeast. Performing a new Y2H screening would therefore only be possible on fresh transformed yeast NMY51 cells.

Keywords
Split-ubiquitin Yeast Two-Hybrid system, Ceramide transporter, Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common form of dementia (1). The need for research into AD is increasing very fast due to the ageing of the overall population, but also because of the fact that no treatment is available for AD (2).

The biological function of ceramides

Ceramide is the backbone of all sphingolipids (SLs). SLs are complex lipids, which are present in the human brain. An impaired regulation of ceramide was associated with neuronal cell death in AD (3). Moreover, AD is characterized by aggregates of Amyloid-ß peptides (Aβ) and synthetic ceramide analogues were found to be capable of inhibiting Aβ production in human neurons (4). Sphingomyelin (SM) is the most common phosphosphingolipid in mammalian cells. Ceramides which are destined to synthesize SM are transported in a non-vesicular manner using the ceramide transfer protein (CERT) (5). The ceramide transporter long isoform ($CERT_L$) is also known as the Goodpasture antigenbinding protein (GPBP), which was discovered in the Goodpasture's syndrome. The GPBP/$CERT_L$ protein consist of three functional domains the plechstrin homology domain, the middle region (MR) and the steroidogenic acute regulatory protein-related lipid transfer region (6). Since the MR domain is responsible for the binding of $CERT_L$ with other proteins, it was decided to use this part as bait.

The Yeast Two-hybrid system

In this report a split-ubiquitin Yeast Two-Hybrid (Y2H) technique (7) was used to detect and identify interactions of the MR domain of $CERT_L$ with other proteins. Using adapted protocols from the DUALhunter system (8), the goal was to detect protein interactions that could not be visualized using the normal Y2H system. The technique is based on bringing the two parts of one complete ubiquitin molecule together. Together these two parts of ubiquitin, the C-terminal Ubiquitin (CUb) and the N-terminal Ubiquitin (NUb) have a strong affinity for each other. Mutating the NUb protein diminishes this affinity. In the DUALhunter system this mutated NUb (NUbG) is used because of its low affinity for CUb (7). Proteins are attached to each part of the ubiquitin. The bait (MR of $CERT_L$) is attached by its N-terminus to a small ER membrane anchor (the ER protein Ost4). By its C-terminus, the bait is attached to a reporter casset, which consists of the CUb and the transcription factor (TF) LexA-VP16 (Figure 1a). The prey protein (possible interactor) is fused to NUbG (Figure 1b). When no interaction between the bait protein and one of the prey proteins is present, no increased transcription of reporter genes is measured (Figure 1a). However, if an interaction between the bait and prey is present, the two proteins will come in close contact with each other. Their attached ubiquitin halves meet, which results in the formation of one complete ubiquitin molecule, the split-ubiquitin. The formation of the split-ubiquitin activates ubiquitin proteases, which make a cut between the CUb and the LexA-VP16 TF. The TF is released and diffuses into the nucleus to turn on transcription of the reporter genes (Figure 1c).

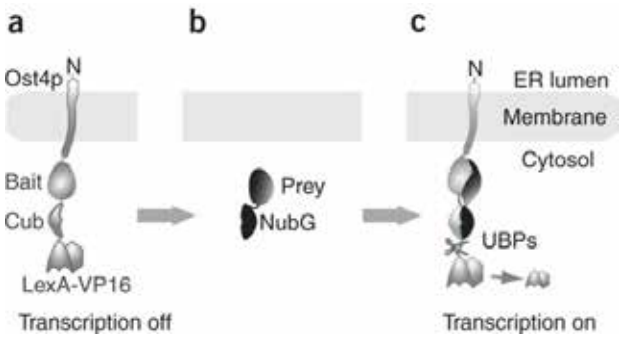

Figure 1. The split-ubiquitin Y2H system (7)

The DUALhunter kit

To perform the Y2H screenings, the DUALhunter kit (P01601-P01629) from Dualsystems Biotech was used (8). The DUALhunter screening comprises three major steps, which are divided in multiple other steps. The first major step was the construction of the bait (MR of the $CERT_L$ protein) and verification of its expression. The second step was the library transformation and the screening for positive clones. After this step positive interactors were selected and glycerol stocks of these interactors were prepared to perform a new Y2H screening. These first two major steps of the Y2H system were executed in a previous Y2H screening (9). The third step was the confirmation of the positive interactors and the analysis of the sequence. This third major step was partly performed in this report.

The previously made glycerol stocks were grown on Synthetic Dextose (SD) plates deficient for tryptophan and leucine (SD-trp-leu) for 3 to 4 days. The absence of leucine selected for the presence of the bait and the absence of tryptophan selected for the prey. One colony was picked from each plate and inoculated in liquid SD-trp-leu medium to grow overnight. Then a DNA minipreparation (miniprep) and a β-galactosidase assay were performed. A DNA miniprep is another name for the plasmid purification from bacteria or yeast (8). The isolated plasmid DNA (pDNA) was transformed in E.coli. Two colonies were selected and a stock of these colonies was made. These colonies were used to isolate the pDNA out of the E.coli competent cells. The E.coli pDNA (EpDNA) was visualized using an 1% agarose gel. At the same time the EpDNA was re-transformed in yeast cells. To select for positive interactors, the yeast was grown on selective and non-selective plates. If the interaction between bait and prey was confirmed, then the EpDNA that was isolated out of the DH5α E.coli competent cells, was send for sequencing (GATC Biotech AG, Konstanz, Germany) and identified using the nucleotide BLAST algorithm (National Library of Medicine).

Aim of the study

The goal of this study is to gain more understanding about the involvement of CERT in AD. Investigating the binding properties of $CERT_L$ in the human brain will help in accomplishing this goal. The protein binding activity of $CERT_L$ in the human brain will be examined using the split-ubiquitin Y2H system. In a previously executed Y2H screening (9), glycerol stocks were made of the yeast NMY51 containing the bait construct and the prey plasmid. To confirm the results from this previous screening, a new Y2H screening had to be performed on these glycerol stocks. The previously made glycerol stocks were grown on SD-trp-leu selective plates and one colony was picked and inoculated in liquid SD-trp-leu medium to grow overnight. With the use of the EZNA plasmid DNA minikit I, a DNA miniprep was

performed on this overnight culture to purify the prey plasmid. Subsequently, the isolated pDNA had to be transformed in DH5α competent E.coli cells.

Technical problems were encountered with these parts of the Y2H technique. When a DNA miniprep was performed on the overnight culture of the yeast NMY51, very low amounts of pDNA were obtained. If this low amount of pDNA was used, it was not possible to successfully transform the prey plasmid in DH5α competent E.coli cells.

In this report a troubleshooting was performed to investigate if there was a problem with the plasmid purification technique that has been used. Initially it was believed that there was a problem using the EZNA plasmid DNA minikit I.

Material and methods

β-galactosidase assay

To measure the strength of the interaction between bait and prey, the yeast colony β-galactosidase assay protocol (quantitative method) was performed using the yeast β-galactosidase assay kit (Life technologies, Rockford, United States). A negative control was included using the yeast NMY51 that only contained the bait.

Plasmid purification from yeast using EZNA plasmid DNA minikit I

The EZNA Plasmid DNA Mini Kit I (Omega bio-tek, Norcross, United States) was designed for plasmid isolation out of E.coli strains. A modified protocol of the centrifugation protocol (10) was used to purify the plasmid out of the yeast.

Troubleshooting EZNA plasmid DNA minikit I

Glycerol stocks from a previous screening (9) were grown on SD-trp-leu selective plates. One colony was picked and inoculated in liquid SD-trp-leu medium to grow overnight. Technical problems were encountered with the prey plasmid purification of the yeast NMY51 and with the transformation of the prey plasmid in competent E.coli cells using the EZNA plasmid DNA minikit I. Troubleshooting was executed to identify the problem. Aliquots were made from each step during the plasmid purification from yeast using the EZNA plasmid DNA minikit I protocol, to examine in which step the pDNA could have been lost. An 1% agarose gel was made to see if a plasmid band could be visualized in the aliquots. Other methods were tried to lyse the yeast. Next to this, other methods to isolate the pDNA, a QIAprep Spin miniprep Kit protocol (Qiagen, Hilden, Germany) and a phenol extraction and ethanol precipitation (adapted from Nori (11)), were performed.

Fresh yeast transformation of the yeast NMY51 with pDNA of a positive interactor

The yeast NMY51 containing the MR of $CERT_L$ was plated on SD-leu plates. A negative control was added by plating the yeast NMY51 containing the bait on a SD-trp-leu plate. The plate was streaked and inoculated into 25 ml liquid YPAD medium to grow overnight at 30 °C. The liquid culture was centrifuged down for 5 minutes (700 x g). The cell pellet was re-suspended in a twentieth volume of sterile water. 300 µl PEG/LiOAc master mix was prepared and added to 1.5 µg of prey EpDNA (sample ID 70.1) and 100 µl of re-suspended yeast NMY51 culture in an Eppendorf tube. The EpDNA was obtained during the previously executed Y2H screening (9). After vortexing for 1 minute the mixture was incubated for 45 minutes in a 42 °C water bath. The supernatant was removed after the mixture was centrifuged. The cell pellet was re-suspended in 200 µl of 0.9% NaCl. 100 µl of the transformed yeast was plated on a SD-trp-leu plate to select for succeeded transformations and 100 µl was plated on a YPAD plate to check if the PEG/LiOAc treatment was not to severe and killed the yeast. The plates were incubated for 3 days at 30 °C.

Transformation of prey plasmid in E.coli

40 µl of homemade DH5α E.coli competent cells were defrosted and added to 10 ng of yeast pDNA. The tubes were mixed by tapping and incubated for 15 minutes on ice. The cells were heat shocked for 45 seconds in a 42 °C water bath and placed on ice for 2 minutes. 900 µl of room temperature LB medium without ampicillin was added to help the cells regenerate. The cells were incubated in a heat block for 1 hour at 37 °C and centrifuged for 1 minute at 5000 RPM to form a pellet. The supernatant was removed and the cells were re-suspended in 2% LB medium. 100 µl of the re-suspended cells was plated on LB plates supplemented with 100 mM ampicillin per ml LB. The plate was incubated overnight at 37 °C.

Results

The 1% agarose gel of the aliquots showed that the problem was probably due to incorrect lysis of the yeast. The lysis of the yeast was investigated using a light microscope. Using the modified centrifugation protocol the yeast was lysed. Extracting the plasmid using the EZNA plasmid DNA minikit I from glycerol stocks from the yeast NMY51 containing both bait and prey did not succeed. Other methods to isolate the plasmid DNA were also unsuccessful. The manual of the DUALhunter Y2H technique was studied and compared to the protocols that were used in the previous Y2H screening (9). An error was found in a protocol used in the previous screening. After the library screening, which was performed in this previously executed Y2H screening, glycerol stocks were made of the yeast NMY51

and stored in the -80 °C freezer. The production of these glycerol stocks was not mentioned in the DUALhunter manual (8). The low yields of pDNA were possibly due to the yeast being stored as a glycerol stock. In order to correct for this, a freshly transformed yeast sample was made.

Miniprep fresh transformed yeast

A freshly transformed yeast sample was made by transforming a yeast NMY51 containing the bait, with the EpDNA (sample ID 70.1) of a previously identified positive interactor (9). By using the EZNA plasmid DNA minikit I and the QIAprep spin miniprep kit, pDNA was isolated and purified from the freshly transformed yeast. The use of both kits resulted in low yields of pDNA (~1 ng/µl). Both the 260/280 and 260/230 ratios were not within normal ranges (12). The same low pDNA yields were seen when the plasmid purification was performed on yeast that was stored in a glycerol stock.

Transformation in E.coli and E.coli miniprep

Ten transformations in DH5α E.coli competent cells were performed using pDNA that was isolated from yeast samples, which were stored in glycerol stocks. One transformation showed positive results using the pDNA of sample 56, which was stored in a glycerol stock. One very small colony could be obtained to perform a plasmid purification on. The pDNA yield from this plasmid purification was very low (~3 ng/µl; Table 1). The 260/280 and 260/230 ratios were not within normal ranges (12) and indicate for the presence of protein.

Table 1. Results Nanodrop1000 E.coli miniprep using Qiaprep spin miniprep kit

Sample ID	ng/µl	260/280	260/230
56	2.53	–2.65	2.63
70.1	207.2	1.93	2.4

The pDNA that was isolated from the freshly transformed yeast was successfully transformed in DH5α E.coli competent cells. One colony was obtained from this LB plate and used to perform a plasmid purification. The plasmid purification from the competent cells that were transformed with the pDNA from the fresh transformed yeast succeeded. This resulted in a normal EpDNA yield (~207 ng/µl; Table 1). The 260/280 and 260/230 ratios were within normal ranges (12).

The EpDNA that was extracted from the E.coli competent cells that were transformed with the pDNA from the fresh yeast was visualized using an 1% agarose gel (Figure 2; F). To rule out cross contamination, the EpDNA from the same plasmid that was used to transform the fresh yeast was also loaded in the gel (Figure 2; 70.1). When the EpDNA was loaded in an 1% agarose gel that was extracted from sample 56, which was stored in a glycerol stock, no plasmid band was visualized (Figure 2; 56).

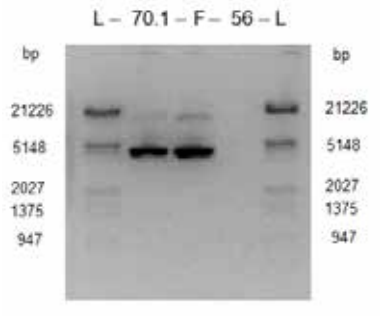

Figure 2. Result 1% agarose gel. The layout of the gel: EcoR1HindIII ladder (L) – EpDNA 70.1 used for the fresh yeast transformation (70.1) – EpDNA from the E.coli cells transformed with the pDNA from the fresh transformed yeast (F) – EpDNA sample 56 (56) – EcoR1HindIII ladder (L). bp; base pairs.

β-galactosidase assay

The yeast colony β-galactosidase assay was performed on the freshly transformed yeast NMY51 with the bait and a prey plasmid (sample ID 70.1) and on the yeast that was stored in a glycerol stock (sample ID 56). The β-galactosidase assay confirmed that the yeast cells from both the freshly transformed yeast and the yeast that was stored in a glycerol stock contained the galactosidase gene, meaning that both yeast samples contained the prey plasmid.

Discussion and Conclusion

When a DNA miniprep was performed, both the fresh transformed yeast and the yeast that was stored in a glycerol stock produced low yields of pDNA. The yeast NMY51 had a very low plasmid copy number before it was frozen into a glycerol stock. It is believed that it is not possible to store the yeast NMY51 containing the bait construct and the prey plasmid in glycerol stocks, because the plasmid yield decreases too much overtime. This could be due to integration of the plasmid into the genome. To confirm this hypothesis, a polymerase chain reaction (PCR) technique could be executed. If the pDNA has integrated

into the genome of the yeast, conducting a PCR using primers complementary to the pDNA should produce positive results.

Yeast strains can be stored in 15% glycerol stocks for more than three years as long as the temperature is maintained below - 55 °C (8). It is not desirable for the prey plasmid to integrate into the yeast genome during storage. Moreover, the prey plasmid should not effectively integrate into the genome since the plasmid is uncut and the library vector contains a yeast origin of replication (13, 14). Working with yeast takes a long time due to its long incubation times, thus the production of glycerol stocks might be of importance in further studies. The use of other yeast strains, which are less prone to integration into the genome might improve the Y2H system.

As described previously, there is a strong link between AD, ceramide and CERT. The discovery of new protein interactors of CERT might provide more insight into the role of CERT in AD. Next to this, the identified protein interactors may also play an important role in AD. Moreover, possible therapeutic candidates for AD can be explored and investigated. This creates possibilities and opportunities for future research.

Next to a new Y2H screening, other systems are available to investigate the protein binding activity of CERT with its possible interactors. As a continuation of the project, the protein binding could be re-confirmed in a in vivo co-immunoprecipitation model. The next step would be to clone the cDNA of the possible protein interactors of CERT in mammalian cells to investigate if these protein-protein interactions also take place in mammalian cells. Moreover, knockout studies could be performed to gain more insight into the biological functions of these proteins.

Initially, It was hypothesized that there was a problem with the EZNA plasmid DNA minikit I. It can be concluded that this initial hypothesis was not correct. At this point it is believed that it is better to use a fresh transformed yeast to perform a Y2H screening. It is not possible to store the NMY51 yeast containing the prey plasmid in glycerol stocks, because the plasmid yield decreases too much overtime. This could be due to integration of the plasmid into the genome. To confirm this hypothesis, a polymerase chain reaction (PCR) technique to amplify pDNA in the genomic DNA could be performed.

Role of the student

Anne Jansen is a bachelor student working under the supervision of associate professor P. Martinez-Martinez. The design of the troubleshooting, the processing of the results as well formulation of the conclusions and the writing were performed by the student.

References

1. Mayeux R. Epidemiology of neurodegeneration. Annu Rev Neurosci. 2003;26:81-104. 2.
2. Association As. Alzheimer's Disease Facts and Figures. 2014.
3. Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, et al. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. Proc Natl Acad Sci U S A. 2004;101(7):2070-5.
4. Puglielli L, Ellis BC, Saunders AJ, Kovacs DM. Ceramide stabilizes beta-site amyloid precursor protein-cleaving enzyme 1 and promotes amyloid beta-peptide biogenesis. J Biol Chem. 2003;278(22):19777-83.
5. Mencarelli C, Martinez-Martinez P. Ceramide function in the brain: when a slight tilt is enough. Cell Mol Life Sci. 2013;70(2):181-203.
6. Mencarelli C, Losen M, Hammels C, De Vry J, Hesselink MK, Steinbusch HW, et al. The ceramide transporter and the Goodpasture antigen binding protein: one protein--one function? J Neurochem. 2010;113(6):1369-86.
7. Möckli N, Deplazes A, Auerbach D. Finding new protein interactions using the DUALhunter system. Nature Methods. 2008 Feb.
8. Biotech D. DUALhunter starter kit user manual (P01601-P01629).
9. Karpati A. Identification of novel protein-protein interactions of the ceramide transporter in the human brain [Junior Thesis]. Maastricht: Maastricht University; 2014.
10. OMEGAbio-tek. Product Manual E.Z.N.A.® HP Plasmid DNA Mini Kit I2013.
11. Nori DV, McCord BR. The application of alkaline lysis and pressure cycling technology in the differential extraction of DNA from sperm and epithelial cells recovered from cotton swabs. Anal Bioanal Chem. 2015.
12. Technologies N. 260/280 and 260/230 Ratios NanoDrop® ND-1000 and ND-8000 8-Sample Spectrophotometers2007.
13. Clontech Laboraties I. Yeast Protocols Handbook. 2009.
14. Bergman L. Two-Hybrid Systems. 2001. In: Methods and Protocols [Internet].