

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Recognition and confirmation of the RNA-binding properties of
human transglutaminase 2

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transglutaminase 2**

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1. Introduction

Transglutaminases catalyze post-translational modifications of proteins and are therefore of great importance in both physiological and pathological conditions.

One of the best-studied members of this family is tissue transglutaminase (transglutaminase 2, TG2), which is a multifunctional protein. It has various enzymatic and non-enzymatic activities. One major group of its enzymatic activities is Ca^{2+} -dependent transglutaminase activities, which include protein cross-linking, amino acid incorporation into proteins, deamidation, and isopeptidase activities. In addition, it also has protein kinase, protein disulfide isomerase, GTPase, and ATPase activities. TG2 has two main conformations, open and closed, the ratio of which is determined by the relative proportions of Ca^{2+} and nucleotides that stabilize them. Nucleotides stabilize the closed conformation of the protein and are negative regulators of transglutaminase activity. Ca^{2+} , on the other hand, relaxes the TG2 structure, and the resulting open conformation facilitates the binding of the appropriate transglutaminase substrate to the catalytic site of the enzyme.

Endothelial cells, such as the human umbilical cord vein derived endothelial cells (HUVEC) used in our experiments, are particularly important in vascular homeostasis and express TG2 protein in large quantities. TG2 may play a role in endothelial cell adhesion, angiogenesis, inflammatory responses, and blood pressure regulation by interacting with various proteins and influencing blood vessel stiffness. In addition, it may contribute to a number of other biological processes (e.g., signal transduction, phagocytosis, apoptosis) and has been linked to immunological, tumor, and neurodegenerative diseases.

To gain a mechanistic understanding of the biological role of TG2, we identified TG2-associated proteins, which raised the possibility that TG2 may influence post-transcriptional regulation. It was already known that TG2 protein knockout can cause changes in the expression of several genes, but the mechanism behind this was unclear. We also revealed that TG2 is capable of RNA binding. Thus, it can regulate RNA metabolism both through its binding to RNA and by binding to RNA-binding proteins as a scaffolding protein.

1.1. Transglutaminase enzyme family

Transglutaminases (TGs) are members of a family of enzymes that share the common feature of catalyzing the formation of covalent isopeptide bonds between the glutamine and lysine side chains of proteins in a Ca^{2+} -dependent manner. This transamidation reaction creates ϵ -(γ -glutamyl)lysine isopeptide bonds between proteins and releases ammonia. The resulting cross-linked protein polymers are extremely stable and resistant to proteolysis, which is why

transglutaminases are also called "nature's biological glues." In the presence of primary amines, TGs can also incorporate these amines into the glutamine side chains of proteins or, in the absence of suitable amine substrates, catalyze the deamidation of glutamine to glutamate. Calcium is required as an activating cofactor for transglutaminase activity, but some microbial transglutaminases are Ca^{2+} -independent. Nine TG family members have been identified in humans, eight of which (coagulation factor XIII (FXIII-A) and TG1–TG7) have catalytic activity. The only member of the human enzyme family that does not possess enzymatic activity is the 4.2 band protein, which is found in the human erythrocyte membrane (human erythrocyte membrane 4.2 band protein). Its enzymatic activity was inactivated by a change in cysteine in the catalytic triad. Overall, TGs play a variety of roles in physiological processes by cross-linking proteins, thereby modifying their structural and functional properties and thus influencing and regulating, for example, cell adhesion, survival, and cell membrane integrity, or even red blood cell oxygen binding.

1.2. The transglutaminase 2

Transglutaminase activity was first demonstrated in liver extract by Waelsch and colleagues, who identified that the incorporation of primary amines requires metal ions. This protein was tissue transglutaminase. It was subsequently studied in detail and it was described that Ca^{2+} ions are the metal ions that most effectively activate the transamidase activity of the TG2 protein. TG2, like other members of the TG family, has four domains. The fibronectin and integrin binding sites are located in the N-terminal β -sandwich domain, the central catalytic domain is responsible for its enzymatic activities and Ca^{2+} binding site, and the second of the two C-terminal β -barrel domains contains the phospholipase C binding site. The unique purine nucleotide binding site is located in the cleft between the catalytic domain and the first C-terminal β -barrel domain.

1.2.1. Enzymatic and non-enzymatic activities of TG2 protein

In addition to transglutaminase (incorporation of amino acids into proteins, cross-linking of proteins, deamidation, and isopeptidase activity) protein kinase, GTPase, and ATPase activities, which are enzymatic, Ca^{2+} -dependent activities, TG2 protein also has non-enzymatic functions. Through its enzymatic reactions, it promotes, for example, the stabilization of extracellular matrix (ECM) proteins through cross-linking. Its non-enzymatic reactions perform functions based on physical interaction with proteins rather than chemical modifications of substrates. As a G protein in cells, it can influence G protein-coupled receptor (GPCR) signaling pathways by

hydrolyzing GTP, and it can also play a role in NF- κ B, EGF/EGFR, and FAK signaling pathways. It can function as a co-receptor by binding to certain subunits of the integrin molecule, facilitating cell adhesion and migration. It binds and activates the phospholipase C (PLC) molecule, thereby increasing the level of Ca^{2+} as a secondary messenger. TG2 can also influence numerous biological processes through protein interactions, which were the focus of my research into the TG2 interactome.

1.2.2. Conformational states of TG2

The TG2 protein has two known conformational states, which are formed and stabilized depending on the ratio of Ca^{2+} to nucleotides. Nucleotides present in the cell, such as GTP, GDP, and ATP, negatively regulate the transglutaminase activity of the protein, while Ca^{2+} positively influences it. The binding of nucleotides in a closed conformation stabilizes the structure of TG2, resulting in the catalytic site being covered. Ca^{2+} ions exert their effect through electrostatic interactions. Ions bind to negative surfaces in the catalytic domain, causing the N-terminal β -sandwich and C-terminal barrel domains to move apart, thereby relaxing and opening the protein structure. The formation of the open conformation makes the active site accessible to the substrate molecule, facilitating substrate binding. So the TG2 enzyme has a GTP-bound, catalytically inactive form and a Ca^{2+} -bound, open, catalytically active form. The GTP-bound state can be suspended by adding Ca^{2+} , which results in an enzyme with an open conformation and transglutaminase activity. A third conformation can also develop in which oxidation causes a disulfide bond to form in the Ca^{2+} -bound open, transglutaminase-active form, thereby inactivating the otherwise open form. The decrease in activity caused by oxidation can be prevented by adding thioredoxin.

1.2.3. Interactome of transglutaminase 2

Its multi-domain structure, structurally disordered regions (IDRs), short linear motifs, and alternating enzymatic and non-enzymatic functions enable TG2 to act as a versatile molecular cross-linker and signal transduction modulator in cells through its binding to partner proteins. This functional versatility explains why TG2 is such an important protein in the human body and why its dysregulation can lead to the development of numerous diseases, such as cancer, fibrotic conditions, cardiovascular and neurodegenerative diseases.

TG2 can interact with integrin (ITGB1), syndecan-4 molecules, and growth factor receptors (PDGFR, VEGFR) to regulate cell adhesion, migration, and ECM stabilization. Through

interaction with matrix metalloproteinases (MMPs), it contributes to the degradation of the extracellular matrix (ECM). Through these various protein interactions, it regulates physiological processes in the body, such as ECM stabilization (e.g., integrin, fibronectin), tissue regeneration (e.g., syndecan-4, PDGFR), apoptosis (e.g., BAX, BAK), phagocytosis (e.g., MFG-E8), and cell survival (e.g., HSP27, HSP20).

1.2.4. Role of TG2 in HUVEC cells

Almost all cells of the cardiovascular system, smooth muscle cells, endothelial cells, including human umbilical vein endothelial cells (HUVEC) used by us, express high amount of TG2 protein.

The TG2 protein may play a role in blood pressure regulation with the help of endothelial and smooth muscle cells. It has been investigated whether TG2 cross-linking activity can be inhibited in endothelial cells by the effect of NO produced in a reaction catalyzed by endothelial nitric oxide synthase (eNOS) through the resulting S-nitrosylation modification. NO production decreases with age, thus increasing TG2 cross-linking activity in endothelial cells, which may contribute to the cross-linking of extracellular matrix proteins. This process can lead to the development of cardiovascular diseases by increasing stiffness of the blood vessels.

TG2 is present in HUVEC cells, which are therefore a good model for studying endothelial TG2 functions. It has been shown that silencing the TG2 protein reduced the amount of focal adhesion kinase (FAK), thereby reducing the adhesion ability of cells. Following flow cytometric measurements, it was observed that TG2 silencing increased the proportion of apoptotic endothelial cells.

However, further experiments are required to clarify the exact biological function of TG2 in endothelial cells.

1.2.5. RNA-binding proteins

RNA-binding proteins (RBPs) are proteins that bind to RNA and modulate the function of the bound RNAs. RBPs bind to sequences or structural motifs within RNA through RNA-binding domains such as RNA-binding motifs, K-homology, zinc fingers, intrinsically disordered regions (IDRs), and DEAD box helicase domains. RNA-binding proteins (RBPs) may participate in the formation of ribonucleoprotein (RNP) complexes, which are essential for gene expression.

Interactions between RNAs and proteins trigger complex regulatory mechanisms in cells. RBPs are key regulators of cell differentiation and organ development and play a central role in many pathophysiological processes. In the cytoplasm, RBPs regulate the proper localization and

stability of mRNAs, as well as the efficiency of mRNA translation. In the nucleus, some RBPs function as alternative splicing regulators, particularly during nervous system development or in tissue-specific processes. In addition, RBPs mediate post-transcriptional regulation of mRNAs, which stabilizes the efficiency of gene expression.

RNA-binding domains (RBDs) are structural elements of RBPs that enable interaction between RNA and protein. The domains consist of fewer than 100 amino acids, and only a few amino acids are involved in direct binding to RNA. For specific binding, RBPs can bind to multiple RNA-binding regions through hydrogen bonds or weaker interactions. However, TG2 does not contain canonical calcium or nucleotide binding domains, and bioinformatics approaches cannot predict any RNA-binding domains.

2. Aims

Transglutaminase 2 protein is a conserved protein that plays a role in numerous biological processes. It is expressed in large amounts in endothelial cells, but the mechanism of its physiological roles is not fully understood.

In order to gain a more thorough understanding of the biological and potential therapeutic significance of the TG2 protein, our research aims to investigate the following:

- exploration of the TG2 interactome from HUVEC cell extracts using two approaches:
 - using site-specifically biotinylated human recombinant TG2 protein and neutravidin agarose, and
 - co-immunoprecipitation of TG2-associated complexes assembled in cells from HUVEC cell extract expressing silenced endogenous TG2 and transgenic 3xFlag-TG2 protein using anti-Flag agarose and identification by mass spectrometry.
- confirmation and characterization of the RNA-binding ability of TG2.

3. Materials and methods

3.1. Cell lines used

In our experiments, we used previously established human umbilical cord vein derived endothelial cells (HUVECs) immortalized by overexpression of the telomerase gene.

For the cell lines, we used M199-based medium for cell culture, supplemented with 2% HEPES, 1% penicillin-streptomycin, 1% amphotericin B, 10% EGM-2, and 10% FBS reagents. Cell culture was performed in a 37°C thermostat with 5% CO₂.

3.2. Design and cloning of an inducible endogenous TG2 protein silencing vector

First, the pLKO Tet-On (Puro) empty vector was opened with EcoRI-HF and AgeI-HF restriction endonucleases, then separated by 1% agarose gel electrophoresis, and the linearized vector was purified from the gel. The primer pairs encoding the silencing oligonucleotide sequence were hybridized and then ligated into the opened vector with T4 DNA ligase. The end product of the ligation reaction was transformed into chemically competent DH5 α bacterial cells. The isolated vectors were checked by XhoI restriction endonuclease digestion and sequencing.

3.3. Construction and cloning of the vector expressing the TG2 transgene

The pENTR-4-3Flag vector, previously constructed by insertion mutagenesis, was opened with EcoRI restriction endonuclease. The phosphate at the 5' end was removed with alkaline phosphatase. The opened vector was purified from the gel after 1% agarose gel electrophoresis. The TG2 cDNA sequence was cut out from the pET30ek/LIC-hTG2 vector previously developed by the working group using EcoRI restriction endonuclease. The cDNA sequence encoding the TG2 protein was then separated by 1% agarose gel electrophoresis. The TG2 insert was purified from the gel. The TG2 cDNA sequence was ligated into the opened pENTR-4-3Flag vector using T4 DNA ligase. The transformation process, plasmid isolation, and verification were performed similarly to the endogenous TG2 silencing vector.

The resulting pENTR4-3xFlag-TG2 and pCW57.1 vectors were recombined using Gateway LR Enzyme Mix II. The pCW57.1 vector contains the genetic elements necessary for doxycycline-inducible transgene expression. The mixture was transformed into Stbl3 bacterial cells. After plasmid isolation, the clones were verified by XbaI restriction endonuclease digestion followed by sequencing.

3.4. Establishment and selection of TG2 silenced and transgenic TG2 expressing HUVEC cell lines

Cells were infected with the endogenous TG2 protein silencing vector on 12-well plates after reaching 70% confluence following medium exchange. Cell selection was performed by replacing the medium with M199-based medium containing 1.5 µg/ml puromycin after 48 hours. After another 48 hours, we replaced the medium with 4 µg/ml puromycin and continued until the control cells, which had only been replaced with basic medium, died. After the cells had grown into the trough following selection, we transferred them to 6-well plates and then to T25 and T75 flasks. In the case of the vector expressing the TG2 transgene protein, the infection of cells previously checked for TG2 silencing (TG2-KD) was performed in a similar manner as with the silencing vector, but during selection we used a culture medium containing 6 µg/ml puromycin. The cell line thus established was named TG2-KD+3F-TG2 HUVEC cell.

3.5. TG2 silencing and transgene induction in the TG2-KD+3F-TG2 HUVEC cell line

Silencing of endogenous TG2 protein and expression of transgenic TG2 protein in double-infected cells was performed by adding 4 µg/ml doxycycline. The culture medium containing doxycycline was replaced every two days in the cell lines developed. At least 3 weeks were required for proper induction of silencing.

3.6. Recombinant protein production and purification

For the production of site-specifically N-terminally biotinylated human recombinant TG2 protein (N-BAP-rhTG2), we used the pET-30ek/LIC-hTG2 bacterial expression vector encoding the human TG2 cDNA sequence, and then expressed the N-terminally biotinylated rhTG2 protein in *E. coli* Rosetta 2 (DE3) bacterial cells by adding IPTG. A His GraviTrap column was used for protein purification.

The recombinant His6-tagged human TG2 protein and the expression-terminated (TEV protease) TG2 protein were produced in *E. coli* Rosetta 2 (DE3) cells by IPTG induction according to a previously established and described method, and then purified using a His GraviTrap column.

The C-terminal β-barrel domain truncated TG2 (TG2 1-584) protein was cloned from the pET-30ek/LIC-hTG2 vector, and the mutants were generated in the same vector using the QuikChange Site-Directed Mutagenesis Kit.

3.7. RNA isolation and RT-qPCR measurement

For RNA isolation, cells were lysated by trypsin-EDTA treatment (5 min, 37 °C) and washed twice with ice-cold PBS. The cell pellet was suspended in TRI reagent and incubated at room temperature for 10 minutes. After adding chloroform, isopropanol was added proportionally to the upper, colorless phase containing the RNA molecules, and the mixture was incubated at room temperature for 10 minutes. After centrifugation, the RNA pellet was washed with 70% ethanol cooled to -20°C, then dried after centrifugation. For reverse transcriptase PCR, 200 ng/μl RNA was transcribed into cDNA. TaqMan assays were used for qPCR measurement. Transcription levels were normalized to actin B levels and gene expression was determined using the CT method.

3.8. Western blot

Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then semi-dry blotting was performed. A 5 (w/v) % low-fat milk powder 1x TTBS solution was used for blocking. A 0.5% (w/v) low-fat milk powder 1x TTBS solution was used for dilution of primary and secondary antibodies.

3.9. Adhesion assay

On the day before the measurement, the culture medium was replaced with starvation medium containing only 1% FBS instead of 10%. The F16 Black Maxisorp plate was coated with fibronectin, then 1000 cells/200 μl HUVEC cell suspension was measured into the wells, followed by 1 hour of incubation. Non-adherent cells were removed by washing with PBS, and after adding medium containing Cell-Titer Blue reagent, we measured the increase in fluorescence intensity proportional to the number of adherent cells using a BioTek Synergy HT device (Ex:530 nm/Em:590 nm) and determined the change in intensity.

3.10. Flow cytometry measurement

After trypsin-EDTA treatment, the cells were washed with PBS solution. The cells were stained with Annexin-V and propidium iodide reagent for 15 minutes in the dark. In the control case, only Annexin-V binding buffer was used. The cells were then analyzed using a FACSAria™ III flow cytometer.

3.11. Isolation of proteins associated with site-specifically biotinylated human recombinant TG2 protein using High Capacity Neutravidin agarose beads and their identification by mass spectrometry

100 µg of site-specifically biotinylated recombinant human TG2 protein (rhTG2) was immobilized on 250 µl of equilibrated Pierce High Capacity Neutravidin agarose beads. After biotin blocking, affinity purification was performed by adding 2.5-3 mg of HUVEC cell extract overnight with continuous rotation at 4 °C. The cell pellet was suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5% NP-40, 1 mM PMSF), which contained PIC (Protease Inhibitor Cocktail) at a 100-fold dilution and PhosSTOP (Phosphatase Inhibitor Cocktail Tablets, Roche) at a 1-fold dilution to prepare the cell extract. The next day, the beads were washed three times with CoIP buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 5% glycine, pH 7.4), and then the potentially interacting proteins were eluted with 0.1 M glycine-HCl buffer (pH 3.5). For the elution of strongly bound partners, we also used 6x Denaturation Buffer (300 mM Tris-HCl, pH 6.8; 12 (m/v)% SDS, 20 (v/v)% glycerol, and 0.43 M β-mercaptoethanol) and heat denaturation (10 min, 100 °C). The samples were run on SDS polyacrylamide gel, and after separation and Coomassie blue staining, the unseparated band containing all proteins was cut out and sent for LC-MS/MS analysis.

3.12. Identification of protein complexes associated with transgenic 3F-TG2 protein using anti-Flag M2 agarose beads

3-3.5 mg of protein-containing HUVEC cell extract was measured on 300 µl, equilibrated monoclonal anti-FLAG M2 agarose beads and incubated overnight. To determine non-specific background proteins, mouse IgG agarose beads were used as a control. After three washing steps, potential interaction proteins were eluted with acidic and then denaturing buffer. The eluted proteins were run on a 10% SDS polyacrylamide gel, and after Coomassie blue staining, the band containing the unseparated proteins was cut out and sent for LC-MS/MS analysis.

To identify conformation-dependent interaction partners, the TG2 protein was treated with various modulators. To stabilize TG2 in the open state, cells were pretreated with 15 µM NC9 modulator 24 hours before cell extract preparation. To preserve the closed conformational state of TG2, we added 1 mM GTPγS inhibitor to the cell extract, as GTPγS does not penetrate living cells unlike the NC9 modulator.

3.13. Orthogonal organic phase separation

RNA-binding proteins were isolated by orthogonal organic phase separation (OOPS). RNA-protein complexes in cells were covalently cross-linked using 400 mJ/cm² UV light with a GS Gene Linker UV Chamber. The cells were then lysed with TRI reagent, and phase separation was performed with chloroform added in a 1:5 ratio. The interphase between the upper and lower phases was washed three times (first with TRI reagent, then with chloroform added, and the phases were separated again, repeating the process three times). The interphase, which contained UV-crosslinked RNA-protein complexes, was precipitated with methanol (9:1), sonicated, and treated with 5 µg RNase A reagent. The released RNA-binding proteins were separated with TRI reagent again, followed by chloroform treatment. We then used the organic lower phase and precipitated it with methanol. For Western blotting, we added 6x denaturing buffer supplemented with 50 mM dithiothreitol (DTT) to the samples. We also identified the protein content of the samples by mass spectrometry.

3.14. Magnetic RNA-protein pull-down experiment

The Pierce RNA 3' Desthiobiotinylation Kit and Pierce Magnetic RNA-Protein Pull-Down Kit were used to detect RNA-protein interactions. The 3' ends of the 43-mer RNA molecule (Kit accessory) and total RNA isolated from HUVEC were desthiobiotinylated using the Kit, and the labeled RNA samples were bound to streptavidin magnetic beads. Human (His)₆-labeled recombinant TG2 protein was added to the magnetic beads containing immobilized RNA. After washing, the RNA-bound proteins were eluted using 6x Denaturing Buffer and analyzed by 10% SDS polyacrylamide gel electrophoresis and Western blot.

To examine the conformation dependence of TG2 RNA binding, the recombinant protein was preincubated with 0.1 mM NC9 modulator to stabilize the open conformation in the presence of 5 mM CaCl₂. For the closed conformation, the labeled RNA-bound magnetic bead was incubated in the presence of 0.5 mM GTPγS inhibitor.

3.15. Measurement of TG2 transamidase activity

The method examines the incorporation of dansylcadaverin into N, N'-dimethylated casein by the TG2 protein. The reaction mixture contained 0.2 mM dansylcadaverine, 2 mg/ml N, N'-dimethylated casein, 3 mM DTT, and 10 mM CaCl₂ solution. The activity measurement was initiated by adding TG2 protein at a concentration of 200 nM in 100 µl of reaction mixture. The

activity was calculated based on the nearly linear phase of increasing fluorescence intensity (Ex/Em: 360/530 nm).

3.16. Fluorescent measurement of TG2 isopeptidase activity

Zedira A102 fluorescent substrate was used to determine TG2 isopeptidase activity. The method is that transglutaminases are capable of cleaving the isopeptide bond in the synthesized substrates, while releasing a quencher molecule (2,4-dinitrophenyl), resulting in an increase in fluorescence intensity. The reaction was initiated by adding TG2 protein at a concentration of 150 nM in a total reaction mixture of 200 μ l. The measurement was performed using a BIOTEK Synergy 4 device (Ex/Em: 318/413 nm).

3.17. Detection of RNA-protein interaction using biosensor interferometry

This method is an optical technique that can examine the kinetics and strength of interactions between molecules in real time. For the measurement, we used a Blitz device and the streptavidin biosensor recommended by the manufacturer, filling the sample holder with a binding buffer containing 10 μ g/ml of biotinylated 43-mer RNA molecules. The purified recombinant TG2 protein was then added to the drop sample holder. A tube sample holder was used for the other steps. The switch between the two different sample holders caused a shift in the curve between the experimental steps. Step correction was applied to eliminate these shifts between subsequent experimental steps. We used BLItz Pro 1.2.1.5 software to determine the dissociation constant (KD) of the bonds between molecules.

3.18. LC-MS/MS analysis

To identify RNA-binding proteins after orthogonal phase separation and to identify the TG2 interactome, the samples were run on a polyacrylamide gel containing 5% collecting and 10% separating gels until they entered the separating gel. After Coomassie staining, the unseparated protein bands were cut out, the dye was washed out, and the samples were sent for mass spectrometry analysis to the Proteomics Service Laboratory of the Institute of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen. The results were in Scaffold 5.0 program (Proteome Software).

During the TG2 interactome analysis, we used a 1.0% FDR value in the Scaffold software, while in the case of identifying potential RNA-binding proteins (RBP), we did not use FDR filtering in order to avoid protein loss. We set a unique peptide criterion for each protein in all cases and verified the authenticity of each peptide MS/MS spectrum with the presence of at

least four consecutive b or y fragments (i.e., we accepted the protein as a true hit based on this criterion).

3.19. Bioinformatic analysis

The identified TG2-associated proteins were analyzed using the PANTHER 19.0 and STRING v12 databases. The most enriched GO Molecular Functions were ranked based on the false discovery rate (FDR) and the results were visualized using GraphPad Prism 8.0.1 software.

We examined the RNA-binding proteins identified by us in HUVEC cells using the RBP2GO database and found 20 newly identified RNA-binding proteins, which we analyzed using the STRING database and then listed the most enriched GO Biological functions based on the FDR (false discovery rate) value. The secondary structure of the 43-mer RNA used was predicted using RNAstructure v6.4 software. The potential RNA-binding ability of TG2 was estimated using the catRAPID online software. The visual representation of TG2 was created using Visual Molecular Dynamics Software (VMD).

4. Results and discussion

4.1. TG2 interactome analysis

The immortalized human umbilical cord vein derived endothelial cells (HUVECs) used in the experiments retained their morphological characteristics and, similarly to primary cells, highly expressed the TG2 protein. Before starting the experiments, we also verified by mRNA sequencing that the immortalized cell line retained the expression of classic endothelial marker proteins.

4.1.1. Identification of TG2 interaction partners from HUVEC cell extract using biotinylated human recombinant TG2 protein

First, we used a well-known, traditional technique, biotin-neutravidin agarose-based affinity chromatography. To prevent random biotinylation of the TG2 protein and its unpredictable steric effects on the interactome, we cloned a biotin acceptor peptide (BAP) into the N-terminal region of the vector encoding the recombinant TG2 protein. During affinity purification, the biotinylated recombinant protein was bound to High Capacity Neutravidin agarose beads, and after biotin blocking, HUVEC cell extract was added, from which TG2 interaction partners were bound. The partner proteins were then eluted and potential interaction partners were identified by LC-MS/MS analysis.

After control subtraction (non-specifically bound proteins), we identified 170 TG2-associated proteins. These included previously identified TG2 interaction partners, which confirms the reliability of our method. Based on STRING bioinformatic analysis of the findings, the most abundant GO *Cell Compartments* were *Focal Adhesion*, *Extracellular Exosome*, *Vesicle*, *Membrane surface*, and *Extracellular space*, which supports the presence of TG2 in exosomal vesicles, thus supporting one potential mechanism of TG2 externalization from HUVEC cells. When analyzing N-BAP-biotin rhTG2-associated proteins, the most enriched GO *Molecular Functions* include *Cadherin binding* and *Cell adhesion molecule binding*, which confirm the role of TG2 in cell adhesion and migration. However, the appearance of *RNA binding* as a GO *Molecular Function* is a new observation in the context of TG2 interaction partners.

4.1.2. Development and characterization of a HUVEC cell line expressing Tet-inducible, endogenous TG2-silenced and 3xFlag-tagged transgenic TG2 protein

In order to eliminate the disadvantages of affinity chromatography performed with recombinant protein expressed in bacteria and to take advantage of the high expression of TG2 protein in HUVEC cells, we decided to develop a new model system for the identification of TG2-associated proteins. For this purpose, we used the HUVEC cell line (TG2-KD+3F-TG2) developed during my work, in which the Flag tag on the TG2 protein facilitated the isolation and subsequent detection of protein complexes containing TG2 protein formed under native conditions. In living cells, the formation of functional protein complexes is a regulated process by chaperones. Assembly chaperones, transient co-translational interactions, and subsequent conformational changes facilitate the formation of protein complexes and the incorporation of the individual proteins that compose them in a specific order. Interaction with assembly chaperones helps to bring about the necessary conformational changes, which may be essential for binding to other proteins.

First, endogenous TG2 expression was silenced by lentiviral transduction into the immortalized HUVEC cell line by introducing the Tet-On puro shTG2 vector. After three weeks of doxycycline induction, we confirmed the reduction in TG2 levels at both the RNA and protein levels in the puromycin-selected TG2-knockdown HUVEC cell line (HUVEC TG2-KD). Next, a Tet-inducible vector containing the sequence encoding the 3F-TG2 protein was introduced into the TG2-KD HUVEC cell line (HUVEC TG2-KD+3F-TG2) via a second lentiviral gene transfer. After selection and three weeks of doxycycline induction, RT-qPCR results and Western blots showed that the introduction of the 3F-TG2 transgene restored TG2 levels at both the RNA and protein levels in the generated HUVEC TG2-KD+3F-TG2 cell line. In our experiments, the expression levels of other transglutaminase family members were not affected by silencing and transgene TG2 expression.

4.1.3. Functional characterization of cell lines using adhesion tests and determination of the proportion of apoptotic cells

We examined the adhesion capacity of the developed cell lines on a fibronectin-coated surface. TG2-KD HUVEC cells showed lower cell adhesion properties. The introduction of 3F-TG2 could not compensate for the observed decrease in cell adhesion processes, which may be due to the relatively large, hydrophobic 3xFlag tag of the protein.

We measured the proportion of apoptotic cells, which was higher in TG2-KD HUVEC cells than in the control and 3F-TG2-compensated cell lines, suggesting that the transgene TG2 can compensate for the silencing effect of endogenous TG2.

4.1.4. Identification of 3F-TG2-associated protein complexes assembled in HUVEC cells

Protein complexes containing TG2 were isolated using anti-FLAG M2 agarose beads with the help of 3xFlag labeling using HUVEC TG2-KD+3F-TG2 cell extract. Following LC-MS/MS analysis, control proteins (non-specifically bound proteins) were again removed from the list of hits identified using anti-FLAG M2 agarose beads. After control removal, 356 TG2-associated proteins were identified, 86 of which were identified by both approaches. A total of 114 proteins were detected with at least five unique peptides, which can be considered the most frequently occurring interaction partners.

4.1.5. Molecular functions and localization of proteins associated with the 3F-TG2 protein in the cell

Surprisingly, the most abundant GO *Molecular Function* for both the complete list and the interaction proteins detected with a minimum of 5 unique peptides was *RNA binding*.

Data obtained from protein findings associated with the 3F-TG2 protein also support the involvement of TG2 in vesicular transport in HUVEC cells. The richest GO *Cell Compartments* were *Extracellular Exosome*, *Vesicle*, and *Extracellular Space*, suggesting that TG2 is likely secreted from HUVEC cells by vesicular transport and may also influence the quantity and composition of vesicles.

4.1.6. The effect of conformational change on protein complexes associated with the 3F-TG2 protein

To examine the effect of TG2 conformational change on its interaction partners, we used modulatory treatments before or during co-immunoprecipitation, using anti-Flag M2 agarose resin. The open conformation of TG2 was stabilized by pretreating the cells with NC9, while the closed conformation was induced by incubation with GTP γ S during cell lysis and co-immunoprecipitation, as nucleotides cannot penetrate cells.

In the presence of NC9 or GTP γ S, we identified 294 and 160 proteins, respectively, after control subtraction, which indicated to us that the modulators stabilize and limit the number of conformational forms that can be formed.

Most of the protein hits were detected in all three experimental settings (untreated, NC9-treated, and GTP γ S-treated samples), suggesting that most of the interaction surfaces are independent of the open or closed conformation. Among these proteins, we find a large number of proteins involved in translation (ribonucleoproteins, translation factors), components of the cytoskeleton and focal adhesion complexes, as well as proteins related to metabolism and signal transduction processes. Among the hits binding to both untreated and NC9-treated TG2 protein, we identified numerous hits related to membrane transport in the most abundant GO *Molecular Function* category. This result raises the possibility that the open TG2 protein with transglutaminase activity may directly influence membrane transport processes and affect these transporter molecules.

To confirm the differences observed in the conformation-dependent TG2 interactome, we also performed data analysis using the PANTHER system. After GTP γ S treatment, we observed a change in the ratio of GO-Slim Molecular Functions. The stabilization of the closed conformation reduced the ratio of interaction proteins associated with the *Transporter Activity* category compared to untreated and NC9-treated samples, suggesting that the open conformation may ensure interaction with various membrane transporters.

The large number of partners interacting with the untreated TG2 protein (93 hits) suggests that the protein is flexible in its default state, which facilitates dynamic structural movements and plays a role in the formation of various interactions. Among these proteins, the most abundant GO *Molecular Functions* are related to *RNA binding*, structural, cell-cell and cell-extracellular matrix binding proteins.

The enrichment of RNA-binding proteins among TG2-associated proteins suggests that TG2 may play a previously unknown role in post-transcriptional regulation by forming RNA-binding protein complexes.

4.2. Confirmation of TG2 RNA-binding capacity

During the TG2 interactome analysis, following STRING analysis of TG2-associated proteins, the RNA-binding function was most enriched in the GO *Molecular Function* category in several cases. Since TG2 can be linked to gene expression disorders, it was suggested that it might itself be capable of RNA binding.

In order to determine whether TG2 is capable of RNA binding, the simplest solution was to isolate HUVEC RNA-binding proteins and detect TG2 in them. We used orthogonal organic phase separation (OOPS) for isolation.

To isolate RBPs, we cross-linked RNA-protein complexes using UV light, washed out uncross-linked proteins and nucleic acid molecules, and then applied RNA-degrading treatments (sonication, denaturation, RNase A digestion). Finally, we detected the presence of TG2 among the isolated RNA-binding proteins using Western blot.

To identify RBPs isolated from HUVEC cells, we also sent the samples for LC-MS/MS analysis. A total of 413 RNA-binding proteins were detected, of which TG2 was the 25th most common hit based on the number of unique peptides detected.

There was an overlap of 150 proteins between TG2-associated proteins (356) previously identified by coimmunoprecipitation techniques and RNA-binding proteins (413), which supports the idea that the TG2 protein, as a "scaffolding" or regulatory protein, may be involved in the assembly of RNA-protein complexes.

We checked the identified HUVEC RBPs in the RBP2GO database, where transglutaminases can be found in RNA-protein complexes. Based on the comparison, we identified 20 previously undescribed endothelial RBP among our findings, which, according to STRING analysis, can be linked to endothelial functions such as angiogenesis, tubule formation, and cell migration and tissue development, supporting the relevance of our results.

4.2.1. RNAs derived from HUVEC cells and a synthetic 43-mer RNA bind to the TG2 protein

In order to determine whether there are RNAs in HUVEC cells that bind to the TG2 protein, we used the total RNA fraction isolated from HUVEC cells as a "bait" in a magnetic RNA-protein pull-down experiment. First, the 3'-terminal end of the HUVEC total RNA preparation was labeled with desthiobiotin-conjugated cytidine and immobilized on streptavidin magnetic beads (Pierce RNA 3'-End Desthiobiotinylation Kit). The recombinant TG2 protein bound to the RNA-coated magnetic beads and produced a signal detectable by Western blot. The 43-mer RNA molecule provided by the manufacturer as a positive control for the labeling reaction also interacted with the recombinant TG2 protein, while in the absence of immobilized RNA, no nonspecific protein binding to the magnetic beads was detected.

In subsequent experiments, this 43-mer RNA was synthesized and used in a form labeled with biotin at the 3'-end as a "bait" to bind endogenous TG2 from HUVEC protein extract. Endogenous TG2, which is present in both primary and immortalized HUVEC cells, binds to the 43-mer RNA molecule used, indicating that TG2-RNA complexes are present in HUVEC cells. The secondary structure of the 43-mer RNA contains a loop similar to an AU-rich element

(ARE) and another, more prominent loop, which provide potential structural elements for interaction with RBP.

One biological significance of RNA binding to proteins may be riboregulation, where RNA binding regulates the biological activity and functions of the protein. Thus, the RNA molecule bound to the TG2 protein may provide a link between the various activities of TG2.

4.2.2. Characterization of the RNA-binding properties of recombinant TG2

A competition experiment was performed to characterize the interaction between TG2 and RNA. In the RNA-protein pull-down assay, recombinant TG2 protein was incubated in the presence of free, unlabeled 43-mer RNA and labeled 43-mer RNA molecules immobilized on magnetic beads. As the concentration of unlabeled 43-mer RNA increased, the amount of binding recombinant TG2 decreased. This also suggests that immobilized ribonucleic acid is responsible for TG2 binding to the beads.

To determine the overlap between the TG2 RNA and GTP/GDP or ATP/ADP nucleotide binding sites and to examine the sequence and structure requirements of the nucleic acid, we used single-stranded poly(dG) and poly(dA) homo-oligomer deoxyribonucleic acids in the pull-down experiments. No interaction was detected with poly(dA), while poly(dG) interacted with TG2. When performing the experiment with free 43-mer RNA and immobilized poly(dG) molecules, we observed competition between the two molecules, which reduced the binding of TG2 to the poly(dG) molecule. These results indicate that the guanine base binding site in the TG2 protein may be involved in the formation of the RNA-TG2 interaction.

To investigate whether the conformation of TG2 influences its binding to RNA, we performed further RNA-protein magnetic pull-down experiments with immobilized 43-mer RNA, poly(dG), or total RNA isolated from HUVEC cells in the presence of GTP γ S and under treatment with NC9 inhibitor. GTP γ S stabilizes TG2 in the closed conformation, while NC9 stabilizes it in the open conformation. Based on the results, GTP γ S prevented the interaction between TG2 and nucleic acids, while NC9 treatment did not affect RNA binding. This suggests that the RNA-binding surface of the TG2 protein is only accessible in its open conformation and is covered in its closed form.

4.2.3. Confirmation of the RNA-TG2 interaction using bio-layer interferometry

Bio-layer interferometry is an optical technique used for the real-time detection of molecular interactions. The strength of the interaction is inferred from changes in reflection on the sensor

surface during immobilization and complex formation. We used a Blitz instrument for our measurements, which can measure only one sample at a time.

To determine the binding affinity, we used recombinant, unlabeled, expression tag-free TG2 protein in increasing concentrations ranging from 2.5 to 200 $\mu\text{g/ml}$ after coating the streptavidin biosensors with an equal amount of biotinylated 43-mer RNA. Increasing TG2 concentration resulted in increased detected saturation signals. The K_D value calculated using BLItz Pro 1.2.1.5 software was 88 nM, using a 1:1 binding model.

When the poly(dG) molecule was immobilized on the sensor surface, the binding curves and K_D values were similar to those obtained with 43-mer RNA. Bio-layer interferometry also confirmed our Western blot results, showing no interaction between poly(dA) and TG2 protein. A study compared the RNA binding affinities of several proteins and identified a relatively wide binding range. For example, the K_D between iron regulatory protein 1 and ferritin H IRE RNA is 45 pM, while the K_D between nuclear RNA export factor 1 protein and CTE RNA is 90 nM. In our experiments, we determined a K_D value in the nanomolar range between TG2 and a biologically irrelevant RNA molecule, which is likely to be even higher when examining the interaction in natural cells.

4.2.4. The effect of 43-mer RNA on transglutaminase activity

Although RBPs can modulate the function of RNA during binding or release, the interaction may also affect the biological activity of the RNA-binding protein. We examined the potential effect of 43-mer RNA binding to TG2 protein on the activity of two different types of transglutaminase. RNA binding did not affect the transamidase or isopeptidase activity of the TG2 protein, even at high RNA concentrations. However, TG2 riboregulation *in vivo* cannot be ruled out in the presence of RNAs and substrates found in cells in their natural environment. RNA binding may be responsible for the fine regulation of TG2, influencing its Ca^{2+} -sensitivity, GTP/GDP binding, and sensitivity to reducing/oxidizing effects. RNA binding may influence the localization of proteins and thus their interactions with transport proteins.

4.2.5. In silico prediction and verification of the potential RNA binding site of TG2 by site-specific mutagenesis

In silico analyses were performed to identify the RNA binding site on the surface of TG2. The catRAPID algorithm calculates the probability of a protein binding to RNA. Based on the values calculated by the algorithm, the human TG2 sequence is unable to bind to the RNA molecule.

TG2 probably does not have typical RNA-binding domains, and its nucleotide and calcium binding sites are not canonical, but its disordered regions (IDRs) are often combined with RNA-binding motifs and may therefore participate in RNA binding.

Using VMD visualization software, we analyzed the structure of TG2 from the perspective of potential RNA-binding sites and motifs. First, we selected sections containing positively charged amino acids (similar to known RNA-binding motifs). Since our results showed that poly(dG) is also capable of binding to the TG2 protein, it was suggested that nucleotide-binding amino acids may also play a role in RNA binding. The known GTP-binding side chains are accessible in the open form, while in the closed form they are partially hidden and close to each other (they cannot bind to RNA), so the side chains in these regions may even function as potential RNA-binding sites and participate in RNA binding, based on our experiments. The GTP-binding amino acid side chains K173, F174, I175, K176, N177, I178, P179, N181, and Q186 located in the catalytic domain are not fully accessible in the closed conformation and are partially covered, so they may act as RNA-binding sites. During sequence analysis, we found several basic amino acids at the C-terminus that resemble typical RNA-binding motifs (H658, H662, K663, N667, 672K, K674, and K677), although these are also in surface positions in the closed conformation. In addition to these, there are several IDRs containing side chains that are often involved in RNA binding in the catalytic domain and C-terminal (R433, D434, E435, R436, E437, H441, K444, P446, E447, G448, S449, K598, Q599, K600, R601, and K602), which are also in surface positions in both conformations. In addition to these, there are several IDRs containing side chains that frequently participate in RNA binding in the catalytic domain and C-terminal (R433, D434, E435, R436, E437, H441, K444, P446, E447, G448, S449, K598, Q599, K600, R601, and K602), which are also in surface positions in both conformations, so they can also be excluded from RNA binding with high probability. Based on these findings, we prepared TG2 proteins with a truncated C-terminal domain and nucleotide-binding mutants containing an increasing number of substitutions at the nucleotide site.

The RNA-protein magnetic pull-down experiment showed that the removal of the last C-terminal domain did not affect the interaction between the truncated TG2 (1-584) protein and RNA, while in the case of nucleotide-binding site mutants, the removal of positively charged amino acids (by mutating amino acids 173KF174 to 173EA174 or amino acids 173KFIKN177 to 173EASED177), the binding of TG2 and RNA to each other was reduced. This suggests that these side chains may play a crucial role in RNA binding. The retained transglutaminase activity of the mutant TG2 proteins proves that the mutations did not result in a decrease in RNA binding due to altering the native structure of the protein.

Overall, our results suggest that TG2 protein has a previously unknown property, namely the ability to bind RNA molecules in a conformation-dependent manner. The RNA binding affinity of TG2 is relatively high, which may be even stronger with natural ligands and in a cellular environment. In order to gain a more accurate understanding of the RNA-binding properties of the TG2 protein and to explore its biological significance, it is necessary to study the structure of the TG2 protein in more detail, map the RNA binding sites, and identify the RNA molecules present in the cells that actually bind to it.

5. Summary

The most studied member of the transglutaminase enzyme family is tissue transglutaminase (TG2), which is a multifunctional protein. It has multiple enzymatic activities and may be involved in numerous biological processes (e.g., signal transduction, cell adhesion, apoptosis). To determine the precise physiological role of TG2 in endothelial cells, we used the human immortalized HUVEC cell line, which expresses the TG2 protein in large amounts under natural conditions.

Two approaches were used to explore the TG2 interactome. In one approach a site-specifically biotinylated recombinant TG2 immobilized on Neutravidin agarose resin was used to capture TG2 interaction partners from HUVEC cell extracts. In the other method, we developed a HUVEC cell line in which the TG2 protein was silenced and then restored by introducing a vector expressing a transgenic TG2 protein (TG2-KD+3F-TG2). Using the Flag tag, we bound TG2 together with its associated proteins to anti-FLAG M2 agarose resin. Following LC-MS/MS analysis, a total of 356 proteins were identified using the two methods, which were then analyzed using databases (STRING, PANTHER). Based on the number of unique peptides detected, the most enriched GO *Molecular Functions* were *Cell Adhesion* and *RNA Binding*. The role of TG2 in cell adhesion processes has already been proven, but its involvement in RNA-dependent processes has not yet been investigated.

The OOPS technique was used to identify RNA-binding proteins in HUVEC cells. Following LC-MS/MS analysis, 413 RNA-binding proteins were identified based on the number of detected unique peptides, including the TG2 protein. Compared to the proteins identified in the interactome study, 150 protein hits (42%) were detected in both cases, confirming the role of the TG2 protein in the functioning of RNA-protein complexes. We used a magnetic pull-down method to demonstrate the RNA-binding ability of TG2. Based on the results, the TG2 protein is able to bind to the RNA molecule when TG2 is stabilized in an open conformation. It is likely that in a closed conformation, the RNA-binding surfaces are hidden within the TG2 structure. In a bio-layer interferometry experiment, we determined the affinity of TG2-RNA binding to be 88 nM. This value may be even higher in a cellular environment with biologically relevant RNA molecules.

To gain a more accurate understanding of the RNA-binding properties of the TG2 protein, it is necessary to study the structure of the TG2 protein in more detail, map the RNA-binding sites, and identify biologically relevant RNA targets.

6. New scientific results

1. A new immortalised HUVEC cell line was generated using lentiviral gene transfer, in which endogenous TG2 expression was inducibly silenced and replaced with a 3xFlag-tagged transgenic TG2 protein. We demonstrated that the cell line can be reliably used to study the cellular functions of TG2, except for adhesion properties.
2. 495 previously unidentified TG2-associated proteins were identified in HUVEC cells using two different approaches. Based on the STRING analysis TG2 may influence GO *Molecular Functions* in HUVEC cells such as *Structural Molecule Activity*, *Cadherin Binding*, *Cell Adhesion Molecule Binding* and *Vesicular transport processes*.
3. We have revealed that the most enriched GO *Molecular Function* of TG2-associated proteins is *RNA binding*, suggesting that TG2 may influence RNA-dependent processes.
4. We have demonstrated that the use of inhibitors, probably by limiting the conformational states of TG2, reduces the number of potential interaction partners. In the presence of GTP γ S, which stabilizes the closed TG2 form, the number of interaction partners decreased to a greater extent than in the presence of NC9, which stabilizes the open form.
5. In the case of the NC9 inhibitor, the proportion of proteins involved in transport processes among TG2-associated proteins increased significantly, suggesting that TG2 in its open conformation may directly influence transporters and membrane transport processes.
6. RNA-binding proteins were identified in immortalized HUVEC cells using the OOPS method. The result suggested that TG2 itself possesses RNA-binding capability.
7. The RNA-binding ability of TG2 were confirmed using RNA-protein pull-down and bio-layer interferometry.
8. By characterizing the RNA-binding ability, we found that the open conformation is capable of binding RNA molecules, and that the RNA sequence and structure likely influence binding.
9. Using modeling and mutagenesis studies, we demonstrated that the GTP-binding site of the catalytic domain significantly influences the RNA-binding ability of TG2. There is likely to be overlap between the two binding sites.
10. Based on the high degree of overlap between RNA-binding proteins in HUVEC and TG2-associated proteins, TG2 may be a central player in the regulation of RNA metabolism.



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List of publications related to the dissertation

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