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Human transglutaminases in extracellular fluids: biochemical characterization of blood coagulation factor XIII isopeptidase activity and transglutaminase 4

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1. Introduction

1.1. The transglutaminase family members and their activities

Transglutaminases (TGs; EC 2.3.2.13) are enzymes catalyzing the Ca^{2+}-dependent posttranslational modification of proteins by introducing N-ε(γ-glutamyl)lysine covalent isopeptide bonds between lysine and glutamine residues of proteins.

Transglutaminases are characterized by a catalytic triad of Cys-His-Asp amino acids at their active site. Out of the 9 human transglutaminase family members in the case of the erythrocyte band protein 4.2, Cys-Ala replacement results in inactivity. The eight catalytically active human transglutaminases are the human transglutaminase 1-7 (hTG1-TG7) and human blood coagulation factor XIII (hFXIII-A). They can catalyze the post-translational modification of various proteins, and can serve as scaffolds, maintain membrane integrity, regulate cell adhesion, and modulate signal transduction.

The best-studied family member is hTG2, which plays a role in cytoskeletal regulation, cell adhesion, and cell survival or apoptosis, depending on the circumstances. The hTG1, hTG3, and hTG5 are mainly present in the skin and play an important role in cornification. The hFXIII-A plays its best-known role in blood clotting, where it converts soft clot to hard clot by developing covalent isopeptide bond between fibrin molecules. Very little information is available on the hTG6 and hTG7 proteins. In rodents, TG4 is involved in the formation of the copulatory plug, and hTG4 has been associated with the development of prostate tumors and type 1 autoimmune polyendocrine syndrome (APS1).

Transglutaminase activities can be very diverse and can result in multiple Gln modifications. Most often, via their Ca^{2+}-dependent transglutaminase activity, they form an isopeptide bond between the glutamine and lysine side chains of proteins, but they can also catalyze the incorporation of mono- and polyamines into the glutamine side chain of proteins (transamidation, amine incorporation). The poorly studied isopeptidase activity is not a complete reversal of transamidation, as glutamine, which originally functioned as a substrate, is converted to glutamate in the protein, which is no longer able to react with the enzyme.

1.2. The human FXIII-A

The human FXIII-A can be found in the blood plasma and also intra- and extracellularly. It is present in the blood as a heterotetramer, composed of two A and two B subunits.

The hFXIII-A becomes a catalytically active transglutaminase after limited proteolysis by thrombin. The cleavage removes the activation peptide resulting in the dissociation from the subunit B. Subunit B participates in the control of subunit A and performs a scaffolding function.

Human FXIII-A has a complex function, its best-known and best-studied function is maintaining the hemostasis. As the last member of the coagulation cascade, the hFXIII-A, activated by thrombin and Ca^{2+}, forms isopeptide bonds between the fibrin molecules, previously held together only by secondary bonds, thus ensuring the mechanical stability of the blood clot. In addition, hFXIII-A is involved in the regulation of fibrinolysis. Crosslinks reduce the availability of clots to fibrinolytic enzymes and compacts the fibrin fibre diameter and increases fibre density in the clot. It incorporates α2-antiplasmin fibrinolysis inhibitor into the clot. Interestingly, due to the isopeptidase activity of hFXIII-A, it is also able to cleave the previously cross-linked α2-antiplasmin to fibrin molecules via isopeptidase activity, thereby accelerating fibrinolysis.

In addition, hFXIII also plays a role in maintaining gravity, wound healing, angiogenesis, differentiation of adipocytes, cartilage and bone cells, and the regulation of the immune system.

1.2.1. Clinical significance of hFXIII-A activity measurements

It has long been known that hFXIII-A plays an essential role in hemostasis, and hFXIII-A disorders can cause severe bleeding problems. In addition, today, more than 2,000 people die of thrombosis in Hungary every year. Anticoagulants are used to prevent thrombosis, but they affect hemostasis and can cause bleeding. Novel therapeutic approaches with minimal or no bleeding risk are desperately needed.

For decades, vitamin K antagonists were the only available anticoagulants administered orally. Only recently, a new generation of direct-acting oral anticoagulants that block thrombin directly or indirectly via upstream factor Xa has become available. However, thrombin can promote or prevent blood clotting, depending on its activation state. An enhanced bleeding risk characterizes interference with thrombin activity by the currently available anticoagulants, thus excluding many patients from beneficial treatment.

Inhibition of hFXIII-A is an untapped potential in the field of anticoagulants. The hFXIII-A is the only coagulation factor that determines clot strength and half-life. A German company, Zedira GmbH, developed a small molecule hFXIII-A inhibitor (ZED3197) that did not increase bleeding and platelet activation time or affect thrombin levels when used in a rabbit model.

Thus, with the use of this inhibitor, bleeding as a side effect would not be a problem, making it appear to be a promising drug candidate; however, its clinical trial is yet to come.

Another coagulation disorder, hFXIII-A deficiency, is caused by an autosomal recessive disorder characterized by lifelong hemorrhage and impaired wound healing. The molecular background of the disease is characterized by high heterogeneity, which contributes to the varying degrees of manifestation of the disease. More than 60 hFXIII mutations have been identified, as well as unique nucleotide polymorphisms that may affect hFXIII-A activity. Determination of hFXIII-A levels and activity in these patients is essential.

Measurement of isopeptidase activity allows accurate, sensitive measurement of hFXIII-A activity, which is a major challenge, especially in patients with hFXIII-A deficiency. Measurement of hFXIII-A isopeptidase activity provides a good opportunity for the accurate and sensitive determination of hFXIII-A activity in the plasma. Several assays have been developed to measure hFXIII-A activity, but only a fluorimetric method allows to measure the enzyme isopeptidase activity. Automated measurement of hFXIII-A isopeptidase activity may greatly assist in diagnosing hFXIII-A deficiency. The unique advantage of detecting isopeptidase activity is that there is no alternative, parallel reaction in the assay. The substrate can be converted only in one direction because the glutamate can no longer react with the enzyme. However, transglutaminases often act on proteins; thus, their reaction kinetics cannot be predicted using peptides or small molecule amine substrates. This is evidenced by the fact that we have previously developed hTG2 mutants that show elevated isopeptidase activity with peptide substrates. Still, the opposite was observed using the protein-peptide-based substrate assay. There is a great need for protein-peptide-based kinetic isopeptidase activity measurements in the field, which are also feasible for testing potential anticoagulants that exert their effects by inhibiting hFXIII-A.

1.3. Human transglutaminase 4

The only well-known biological function of TG4 is the formation of the copulation plug in rodents through calcium-dependent transamidase activity. Following ejaculation, proteins in rodent semen clot in the female vagina due to the cross-linking activity of the TG4, secreted by the male coagulating gland, and the formed hard plug remains close to the cervix for days. Removal of the seminal vesicle and coagulating gland in rodents prevents the formation of the copulatory plug and reduces litter size. The copulatory plug also affects the events after fertilization (implantation, pregnancy).

Rodents are good experimental animals for mammalian reproduction, so rodent TG4 has been studied extensively. The greatest advancement was recognising that vesiculase, or dorsal protein-1, involved in the formation of the copulatory plug in rats, is a member of the transglutaminase family. Rat TG4 transamidase activity was characterized, and its post-translational modifications were identified. It was recognized that 0.1 mM SDS increases the enzyme's activity, which led to the conclusion that TG4 is a membrane protein that requires a lipid anchor to function. The expression of rat TG4 was shown to be androgen-dependent, as the mRNA expression of the enzyme was reduced by 80% after castration of the rat. The GTP-binding property of rat TG4 has also been recognized, and the N-terminal part of the enzyme is responsible for GTP binding. GTP has been shown to inhibit the calcium-dependent transamidase activity of the enzyme. TG4 produced in MDCK (Madin-Darby canine kidney cells) cells was not secreted into the cell medium, presumably due to post-translational differences.

The physiological function of human TG4 is not known. It is mainly expressed in the luminal epithelial cells of the prostate. However, outside the prostate, it is present in smaller amounts in the urinary glomerular membrane vesicles, pancreatic cells, and saliva. In the human saliva, hTG4 can be detected in full length and with an approx. 55 kDa fragment. TG4 has also been shown to be present in rat and mouse aortic and venous cava tissues; however, there is no evidence in the literature that human aortic and vena cava tissues also contain hTG4. Under pathological conditions, breast, bladder, lung, and colorectal tumor cells contain hTG4.

The antigenic properties of sperm cells are well known, and when they enter the female genital tract, they should elicit an immune response as a foreign substance. This would damage the sperm to such an extent that fertilization could not occur. However, this is not the case, in part due to the activity of TG4. In rats and mice, TG4 has been shown to bind immunosuppressive agents to the surface of sperm that inhibit the immune response of females.

In humans autoantibodies against hTG4 have been detected in most adult autoimmune polyendocrine syndrome type 1 (APS1) male patients. In male mice modelling the disease, immunity to TG4 was associated with destructive prostatitis and secretion of damaged TG4.

Several anatomical features are associated with mating habits in primates. Several sperm proteins, including TG4, develop adaptively in primates. The apparent loss of function of genes involved in the copulatory plug formation in rodents has been shown in several studies in species where sperm competition is low or non-existent. Sperm competition is unlikely to occur frequently in humans, as monogamy or polygamy is common in most human societies. Human sperm forms a gel only for a few minutes after ejaculation and then re-liquefy. The main

components of human seminal fluid are semenogelin I and II proteins. TG4 and transamidase activity is also present in human semen, but no cross-linking of semenogelins was observed *in vitro*. In the adaptive evolution of these genes, sperm competition was the guiding factor. Copulatory plug is not formed in humans, and thus human homologs of proteins involved in the copulatory plug formation in rodents have undergone a significant evolutionary change.

Human TGM4 and F13A1 have a high mutation rate within the transglutaminase enzyme family and relative to other genes. The allele frequencies of the most common non-synonymous single nucleotide variations are the highest in hTG4. This suggests that the evolutionary pressure of hTG4 is lower than other transglutaminases. It is conceivable that, due to the mating strategy of humans, in the absence of sperm competition, the proteins involved in the copulatory plug formation have undergone an evolutionary change and lost their function.

High levels of hTG4 are associated with increased invasiveness of prostate tumor cells. The hTG4 plays a role in cell migration, membrane micromovements, and cell-matrix adhesion. Treatment with recombinant hTG4 or overexpression of hTG4 in prostate tumor cells caused a reduction in E-cadherin level, and mesenchymal features became dominant over epithelial features and cell motility was increased. In PC-3 cells, overexpression of hTG4 promoted cell adhesion to endothelial cells, suggesting that hTG4 may play an important role in the interaction between tumor cells and the endothelium. The hTG4 can antagonize the effect of IL-24 on cell growth and migration in prostate tumor cells and in cooperation with RON, increases the migration of prostate tumor cells.

According to the Cancer Genome Atlas, compared to other human tumor types the highest levels of hTG4 mRNA expression were found in prostate adenocarcinomas. Expression of hTG4 in primary prostate tumors is associated with disease progression. Patients with high hTG4 expression in their primary tumors significantly reduced survival time. In these tumors, hTG4 may be a potential chemotherapeutic target. In the sperm plasma of patients (>50 years of age, >4 ng/ml serum prostate-specific antigen level) with prostate tumors hTG4 levels were found to be higher than in samples from healthy individuals. The hTG4 may serve as a biological marker of prostate cancer.

2. Objectives

- Detection of the isopeptidase activity of hFXIII-A using protein substrate.

- Development of a kinetic protein-peptide and anisotropy based assay to test hFXIII-A isopeptidase activity.

- Studying the hTG4 transamidase activity and its regulation.

- To reveal the hTG4 biological function in the saliva by the identification of its substrates and interaction partners.

3. Materials and methods

3.1. Western blot analyses

SDS-PAGE with 10% gel was followed by semidry blotting. 5 ($^{m}/_{v}$)% low-fat milk powder in TTBS was used for blocking, and antibodies were applied in 0.5 ($^{m}/_{v}$)% low-fat milk powder containing TTBS. WesternBright ECL HRP substrate and Agfa film were used for development.

3.2. Protein production and purification

Production of S100A4(GST) and recombinant human TG2 were performed in *Rosetta2 (DE3)* bacterial cells and purified by affinity chromatography based on earlier published protocols. Recombinant human TG4 enzyme was expressed overnight in *E. coli BL21 (DE3) pLysS* bacterial cells by IPTG induction at low temperature. Cells were harvested by centrifugation, and the pellet was resuspended in lysis buffer. After sonication and centrifugation, recombinant hTG4 was purified from the supernatant by Talon affinity chromatography followed by ion-exchange chromatography using HiTrap Q HP column. The purity (> 90%) of recombinant hTG4 was checked by Coomassie staining and Western blot. *E. coli Rosetta 2 DE3 pLysS* bacterial cells were used to express biotinylated hTG4. Biotinylation was performed with BirA ligase, and then free biotin was removed by dialysis.

3.3. Kinetic measurement of hFXIII-A and hTG2 activity

In the presence of 5 nM hTG2 or hFXIII-A, 100 nM FLpepT26 or FlpepPI2 was incubated with 5 μM S100A4 (GST) protein in HEPES (pH 7.5) transamidase buffer. For isopeptidase reactions, 380 nM FLpepT26-S100A4 (GST) or 259.5 nM FLpepPI2-S100A4 (GST) crosslinked substrate was used in the MOPS (pH 6.5) isopeptidase buffer in the presence of various concentrations of hTG2 or hFXIII-A. Negative control reactions contained 10 mM EDTA and 2 mM iodoacetamide (IA) and the other reactions contained 5 mM Ca^{2+}. Normal human plasma samples were used at 10-fold dilutions using 2 mM GPRP peptide and 5 mg/ml polybrene. For hFXIII-A reactions, the reaction mixture also contained 500 mU of thrombin. The change in fluorescence polarization was detected with a Synergy H1 microplate reader. The reaction rates were calculated in terms of anisotropy per minute. After measuring the fluorescence polarization change, the reaction mixtures were separated by 15% SDS-PAGE, and fluorescence was detected using a PharosFX Plus Molecular Imager.

3.4. Endpoint measurement of hTG4 and hTG2 transamidase activity

The classical microtiter plate assay was used to measure hTG4 transamidase activity with minor modifications. The transglutaminase reaction was usually performed using 2.5 μg of hTG4, 0.5 mM biotin-pentylamine (BPA) substrate, 10 mM DTT, and 5 mM Ca^{2+}. The colour change by Extravidin alkaline phosphatase (1/2500) reaction was monitored at 405 nm using Synergy H1 instrument. The rate of transglutaminase reaction was determined by the change in mAbs per minute of the phosphatase reaction. To adjust for various reducing or oxidizing conditions, the reaction mixtures were supplemented with 2.4 mM GSSG and 0.24, 0.45, 1.25, or 2.4 mM GSH. The ratio of $[GSH]^2/[GSSG]$ was calculated from their final molar concentration.

3.5. Testing the isopeptidase activity of the hFXIII-A on cross-linked fibrin degradation products

Cross-linked fibrin degradation products (xFDP, D-dimer) and fibrin degradation products (FDP) were incubated in 20 mM MOPS (pH 6.8) buffer in the presence of recombinant human FXIII-A or in the control reactions without enzymes. The reaction time was 2 hours at 37°C, then the reaction mixtures were tested by Western blot analysis.

3.6. Checking the hTG4 binding to fibronectin

NuncTM Maxisorp plate was coated with 0.3 μg fibronectin in bicarbonate buffer pH 9.6. Subsequently, hTG2 and hTG4 in Ca-TTBS buffer were added to the wells. The bound recombinant enzymes were detected with anti-His_6/HRP antibody and 3,3', 5,5'-tetramethylbenzidine. Absorbance values were measured at 450 nm with Synergy H1 instrument.

3.7. Testing the guanosine-triphosphate nucleotide-binding of hTG4

Based on previous publications, BODIPY-FL-GTPγS and GTP-agarose were used to test hTG4 nucleotide-binding with minor modifications. Protein-bound BODIPY-FL-GTPγS showed a higher fluorescence value as measured by the Synergy H1 instrument. Nunc black microplates and as a positive control 100 nM recombinant hTG2 were used for the experiment. 5 μg recombinant hTG4 or hTG2 was used for GTP-agarose pull-down measurement.

Transglutaminases bound to GTP-agarose resin were eluted by incubation at 100°C for 10 minutes after the addition of two times diluted denaturing buffers. The presence of recombinant TGs was tested using Anti-His_6/HRP antibody in the Western blot.

3.8. Differential scanning fluorimetry

The thermal stability of hTG4 was tested by Differential Scanning Fluorimetry. The Prometheus NT.48 instrument allows the detection of protein's unfolding without labelling, based on the change in the internal fluorescence of the proteins while the temperature is heated from 20 to 95°C. The hTG4 protein was diluted in 20 mM Tris-HCl buffer (pH 7.2).

3.9. Digestion by dispase

To test the proteolytic stability hTG4 or hTG3 was incubated with dispase I or II in 0.1 M Tris-HCl buffer (pH 8.5) in the presence of Ca^{2+} (1 h, 37°C). Then, the incubated mixture was examined by Western blot.

3.10. Comparison of hTG4 and hTG2 aminoacid sequence

The amino acid sequences of the hTG2 and hTG4 from the Uniprot database were compared using the NCBI Protein BLAST online tool. The presence of known functional sites of the hTG2 in the sequence of hTG4 was checked based on the alignment .

3.11. Transient hTG4 expression in AD-293 cells

Cells were plated on a 6 well plate. After reaching the 70% confluence, the FBS was lowered to 2%, and antibiotics were omitted from the media. The pTriEx-4 Ek/LIC hTG4 vector was constructed using pTriEx™-4 Ek/LIC Vector Kit-Novagen. The transfection was performed using Lipofectamine 2000 based on the manufacturer's instructions. After 3 h, the transfection medium was replaced with a culture medium. The cells were collected 48 h after transfection using lysis buffer.

3.12. Collection of saliva samples and isolation of salivary extracellular vesicles

Donors were asked to refrain from eating for 2 h and drinking 30 min prior to saliva collection. Before saliva collection started, donors were asked to rinse their mouths with clean

water. After saliva collection 1 mM PMSF and 1 mM Proteinase Inhibitor Cocktail were added to the samples. The extracellular vesicles were isolated by ultracentrifugation.

3.13. Search for human TG4 substrate in AD-293 cell extract and saliva

To inhibit endogenous transglutaminases in saliva iodoacetamide and irreversible inhibitors were used. The unbounded inhibitors were removed by dialysis. The success of endogenous transglutaminase inhibition in saliva samples and the dialysis of inhibitors was verified by biotin incorporation assay by Western blot using streptavidin/HRP.

Nuclei Isolation Kit: Nuclei EZ Prep was used to separate the cytoplasmic and nuclear fractions of the AD-293 cell extract according to the manufacturer's instructions. Cytoplasmic or nuclear fractions of the AD-293 cell extract or saliva sample were incubated in the presence of hTG4, BPA and Ca^{2+} (2 h, 37°C). Proteins containing BPA modifications were separated and enriched in the sample using High Capacity Neutravidin Agarose Resin. To identify the BPA modified proteins the samples were sent for LC-MS/MS analysis. To create proper control samples, AD-293 cell extract or untreated saliva was mixed with the NeutrAvidin gel in the absence of hTG4.

3.14. Search for human TG4 interaction partners in the human saliva

After equilibration, biotinylated hTG4 was immobilized on High Capacity NeutrAvidin Agarose Resin (1 h, 4°C with continuous rotation). After biotin blocking, whole saliva sample was mixed with the resin, and incubated for 1 hour at 4°C with continuous agitation. Then the beads were washed three times and incubated at 100°C for 10 minutes after adding 6 times denaturing buffer. After centrifugation, the supernatant was run into a 10% Tris-glycine gel by SDS-PAGE, and the gel sections were excised after Coomassie staining and sent for LC-MS/MS analysis. No biotinylated hTG4 was added to the gel during the preparation of the control samples; the sample preparation started immediately with the blocking step, without any other difference. The identified proteins were grouped using Functional Enrichment Analysis of the String online protein network database.

3.15. Analysis of proteomic databases

The “PSM Provenance” table was exported from the MassIVE database (Mass Spectrometry Interactive Virtual Environment). The interface of the MassIVE allows sorting the peptides

according to their uniqueness by filtering the Matched Genes feature for the peptides. The unique and shared peptides were separated into two tables according to that list. The origin of the samples where the peptides were identified was mined out from the names of the files or based on the dataset identifier. In the case of 76 out of 3499 peptides, their origins were not available. After clicking on the observed TG4 peptides in the PeptideAtlas under the "Sequence Motifs" feature, the data from the "Observed in Experiments" part was collected. Whether a given peptide was unique for hTG4 was checked in each case using NCBI Standard Protein BLAST online tool. The tissues were collected from the Experiment Names, always referring to the sample origin. In ProteomicsDB, under the "Peptides/MSMS" tab, 49 unique and one shared hTG4 peptide can be found, identified in the datasets of the ProteomicsDB. Clicking on the peptide sequences, the "Peptide Details" appear, which were exported to Excel (Microsoft, Redmond, WA, USA) in the case of all 50 peptides. Based on the name of the project, experiment , or file the origin of the sample can be identified. In publicly available datasets, the sample mapping can be reached by clicking on the experiment.

3.16. LC-MS / MS analyses

Samples for LC-MS/MS analysis were run into a 10% polyacrylamide gel but only into the first centimetre of the separation gel. After Coomassie staining, the protein bands were excised from the gel, and the dye was washed out with nanopure water. Then the mass spectrometric analysis was performed by Proteomics Core Facility (Department of Biochemistry and Molecular Biology, University of Debrecen). Protein identification based on the peptide sequences (MS/MS spectra) was made by MaxQuant 1.6.2.10. search engine using SwissProt/Uniprot database. The results were imported into the Scaffold 4.8.9 program (Proteome Software, Inc., Portland, OR, USA), and for the protein identification, the following settings were applied: protein threshold 1.0% False Discovery Rate (FDR), the minimum necessary identified peptide number 3 for each protein, and peptide threshold 0.1% FDR. In the case of determination of BPA labelled proteins, non-FDR filtering mode and one peptide per protein criteria were used. In addition, the MS/MS spectrum of each peptide was verified by the presence of at least four consequent b or y fragment ions. Only those proteins were considered as substrates, in which the modified peptide fulfilled this requirement.

4. Results and discussion

4.1. Characterization of hFXIII-A isopeptidase activity

4.1.1. Testing the isopeptidase activity of hFXIII-A on cross-linked fibrin degradation products

First, we tested the isopeptidase activity of hFXIII-A on protein substrates using an antibody specific for fibrin degradation products containing isopeptide bonds. We incubated the cross-linked fibrin degradation products (xFDP and D-dimer) in the presence of activated FXIII-A. Western blot analysis of the reaction mixture revealed regularly, but not consistently, an apparent 10–20% decrease in the level of the antibody recognized xFDP band The experiment was repeated several times, sometimes with increased incubation time. Still, we concluded that this antibody is not suitable for accurately monitoring the amount of isopeptide bond between fibrin subunits. But, antibody-based detection of cross-linked fibrin degradation products may be a good qualitative marker of hFXIII-A transamidase activity during blood coagulation.

4.1.2. Protein-peptide-based kinetic method for measuring hFXIII-A transamidase and isopeptidase activity

Determining the exact level of coagulation factors in coagulation disorders is essential in the diagnosis of the disease and for monitoring the effectiveness of factor replacement therapy. Measurement of hFXIII-A activity provides an excellent tool for identifying hFXIII-A abnormalities. Fluorescence anisotropy change is a convenient way to monitor transglutaminase activity.

Thus, we developed a kinetic protein-peptide-based anisotropic method to measure hFXIII-A transamidase and isopeptidase activity. We attempted to accomplish this by adapting a protein-peptide-based kinetic anisotropic assay previously developed by our group to measure hTG2 transamidase and isopeptidase activity. The FLpepT26 peptide was not a suitable substrate for the kinetic detection of hFXIII-A transamidase activity, because the generated S100A4(GST)-FLpepT26 crosslinked substrate did not generate a sufficient signal for the kinetic detection of hFXIII-A isopeptidase activity.The replacement of FLpepT26 peptide for FLpepPI2 resulted in a good substrate for measuring the activity of the hFXIII-A transamidase and isopeptidase activity both in the formation of cross-linked substrate and cleavage of the previously generated cross-linked product. Later, due to the advantages of isopeptidase measurement, we focused on its applicability and validity.

4.1.3. Sensitivity, linearity, and detection limit of the protein-peptide-based kinetic isopeptidase hFXIII-A activity measurement

To test the potential applications of the protein-peptide-based hFXIII-A kinetic isopeptidase assay, first, the effect of increasing FLpepPI2-S100A4(GST) concentration on the reaction rate was measured in the presence of 150 nM hFXIII-A. The activity values followed saturation kinetics, and a curve was fitted using the Michaelis-Menten equation (coefficient of determination: R^2=0.928). The activity attained a plateau phase, and Km and Vmax parameters were calculated as 4.10±0.76 nM and 54.92±1.7 mr/min/nM hFXIII-A, respectively, using GraphPad Prism. This Km is extremely low compared to other published values, which were 19.8±2.8 μM and 530 μM. One of the simplest explanations could be that protein-peptide-based substrates have a higher affinity for the enzyme than small peptides or amines due to a higher number of potential interacting residues between them.

The next step was to examine the sensitivity and linearity of the assay. We measured the hFXIII-A activity up to 150 nM enzyme concentration, and the Pearson correlation coefficient was 0.9387 with a value of 0.8811 R^2, which shows a strong but not exactly linear relationship between hFXIII-A concentration and activity over the entire studied concentration range. The lowest measurable hFXIII-A concentration was 1.0 nM. When the data were retested in a lower concentration range up to 15 nM hFXIII-A, the correlation coefficient was 0.9907 with a value of 0.9816 R^2.

To further evaluate the applicability of the assay, normal human plasma was added at a 1:10 dilution to the reaction buffer, which contained various concentrations of recombinant human FXIII-A. To obtain an appropriate blank value in the presence of plasma, the transglutaminase activity of endogenous hFXIII-A was inhibited by chelating Ca^{2+} using 10 mM EDTA and adding iodoacetamide. In the presence of normal human plasma, corrected activity values were lower but showed better correlation parameters. Up to a concentration of 50 nM recombinant hFXIII-A, the Pearson correlation coefficient was 0.9950 with $R^{2,}$ and the coefficient determined for the fitted curve was 0.9422. The smallest, clearly measurable amount of hFXIII-A was 5 nM hFXIII-A. The anisotropy change at lower concentrations was inseparable from the negative control reaction. The mean of physiological hFXIII-A concentration is approximately 68 nM, and the reference interval is between 69 and 143%, which by taking into account the 10 times dilution, overlaps with the linear range of our protein-peptide based assay. It is suggested that it could determine the physiological hFXIII-A level in the human plasma however, it is not feasible to measure enzyme activity in severe hFXIII-A deficiency.

As inhibition of hFXIII-A is a therapeutic goal to prevent unwanted blood clot stabilization, applying a protein-peptide-based assay for drug testing would be useful because in vivo hFXIII-A acts on protein substrates. The effect of iodoacetamide (IA), a general transglutaminase inhibitor, and ZED1301, an hFXIII-A specific peptidomimetic inhibitor, were tested in the anisotropy based hFXIII-A assay. Both in the presence of IA or ZED1301, dose-dependent inhibitions were detected with 1.89±0.29 μM and 268±56 nM IC50 values, respectively. The use of a kinetic fluorescence anisotropy-based method for testing inhibitors is also desirable because effective inhibitors often have fluorescent properties and do not significantly affect the change in anisotropy.

The assay could be developed further with the optimization of the assay for a simple microfluidic platform or by further purification of the isopeptidase activity substrate. In addition, the selection of substrate molecules that are similar to S100A4 (GST) would allow the development of widely applicable assays to measure the kinetic activity of the transglutaminase enzyme family members. Our experiments raise the possibility of developing isoenzyme-specific protein-peptide-based assays to measure the activity of several co-expressed transglutaminases in biological samples.

4.2. Characterization of human transglutaminase 4 transamidase activity and investigation of its role in saliva

4.2.1. Analysis of proteomic databases to map human body hTG4 expression

Human transglutaminases are receiving increasing attention due to their biological function and potential medical relevance. Still, we have a small black spot in our knowledge regarding the biochemical properties, tissue distribution, substrates, and interaction partners of human TG4. To collect the hTG4 expressing tissues, we examined the MassIVE, PeptideAtlas, and ProteomicsDB databases. By reviewing these databases and analysing the tissue origin of the samples and the metadata of each detected peptide, 41 tissues were collected where hTG4 was detected. Then, after excluding non-unique peptides, 9 tissues remained, where the detection of unique peptides confirmed the presence of hTG4. These data support that in addition to the male genital tract, hTG4 is also present in the heart, spleen, salivary glands, colon, and bladder.

4.2.2. Biochemical characterization of hTG4

To characterise hTG4 experimentally, we produced recombinant hTG4 and tested its transamidase activity by applying the classical microtiter plate biotin-pentylamine (BPA) incorporation assay. The transamidase activity of 0.5 μg hTG4 was 0.62±0.29 mAbs/min,

which is extremely low compared to the positive control recombinant human TG2 (29.25±5.93 mAbs/min; at 5 mM [Ca^{2+}]). The transamidase activity of commercial recombinant hTG4 (Zedira GMBH.) was similar to the recombinant enzyme produced in our laboratory. The activity values, available for human recombinant transglutaminases sold by Zedira GmbH, support and confirm our observation that hTG4 has very low transamidase activity compared to other transglutaminases. Still, when the effect of increasing amounts of hTG4 was measured on the transamidase activity, it demonstrated a good linear correlation reaching 7.31±0.33 mAbs/min at 4 μg protein. The hTG4 activity demonstrated an optimal [Ca^{2+}] at 5 mM.A higher Ca^{2+} level (10 mM) showed an inhibitory effect on the activity, probably due to the aggregation of the protein.

As a next step, to search for a regulatory mechanism that could activate hTG4, resulting in higher enzymatic activity we further characterized the transamidase activity of hTG4 under several different conditions. The hTG4 showed slightly higher activity at lower pH (below pH 7), in line with human vaginal conditions, in contrast to mouse TG4, which is active in basic sperm when the copulatory plug is formed. This property of hTG4 may be related to a change in human sexual strategy leading to the elimination of sperm competition.

The transamidase activity of hTG4 was measured at different reduced and oxidized (GSH/GSSG) glutathione ratios, and hTG4 was found to show higher transamidase activity under reducing conditions. The preference of hTG4 for reducing conditions suggests that transglutaminase activity may play a role in intracellular processes. Examining the effect of increasing SDS concentration, we found that 0.1-0.5 mM SDS concentration resulted in an approximately 3-4-fold increase in hTG4 transamidase activity, whereas higher SDS concentrations showed an inhibitory effect. This increased activity is still much lower than in hTG2-catalyzed reactions. Further increase in SDS concentration leads to complete denaturation of hTG4. SDS activation may mimic the binding of other amphipathic biomolecules by regulating hTG4 activity.

Due to the low activity value, the stability of hTG4 was suspected to be also relatively low. Nano differential scanning fluorimetry was used to characterize the protein's thermal stability. Using a dilution series, the mean Tm values for hTG4 were 62.6±0.23 °C. The temperature at which the protein began to unfold was 56.1±1.34 °C. No other structural (eg.: dissociation, aggregation) transformation was observed in the range of protein concentrations used. Considering the general temperature of the human body, hTG4 shows high thermal stability, which suggests a stable native structure under physiological conditions.

Using the Protein BLAST online tool, we aligned the sequences of hTG2 and hTG4 and then tried to predict the presence or absence of fibronectin and GTP binding sites in the sequence of hTG4. By comparison, the conservation of the catalytic triad is clear. Based on sequence similarity, the amino acids responsible for fibronectin and GTP binding to hTG2 are not found in the amino acid sequence of hTG4. Following the sequence comparison, we experimentally tested the GTP and fibronectin-binding of hTG4. The GTP binding property of the enzyme was then tested using GTP analogues, fluorescent BODIPY-FL-GTPγS reagent and GTP-agarose gel. The results obtained by the two independent methods demonstrate that hTG4 does not have a guanine nucleotide-binding site, thus GTP can not affect the activity of hTG4, in contrast to the rat orthologue (sequence similarity 53.3%). The lack of negative regulation of hTG4 by GTP promotes the effect of the enzyme to increase tumor invasiveness. The presence of hTG4 in prostate tumor cells is a negative prognostic marker.

The *in vitro* observed low hTG4 catalytic activity suggests that hTG4 could need a still unknown activation. Limited proteolysis is required for activation in the case of several members of the transglutaminase family. Therefore, we tested the potential activation of hTG4 by thrombin or dispase. Based on in silico analysis, the sequence of hTG4 does not contain thrombin cleavage sites, which was confirmed experimentally. Limited proteolysis with dispase I and dispase II was experimentally also tested on recombinant His_6-tagged hTG4 and hTG3. Western blot analysis showed that dispase I completely degraded hTG4 and dispase II only reduced the amount of protein compared to the control, suggesting that dispase can cleave hTG4 but does not generate detectable stable fragments. Recombinant hTG3 was tested as a positive control, in which stable proteolytic hTG3 fragments were detected.

In AD-293 cells a protease can activate TG5 by limited proteolysis. We tested whether hTG4 also could be activated by a protease in AD-293 cells and the cells were transfected by a transient hTG4 expressing plasmid. The protein extract of the transfected cells contained only full-length hTG4, indicating that no active protease was present in the cells that would modify the protein by limited proteolysis. However we can not close out, but our results suggest that limited proteolysis is unlikely to be involved in the processing and activation of the hTG4 enzyme.

4.2.3. Investigation of the presence and biological role of hTG4 in saliva

To confirm the presence of hTG4 in saliva, whole saliva samples collected from 8 healthy individuals were examined by Western blot analysis. In saliva samples, hTG4 can be detected as a fragment of about 55 kDa and in high molecular weight protein complexes, possibly held

together by crosslinks or other covalent bonds. However, the hTG4 content of the samples varies from individual to individual,. Subsequently, the presence of hTG4 in different fractions of saliva was examined. Western blot analysis revealed that hTG4 was enriched in the extracellular vesicle fraction. In addition to the 55 kDa fragment and large protein complexes, full-length hTG4 of approximately 80 kDa could also be detected. This supports the presence of hTG4 in saliva and its vesicular fraction. The re-analysis of proteomic databases also supports the presence of the hTG4 in the saliva.

4.2.4. Search for human TG4 substrates in saliva

The previously known functions of hTG4 are linked to reproduction, but its presence in saliva suggests unknown, new functions. We searched for hTG4 substrates in human saliva samples to explore these functions. 5 mg of salivary protein in which endogenous transglutaminases were inhibited was treated with recombinant hTG4 in the presence of Ca^{2+} and BPA. Then, the hTG4-modified BPA-containing proteins were enriched by NeutrAvidin-agarose affinity chromatography and sent for mass spectrometric analysis (LC-MS/MS) to identify substrate proteins. After subtraction of proteins from control samples, LC-MS/MS analysis identified 43 potential hTG4 substrate proteins, among them recombinant hTG4 has been identified as well. Detection of the BPA modification in the proteins confirms whether the identified protein is indeed a hTG4 substrate. No BPA-modified peptide was detected in the case of the 43 identified proteins, so further analyzes are required to determine reactive glutamine for these proteins. However, in the complete hit list, which also included the proteins identified in the control sample, the Immunoglobulin heavy constant alpha 1 protein was identified by a BPA modified peptide. This protein binds non-specifically to NeutrAvidin agarose, as it has also been detected in control samples, but its BPA modification confirms that it is also a substrate for hTG4. The confirmation of potential hTG4 substrates identified in the saliva is a future task for our group.

4.2.5. Human TG4 incorporates biotin-pentylamine into several proteins in AD-293 cell extract

The existence and presence of efficient glutamine-donor protein substrates of hTG4 were also tested in AD-293 cell extract. Western blot analysis of the AD-293 cellular extracts incubated with recombinant hTG4 in the presence of Ca^{2+} revealed that several proteins had incorporated biotin-pentylamine (BPA). Before the hTG4-dependent BPA labelling, the nuclear and cytoplasmic fractions of AD-293 cells were separated to increase the efficiency of protein identification. To enrich the ratio of BPA labelled proteins, NeutrAvidin-agarose affinity

chromatography was applied, and then the mixtures were sent for mass spectrometry-based (LC-MS/MS) protein identification. After subtracting the non-specifically bound proteins detected in control samples, mass spectrometric analysis of hTG4-treated samples revealed 8 proteins from the cytoplasmic and 230 proteins from the nuclear fractions. These proteins can be substrates of hTG4 or interaction partners of the substrates. The detection of a BPA modified peptide fragment of the protein proved the presence of reactive glutamine and the occurrence of the hTG4-mediated BPA-incorporation in the following proteins: in the cytoplasmic fractions, Keratin, type II cytoskeletal 1 and Keratin, type I cytoskeletal 9 got BPA-labelled, but the unmodified forms of these proteins were also identified in the control samples. Their BPA modifications proved that hTG4 can use them as substrates. In the nuclear fraction, only Insulin-like growth factor 2 mRNA-binding protein 1 was BPA-modified. Further study would be needed to confirm and determine the reactive glutamine in the remaining other identified potential substrate proteins.

While the proteomics dataset's evaluation focused on protein identification, only BPA modifications confirmed the existence of three substrate proteins, another approach for the data analysis focussing on BPA-modified peptides could also reveal existing substrate proteins and determine their reactive glutamine residues. Therefore, we filtered the dataset again for BPA modification in the peptide fragments and removed the previously applied filtering parameters, which are valid for protein determination. We found 105 proteins that contained incorporated BPA. Each peptide's MS/MS spectrum was verified, and 20 peptides were confirmed, in which at least four consequent b or y fragment ions were present. Based on these twenty selected hTG4-modified peptides, we analysed the linear environment of the reactive Gln residues. Unfortunately, it is not possible to deduct an obvious consensus recognition sequence for hTG4 based on these 20 peptides. But it is visible, that hTG4 prefers poly-glutamine tracts and nearby Glu. Leu, Arg, Ser, and Val amino acids also are frequent in the surrounding linear area of the reactive Gln residue. For reference, we checked the TRANSDAB, transglutaminase substrate database. We found that some of our newly identified hTG4 substrates are substrates for other transglutaminases as well: keratin, type II cytoskeletal 1 for hFXIII-A, filaggrin for hTG1 and hTG3. There are some similarities: NF-kappa-B inhibitor alpha, F-box only protein 2, and Myosin-9 are substrates of hTG2, and Coiled-coil domain-containing protein 126 is a substrate of hFXIII-A. Only isoforms of the previous proteins are substrates of hTG4, suggesting that hTG4 has a unique substrate recognition. This is not completely unexpected because mouse TG4 can use F2 (79–259 amino acids) fragment of the seminal vesicle secretion I (SVS I) protein as a glutamine donor while TG2 does not prefer it. Interestingly, the SVS I is an amine

oxidase with two reactive glutamines (Q232, Q254) and, in the case of its human orthologues, the reactive glutamine residues are changed to Arg or His, respectively.

Then, we analyzed the physiological and pathological roles of the identified substrate proteins. We focused on membrane-localized proteins because hTG4 overexpression contributes to the invasiveness of prostate tumor cells, increasing their adhesion and migration. Based on the Human Protein Atlas, Adhesion G protein-coupled receptor L3 is frequently present in the breast, prostate, and colorectal cancers. Protocadherins, members of the cadherin family, are essential to maintain normal cell-cell interaction and are involved in the epithelial-mesenchymal transition. Plexin-A2, as a coreceptor of semaphorins, plays a role in invasive growth and cell migration. Transglutaminase-dependent modification of NF-kappa-B inhibitor alpha could promote its degradation and activation of NF-kappa-B, contributing to cell survival similarly to hTG2. Human TG4 promotes tumor and endothelial cell interaction by bypassing the ROCK pathway. This is supported as no ROCK1 or ROCK2 interaction partners were found as TG4 substrates. But among the 105 identified TG4 substrates, we can find Rho guanine nucleotide exchange factor 28, which can play a role downstream of the ROCK signalling. The role of these identified substrates in cancer may initiate further studies to reveal their exact role in cancer biology, potentially leading to identifying new cancer therapeutic targets.

4.2.6. Search for hTG4 interaction partners in saliva

To elucidate the role of hTG4 in saliva, we attempted to search for protein interaction partners in whole saliva samples. Biotinylated hTG4 was bound to NeutrAvidin agarose gel, and after blocking, it was incubated with salivary proteins. Proteins bound to hTG4 were sent for LC-MS/MS analysis for identification. The identified proteins in the control samples were removed from the list of identified proteins when biotinylated hTG4 was immobilized on the resin, resulting in 280 identified potential hTG4 interaction partner proteins. Functional enrichment analysis was performed to group the proteins identified as potential interaction partners according to their function using the String online protein network database. String grouped only 211 from the 280 identified potential interacting proteins; in the case of 69 proteins, the analysis was not feasible due to the shortcomings of their annotations. 330 GO Biological Process was significantly enriched (FDR <0.05).

As hTG4 is present in salivary vesicles and may play a role in masking sperm antigenicity, we have focused mainly on the related Biological Processes. Secretion, Vesicle-Mediated Transport, Cell Activation, and Immunological Process GO Biological Processes are associated with a total of 98 of the 211 grouped proteins. Based on identified GO Cell constituents, a

significant proportion of the proteins identified in the whole saliva are classified as Extracellular Vesicles and Vesicle GO Cellular components. This is not surprising for salivary proteins but further confirms the possibility that hTG4 can bind to the membraneous fraction of the saliva. Among the interaction partners identified in saliva, 16 proteins overlap with the earlier identified potential salivary hTG4 substrates. Examples of such proteins include the 14-3-3 protein zeta/delta isoform, Antileukoproteinase, BPI fold-containing family A member 2, Cystatin SN, and Vinculin. The 14-3-3 protein zeta/delta has many binding partners, to which it usually binds through their phosphoserine or phosphothreonine motif and usually affects the activity of the binding partner. The antileukoproteinase protein is an acid-stable proteinase inhibitor that can inhibit the activity of trypsin, elastase, and cathepsin G, among others. It is also involved in regulating inflammatory and immune responses in bacterial infections. The BPI fold-containing family A member 2 has a strong antibacterial effect. Cystatin-SN is a cysteine proteinase inhibitor found in saliva and it is an inhibitor of papain. Vinculin binds to actin filaments and is involved in cell-matrix and cell-cell adhesion. It regulates the expression of cell surface E-cadherin and may play an important role in cell morphology and micromotion. The hTG4 reduced the level of E-cadherin in prostate tumor cells; it might be possible through its interaction with vinculin. Confirmation of the interaction partners using other methods is a future task for our research group.

5. Summary

The importance of research on transglutaminases is confirmed by their many industrial and translational medicinal applications. Inhibition of human FXIII-A (hFXIII-A) transamidase activity shows increasing potential in treating thrombosis and preventing unwanted blood coagulation. However, hFXIII-A can also destabilize the clot by its poorly known isopeptidase activity. We have developed a peptide-protein-based anisotropic activity assay that allows studying both directions of reactions catalyzed by hFXIII-A, using the same substrate molecules, as two substrate molecules for measurement of transamidase activity (FLpepPI2 and S100A4(GST)) and as a previously crosslinked substrate for monitoring isopeptidase activity (FLpepPI2-S100A4(GST)). Peptide-protein substrates can mimic the *in vivo* substrates of hFXIII-A during clot modification to study the opposite activities of mutant hFXIII-A enzymes and the effect of regulatory molecules that regulate hFXIII-A activities.

Another secreted family member, the human TG4 (hTG4) is involved in the development of APS1 autoimmune disease and prostate tumours. Rodent TG4 enzymes are well characterized compared to the human orthologue. Characterisation of hTG4 biochemical properties could help to understand its function and role in humans. Reanalysis of proteomic databases revealed that hTG4 is also expressed outside the prostate. The activity and regulation of hTG4 transamidase activity were studied *in vitro* using the classical microtiter plate method. The hTG4 has low transamidase activity, and it prefers a slightly acidic pH and reducing environment. The activating effect of SDS in submicellar concentration was observed, suggesting that the enzyme exerts its activity in membrane-bound form. In contrast to rat TG4, hTG4 can not bind GTP. Proteolytic activities, which are known to activate other transglutaminases were not observed upon the incubation of hTG4 with dispase or expressing hTG4 exogenously in AD-293 cells. In saliva, we identified 280 potential hTG4 interaction partners, mostly involved in secretion, immune processes, and vesicular transport correlating with the enrichment of hTG4 in the vesicular fraction of saliva. Several hTG4 glutamine donor substrates were identified in saliva and AD-293 cell extract. Some of these substrates are involved in cell-cell interactions, adhesion, and proliferation, suggesting that hTG4 could become an anti-cancer therapeutic target.

Our results contribute to a better understanding of the properties of hTG4 and hFXIII-A and confirm that these proteins are real targets in the therapy of common diseases.

6. List of publications

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Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Csobán-Szabó, Z.**, Bécsi, B., El Alaoui, S., Fésüs, L., Korponay-Szabó, I., Király, R.: Biochemical Characterisation of Human Transglutaminase 4.
   Int. J. Mol. Sci. 22 (22), 1-18, 2021.
   DOI: http://dx.doi.org/10.3390/ijms222212448
   IF: 5.923 (2020)
2. **Csobán-Szabó, Z.**, Fésüs, L., Király, R.: Protein-peptide based assay for the characterization of human blood coagulation factor XIII-A isopeptidase activity.
   Anal. Biochem. 600, 1-8, 2020.
   DOI: http://dx.doi.org/10.1016/j.ab.2020.113699
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