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Characterization of amine donor and acceptor sites for tissue type transglutaminase using a sequence from the C-terminus of human fibrillin-1 and the N-terminus of osteonectin

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ABSTRACT

Transglutaminase (TGase)-modified proteins are commonly observed in a wide range of biological systems. Therefore, the identification of TGase substrates and respective consensus sites may contribute to a better understanding of the physiological role of TGase. In this study, we identified enzyme-specific properties of two peptide sequences, EDGFFKI, derived from human fibrillin-1, and the previously characterized APQQEA, derived from human osteonectin. EDGFFKI was identified in a previous publication as an amine donor substrate for tissue TGase; APQ^{3}Q^{4}EA is an amine acceptor for this enzyme. A widely-used lysine donor mimic, monodansylcadaverine (MDC), was used as a control. EDGFFKI crosslinked specifically only to Q^{3} of the acceptor probe. The EDGFFKI sequence also showed enzyme specificity for tissue TGase while no reaction was observed with plasma TGase (Factor XIIIa), consistent with its natural occurrence *in vivo*. Using this substrate in biotinylated form we demonstrate its value as a tracer probe to detect endogenous TGase activity in human tissues as well as to target potential amine acceptor substrates via an enzyme-directed site-specific labeling. The results of this study show natively derived EDGFFKI and APQQEA are better and more specific indicators of endogenous tissue TGase activity as compared to a small molecule probe; this may be important in diagnostic applications. The specificity with which matrix sequences APQQEA and EDGFFKI interact with tissue TGase but not plasma TGase may also be crucial for understanding and controlling the function of these TGases *in vivo* and in tissue engineering.

1. Introduction

Tissue transglutaminase (tissue TGase or TGase2) is expressed throughout the body with diverse biological functions. It has been implicated in many biological processes, such as cellular differentiation [1,2], receptor signaling [3], programmed cell death [1,4], several degenerative diseases [5], and assembly and stabilization of ECM [6]. The Ca^{2+}-dependent TGase catalyses an acyl-transfer reaction between the γ-carboxamide group of a glutamine residue and the ε-amino group of a lysine residue of certain proteins, resulting in the formation of a covalent and protease resistant ε-(γ-glutamyl)lysine bond, thus contributing to the stability of tissues. For example, fibrillin-1, the major fibrillin isoform in elastic fibers, has been reported to be crosslinked by TGase with tropoelastin as the lysine donor, a process fundamental to elastic fiber formation [7,8] and similarly with itself [9]. Therefore, it has been suggested that tissue TGase-mediated crosslinking plays a vital role in strengthening microfibrillar networks [10]. To advance the understanding of the physiological and biotechnological role of TGase2 crosslinks, it is important to identify its acceptor and donor substrate sites in naturally occurring proteins. A considerable number of reactive glutamine residues have been identified in various proteins [11,12], but only a limited number of amine donor substrates have been characterized so far [13–19]. To fill this gap, Tong and co-workers identified a specific region on human fibrillin-1, spanning residues 2800–2806 (EDGFFKI), as an

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active amine donor substrate for TGase2 [40]. A previously characterized glutamine substrate site (APQ^3Q^4EA) derived from osteonectin, a matricellular protein, was used as an amine acceptor probe [20] and a widely-used potent lysine mimic, monodansylcadaverine (MDC) [22–25], was used as a control. Herein, we describe discovery of the sequence (EDGFFKI) that was applied previously [40] and provide more detailed biochemical studies on this sequence and its enzyme-specific properties. In this study, the potential of EDGFFKI to serve as a very specific tracer peptide to detect endogenous TGase activity and as a probe for enzyme-directed site-specific labeling of potential amine acceptor sites in native proteins was determined. The specificity of tissue transglutaminase and its substrates towards a particular glutamine in APQ^3Q^4EA during crosslinking was first examined, followed by specificity of reaction towards tissue transglutaminase or plasma transglutaminase (Factor XIIIa). APQQEA and EDGFFKI were then used as labels for tissue transglutaminase as compared to cadaverine for the specific staining of TGase when used in human skin samples. The results of this study should not only provide a better understanding of TGase2 activity and specificity, but should also inform optimal use of these enzymes in biotechnological and biomedical applications.

2. Materials and methods

2.1. Materials

All chemicals and solvents for peptide synthesis were of analytic reagent grade or higher and were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). All amino acids were of L-configuration and were purchased from Novabiochem (San Diego, CA).

2.2. Peptide synthesis

Peptides were synthesized on an automated Multipep peptide synthesizer (Intavis, Cologne, Germany), using solid-phase method. Briefly, the peptides were assembled on Fmoc-Gly-Wang resin. Stepwise couplings of amino acids were accomplished using a double coupling method with 5-fold excesses of amino acids, equivalent activators, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and N-hydroxybenzotriazole, and two equivalents of base, N-methylmorpholine. The removal of Fmoc was completed by using 20% piperidine in dimethylformamide. Cycles of deprotection, washing, double coupling, and washing were repeated until the desired peptide sequence were achieved. Crude peptides were purified using an Agilent 1100 semi-preparative HPLC (Agilent Technologies, Santa Clara, CA) on an Agilent Zorbax 300SB-C18 reverse phase (RP) column (5 μm particle size, 300 Å pore size, 25 × 1.0 cm) with a linear gradient of buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile) from 10% B to 60% B in 30 min at a flow rate of 4 mL/min. The purity of all peptides was greater than 95% as determined by Shimadzu analytical HPLC (Kyoto, Japan). The peptide mass was verified by LC–MS (Bruker, Bremen, Germany) and matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectroscopy (Bruker, Bremen, Germany). Peptides were dissolved in 100 mM Tris/HCl buffer (pH 7.4) at 2 mM and stored at −20 °C before use.

2.3. Enzymatic crosslinking and analysis

A previously characterized APQQEA was used as an amine acceptor probe [20]. The reaction volume (25 μL) contained 0.5 mM APQQEA, 0.5 mM EDGFFKI or MDC (Sigma–Aldrich), 100 mM Tris/HCl (pH 7.4), 10 mM $CaCl_2$, and 0.5 U/mL guinea pig liver TGase2 (Sigma–Aldrich). A control reaction contained similar compositions, except with no TGase2 added. The reaction cocktail was incubated at 37 °C and stopped by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 10 mM and diluted 2× by using 100 mM Tris/HCl (pH 7.4) at pre-determined time point. To quantify the reactivity of EDGFFKI and MDC towards the amine acceptor probe, the reaction mixtures were analyzed by analytical HPLC on an Agilent Zorbax 300SB-C18 reverse phase (RP) column (5 μm particle size, 300 Å pore size, 25 × 0.46 cm) with a gradient of buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile) from 5% B to 60% B in 20 min at a flow rate of 1 mL/min. Injection volume was 5 μL and the UV detector was set at 220 nm. LC–MS was used to identify each peak. The peak area of the unreacted peptide substrate was compared to that of the respective control (without TGase2) to determine the reaction conversion. MALDI–TOF mass spectroscopy was used to accurately identify the peptide substrates and the crosslinked peptides.

2.4. Factor XIIIa-catalyzed crosslinking

The reaction volume (25 μL) contained 0.5 mM APQQEA, 0.5 mM EDGFFKI or MDC, 100 mM Tris/HCl (pH 7.4), 10 mM $CaCl_2$, 10 mM DL-dithiothreitol (Sigma–Aldrich), 5 μg/mL human plasma coagulation factor XIII (FXIII) (Calbiochem, San Diego, CA) and 0.3 U/mL human plasma thrombin (Sigma–Aldrich). A control reaction contained similar compositions, but without FXIII. Reaction was performed at 37 °C for 18 h to generate active FXIIIa and to initiate crosslinking which was monitored using MALDI–TOF MS.

2.5. Detecting endogenous TGase activity in human plasma clot

Fresh human blood obtained via venipuncture was mixed with ethyleneglycoltetraacetic acid (EGTA) to a final concentration of 3 mM to inhibit coagulation and centrifuged at 4000 rpm for 15 min to isolate clear plasma. 47 μL of plasma were placed on a glass slide and mixed with 3 μL of 200 mM $CaCl_2$, spread over an area of ~1 cm^2, and left to clot and air dry for 3 h on a Leica HI 1220 flattening table (Leica Microsystems, Singapore) at 37 °C. The dry plasma clot was then fixed with methanol at −20 °C for 5 min, treated with 15 mM EGTA (in Tris/HCl, pH 7.4) for 30 min and washed thrice in PBS for 15 min. Plasma clot samples were then incubated for 1.5 h at room temperature with substrate buffer (10 μL of 10 mM biotin-EDGFFKI or biotin-APQQEA or biotin-cadaverine + 25 μL of 200 mM $CaCl_2$ + 965 μL of Tris/HCl) at pH 7.4. Parallel controls contained 25 μL of 200 mM EGTA instead of $CaCl_2$ to inhibit Ca^{2+}-dependent TGase activity. After washing 3 times in PBS, the samples were incubated for 30 min at room temperature with streptavidin-dichlorotriazinyl aminofluorescein (DTAF) (Jackson Immuno Research Laboratories, West Grove, PA) (1:100 in PBS) and washed 3 times in PBS for 15 min and mounted in polyvinyl alcohol (PVA) mounting medium with anti-fading agent. Images were captured using a Zeiss Axio Observer in ApoTome mode, 40× objective and at a fixed exposure time optimal for the control samples.

2.6. Detecting endogenous TGase activity in human skin tissue

As reported previously in detail [21], cryostat sections (5 μm) of human skin were air-dried for 10 min at room temperature, blocked and rehydrated with 1% BSA in 0.1 M Tris/HCl (pH 7.4) for 30 min at room temperature and then incubated for 1.5 h at room temperature with substrate buffer (10 μL of 10 mM biotin-EDGFFKI or biotin-APQQEA or biotin-cadaverine + 25 μL of 200 mM $CaCl_2$ + 965 μL of Tris/HCl) at pH 7.4. Parallel controls were treated 25 μL 200 mM EGTA instead of $CaCl_2$ to inhibit Ca^{2+}-dependent TGase activity. After washing 3 times in PBS, the sections were incubated for 30 min at room temperature with streptavidin-DTAF (1:100 in PBS), counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) to identify the nuclei of cells, and washed 3 times in PBS for 15 min and mounted and observed as above.

2.7. Specific labeling of amine acceptor sites in human skin tissue by exogenous TGase2 using EDGFFKI as a probe

To irreversibly inhibit the endogenous TGase activity, skin sections were incubated with a 10 mM solution of TGase inhibitor iodoacetamide in 100 mM Tris/HCl, pH 7.4, for 1.5 h and then were washed six times in PBS for 5 min each. The inhibition was verified using the protocol for endogenous TGase activity (see above). Iodoacetamide-treated sections were then incubated for 1.5 h at room temperature with enzyme + substrate buffer (200 μL of 1.25 mg/mL guinea pig liver tissue TGase II (Nzyme Biotech, Germany) + 10 μL of 10 mM biotin-EDGFFKI or biotin-APQQEA or biotin-cadaverine + 25 μL of 200 mM $CaCl_2$ + 765 μL of Tris/HCl) at pH 7.4. A control was treated similarly, except that $CaCl_2$ was replaced with 25 μL 200 mM EGTA to inhibit the Ca^{2+}-dependent tissue TGase. The slides were washed 3 times in PBS for 15 min, incubated for 30 min at room temperature with streptavidin-DTAF (1:100 in PBS), counter-stained with DAPI, washed thrice in PBS, and mounted and observed as above.

3. Results

3.1. Identification of the reactive glutamyl residue in APQQEA

A previously characterized APQQEA was used as an amine acceptor probe. EDGFFKI substrate has been shown to be cross-linked to only one of the Gln residues, resulting in the conjugation of EDGFFKI and APQQEA with a 1:1 ratio [40]. However, the preference of TGase2 for either Gln3 or Gln4 was not known. Two alternative substrate peptides, APQNEA and APNQEA, were used to verify whether EDGFFKI bound specifically to one Gln residue or randomly to one of the two adjacent Gln residues. It can be seen that both EDGFFKI and MDC were readily coupled to the