Amino Acids (2013) 44:245–250
DOI 10.1007/s00726-011-1142-5

ORIGINAL ARTICLE

Identification of human salivary transglutaminases

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Received: 30 June 2011 / Accepted: 27 October 2011 / Published online: 12 November 2011

Abstract Transglutaminases (TGs) expression and enzymatic activities in human saliva were investigated. Specific antibodies showed the co-existence of TG1, TG2, TG3 and TG4. TG2 and TG3 were found in native and multiple proteolytic forms. Our data indicate that TG1 and TG2 isoenzymes are highly active with the major activity attributed to TG1. These findings pave the way for future studies on the physiological role of TG in the oral cavity and the potential impact of their deregulation in TG-associated oral diseases.

Keywords Transglutaminase · Saliva fluid · Transamidase activity · TG-specific assay

Introduction

Transglutaminases (TGs) are a family of enzymes that catalyze Ca^{2+}-dependent transamidation reactions, resulting in inter- or intramolecular protein crosslinking, polyamination or deamidation (Beninati and Piacentini 2004). Among the well-characterized isoenzymes there is the keratinocyte TG (TG1), which exists in membrane bound and soluble forms (Kim et al. 1992, 1995), the epidermal TG (TG3) proenzyme, which requires proteolytic activation (Kim et al. 1990; Negi et al. 1985) and TG1 and TG3, that are involved in the terminal differentiation of keratinocytes and have been associated with some pathologies such as laryngeal carcinoma, squamous carcinoma and lamellar ichthyosis, respectively (He et al. 2002; Ohkura et al. 2005; Duvic et al. 1994; Herman et al. 2009). Also among the well-described isoenzymes there are the plasma TG (Factor XIIIa) and the prostatic secretory TG (TG4), which are involved, respectively, in stabilization of fibrin clots and in fertility in rodents (Iismaa et al. 2009; Seitz et al. 1990), and the ubiquitous tissue TG (tTG, TG2), whose role is likely to be more extended. TG2 has been found in different intracellular compartments and also in the extracellular matrix (Griffin et al. 2002). tTG has been involved in a wide range of physiological functions including cell adhesion, formation of extracellular matrix, and apoptosis (Rossin et al. 2011; Fesus and Piacentini 2002; Falasca et al. 2005), and also in a number of pathological states such as fibrosis, celiac disease (Rossin et al. 2011), hepatocellular carcinoma, diabetes and neurodegenerative disorders (Caccamo et al. 2010; Iismaa et al. 2009).

Although the distribution of these isoenzymes has been well established in tissues and body fluids, only few studies reported the presence of TGs in saliva, though without identifying the exact isoform(s) (Bradway et al. 1992; Cocuzzi et al. 1989; Hannig et al. 2005). Recently, using advanced proteomic technology, Bandhakavi et al. (2009) identified peptides with high homology sequence to TG1 and TG3 in saliva.

In contrast to saliva, oral epithelial cells are well known to be a significant source of TGs, with major interest focused on TG1 and TG3 linked to oral diseases. In 1990, an early study of normal and pathological tissue samples from the oral epithelium provided first evidences for the absence of TG1 expression in premalignant oral lesions (Ta et al. 1990). Unfortunately, this study did not survey the status of the isoenzyme in saliva despite its

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potential use in diagnostic. In more recent studies, TG3 has been shown to be substantially expressed in histological samples from leukoplakias in the oral mucosa while suppressed in oral squamous cell carcinoma (OSCC) and some severe dysplasias, but again the saliva was largely disregarded (Ohkura et al. 2005). In celiac disease, where TG2 is considered as the auto-antigen, IgA anti-TG2 antibodies have been detected in saliva; however, the low sensitivity of the current tests has limited the use of this fluid for the diagnostic purpose (Baldas et al. 2004).

With this knowledge and because saliva has gained increasing interest for monitoring general health and for the early diagnosis of diseases, we carried out a preliminary study in which TG isoforms and their corresponding activities were investigated in healthy volunteers in order to better characterize the enzyme family in this fluid.

Materials and methods

Reagents and antibodies

TG2 site-specific inhibitor Z-DON-VPL-OMe, human recombinant TG1 (rhTG1), human recombinant TG2 (rhTG2), human recombinant TG3 and human recombinant TG4 were purchased from Zedira (Darmstad, Germany). Rabbit anti-human TG1 antibody, rabbit anti-human TG2 antibody, monoclonal anti-human TG3 antibody and polyclonal anti-human TG4 antibody, goat anti-mouse horseradish peroxidase and goat anti-rabbit horseradish peroxidase antibody were from Covalab (Villeurbane, France).

Selection of participants

Twenty subjects participated in the study (11 women and 9 men). All the participants underwent complete dental evaluation. Smokers and subjects with bad oro-dental hygiene were excluded.

Collection of saliva

A standardized saliva collection protocol was used. In brief, subjects were instructed to wash their mouth with unionized water to remove any food or debris in their mouth. Unstimulated saliva was collected from all individuals in plastic tubes over a period of not more than 5 min. Whole saliva was stored at −20°C, or centrifuged at 4,000*g* using a bench centrifuge; supernatant and pellet were stored at −20°C, separately.

TG activity assay

Transglutaminase activity was evaluated using the commercially available TG-Covtest kit (Transglutaminase Colorimetric Microassay; Covalab, Villeurbanne, France), which uses immobilized CBZ-Gln-Gly as the first substrate and biotinylated-cadaverine (biotin-Cd) as second substrate of the enzyme. The assay was performed following the manufacturer's instructions. In brief, samples were incubated with 50 µl of biotin-Cd solution containing $CaCl_2$ for 30 min at 37°C. As negative control, 50 mM EDTA (50 mM) or iodoacetamide were added into the corresponding wells. At the end of the incubation period, plates were washed three times with Tris–HCl (pH 8.3) containing 0.1% Tween 20. Then, 100 µl of horseradish peroxidase (HRP) diluted to 1:2,000 was added to the wells and incubated for 15 min at room temperature. The HRP signal was revealed using 100 µl of 0.01% H_2O_2 as substrate of the enzyme and 3,3′,5,5′-tetramethylbenzidine (TMB) as chromogen. The reaction was stopped by the addition of $2NH_2SO_4$ and the absorbance read on a microplate reader at 450 nm.

TG1 and TG2 specific activity assays

Specific TG1 and TG2 isoenzyme activities were evaluated using the TG1-CovTest and TG2-CovTest (Covalab), which utilize immobilized spermine as second substrate of TG and the biotin-HQSYVDPWMLDH peptide (pepT26) or the biotin-YEQHKLPSSWPF peptide (pepK5) as preferred first substrate for TG1 and TG2, respectively. The assay was performed following the manufacturer's instruction. In brief, 50 µl of the reaction buffer was mixed with 50 µl samples and incubated in the microtiter plates at 37°C for 25 min, and at the end of this period, wells were emptied and washed three times with 0.1 M Tris–HCl, pH 8.5 containing 0.1% Tween 20. The cross-linked biotinylated peptides were detected by the addition of 100 µl HRP as described above.

Western blot

Proteins were separated on a 10% (w/v) polyacrylamide gel and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Life Science, UK). The membrane was blocked with 5% (w/v) not-fat dried milk and 0.1% (w/v) Tween 20 in TBS, and then immunoprobed with specific antibodies for the different TG isoenzymes in 1:750 dilution at 4°C overnight. Primary antibody binding was revealed with goat anti-mouse horseradish peroxidase or goat anti-rabbit horseradish peroxidase secondary antibody (Covalab) in 1:1,000 dilution for 1 h at room temperature and visualized using chemiluminescent reagent kit covalight (Covalab).

Results and discussion

TG activity and patterns of the TG isoenzymes proteins in saliva

The presence of transglutaminase activity was measured using a commercially available TG enzymatic assay (TG-Covtest, Covalab, France) which tests crosslinking activity in TG samples, irrespective of the isoenzyme forms. From this screening, Ca^{2+}-dependent crosslinking enzymatic activities were identified in all samples, with no differences between men and women (Fig. 1a). The signal was inhibited by iodoacetamide, a compound known to modify the cysteine-277 residue at the active-site of TG (Lee et al. 1993).

Using specific antibodies against the different members of the TG family, TG1, TG2, TG3 and TG4 were identified in the whole saliva. As shown in Fig. 1b, TG1 and TG3 were found at their expected molecular weights (90 and ≈70 kDa, respectively). In addition, three weak inmunoreactive bands for TG3 (30, 50 and 60 kDa) were also visualized. These bands may correspond to active proteolytic forms of the isoenzyme (Kim et al. 1990; Negi et al. 1985). Immunoblots against TG2 showed the presence of 77, 55 and 26 kDa bands and the specific antibody for TG4 revealed two reactive bands of the enzyme, in both female and male individuals (Fig. 1b). This latter finding is of particular interest taking into account that TG4 is considered to have a prostate-restricted expression pattern (Dubbink et al. 1999).

To understand whether the observed TG isoenzymes were differentially distributed between the soluble and the insoluble component of saliva, samples were centrifuged at 4,000*g* and pellet and supernatant fractions were analyzed separately for the presence or absence of TG isoforms. TG1 and TG4 proteins were detected in pellet samples but absent or in small amount in supernatants (data not shown). This observation suggests that these isoforms are mainly associated with cellular debris in saliva, probably as membrane-bound forms. In contrast, TG3 immunoreactive bands were observed in both, soluble and insoluble

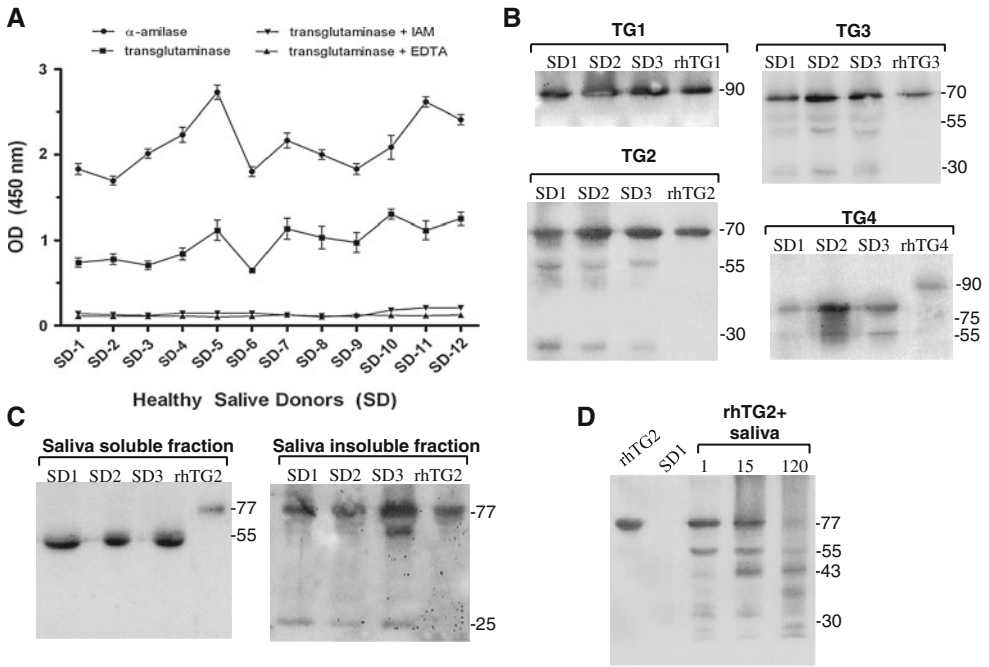

Fig. 1 **a** Transglutaminase crosslinking activity in saliva using *N*-CBZ-L-Gln-L-Gly and biotinylated-cadaverine as TG substrates (TG-Covtest, Covalab). The graph is representative of 12 out of 20 subjects. Salivary alpha-amylase activity (alpha-Amylase Saliva EA assay, GmbH) was used as control of the fluids quality and the flow rate differences. The data shown are the mean ± standard error of mean. **b** Expression of TG1, TG2, TG3 and TG4 isoenzymes in saliva. Aliquots of 10 μg of total protein from the whole saliva of three subjects (*lanes 1–3*) were resolved by SDS-PAGE (10%) and Western blots were performed using anti-TG1 antibody (pab0060, Covalab), anti-TG2 antibody (pab0062 Covalab), anti-TG3 antibody (mab0058) and anti-TG4 antibody (pab0066), respectively. Human recombinant TGs (rhTG1–rhTG4) were used as corresponding control (*lane 4*). **c** Differential expression pattern of TG2 in salivary soluble and insoluble fractions. Whole saliva was centrifuged at 4,000*g* and 10 μg of total protein from either supernatant (*left panel*) or pellet (*right panel*) from three different subjects (*lanes 1–3*) was analyzed by Western blot using anti-TG2 antibody. Human recombinant TG2 (rhTG2) was used as control. **d** rhTG2 proteolysis by component(s) of the saliva fluid. Western blot was performed using anti-TG2 antibody (pab0062) in membranes containing 10 μL saliva soluble fraction (0.1 μg/μL), preincubated at 37°C with either vehicle alone (*lane 2*) or with 3 μg of rhTG2 (*lanes 3*, *4*) at different times, as indicated. Distilled water (10 μL) containing rhTG2 (3 μg) was used as control (*lane 1*)

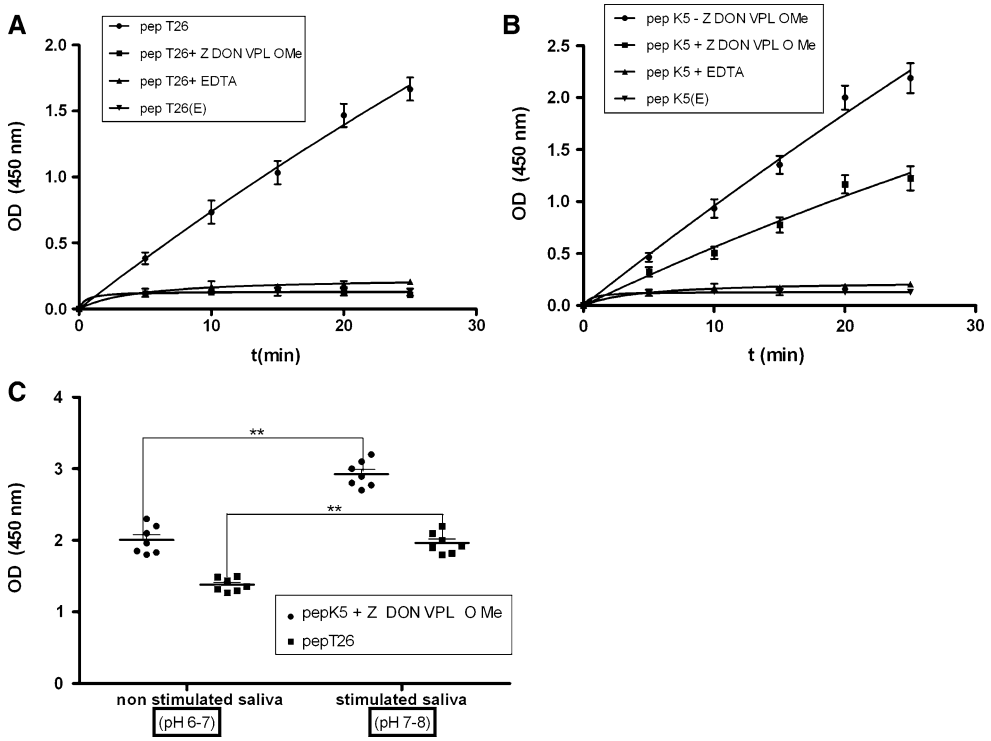

Fig. 2 **a** TG2 crosslinking activity measured in saliva. The graphic shows the isoenzyme specific incorporation of biotinylated-pepT26 into spermine in time-dependent transamidation reaction. The incorporated peptide was detected by using peroxidase-conjugated streptavidin and TMB as described before (Seitz et al. 1990). The specific inhibitor of TG2, Z-DON-VPL-OMe, the quelating agent EDTA and biotin-pepT26 with the glutamine residue substituted by glutamic acid (pepT26 (E)) were used as negative controls of the reaction. **b** Salivary TG1 crosslinking activity. This was carried out as described in A, using the biotinylated-pepK5 as preferred substrate for TG1. **c** TG1 and TG2 crosslinking activities in stimulated vs non-stimulated saliva. The assay was performed in the same conditions as in a and b, using the preferred substrates pepK5 + Z-DON-VPL-OMe and pepT26 for assaying specific TG1 and TG2 activities, respectively (Seitz et al. 1990)

fractions (data not shown). For TG2, the 77 kDa band was detected in the insoluble fractions; whereas, in the supernatants only the single band at 55 kDa was found (Fig. 1c). This band might be the result of a specific cleavage by salivary protease(s), since a similar electrophoretic pattern was observed when human recombinant TG2 (rhTG2) was incubated with 10 μL of saliva supernatant (Fig. 1d).

Prevalent activity of TG1 and TG2 isoenzymes in saliva

In recent works, we have reported the development of highly specific assays for measuring TG1, TG2 and TG3 activities using their preferred substrates: pepK5, pepT26 and E51 peptide, respectively (Hitomi et al. 2009; Perez Alea et al. 2009; Yamane et al. 2009). To specifically assess active TG isoenzyme(s) in saliva, we combined these assays with different TG inhibitors, including Z-DON-VPL-OMe which shows no effect on TG1 activity and high inhibition of TG2 activity (IC50 $\approx$ 0.05 μM).

All saliva samples, either pellet or supernatant fractions, were able to incorporate pepT26 into spermine (Fig. 2a) (Hitomi et al. 2009; Perez Alea et al. 2009). The crosslinking of pepT26 was completely prevented by Z-DON-VPL-OMe or when the glutamine residue in pepT26 was replaced by glutamic acid (pepT26(E)) suggesting that both 77 and 55 kDa TG2 forms present in saliva are enzymatically active. The evidence for the existence of a cleaved soluble and active form of TG2 in saliva might sheds some light into the unknown mechanism by which TG2 is found in the extracellular compartment of some tissues.

Interestingly, TG2 was not the unique active isoenzyme we found in this fluid. pepK5 (preferred substrate for TG1) was also crosslinked into spermine in a Ca^{2+}-dependent manner (Fig. 2b). Substitution of glutamine by glutamic acid residue in pepK5 (pepK5 (E)) did not lead to the formation of the crosslinking product. The addition of Z-DON-VPL-OMe inhibitor with pepK5 resulted in a partial inhibition (50%) of the reaction. As published before, pepK5 can also act as first substrate for TG2, although with lower affinity (Hitomi et al. 2009). Since Z-DON-VPL-OMe does not affect TG1 activity, our data strongly suggest that both enzymes are active in saliva and the decreased activity observed with pepK5 in the presence

of the inhibitor corresponds to the transglutaminase activity of TG1 after TG2 enzymatic activity has been inhibited.

These findings are not surprising if we consider the high content of calcium ion in saliva and the pH range of this fluid (pH 6–7 in non-stimulated saliva and pH 7–8 in stimulated saliva) which are consistent with optimal conditions for the transglutaminase activity (Chae et al. 2005). Indeed, when saliva is stimulated by chewing, a concomitant increase of salivary pH with TG activity was observed (Fig. 2c). Concerning TG3 and TG4 activities, no specific assays are available to date. However, given the fact that TG3 is mainly found at a molecular weight that corresponds to the proenzyme, we hypothesized that the isoenzyme might be mainly inactive in the saliva fluid, while TG4 remains to be further investigated.

Conclusion

Three main findings emerged from our study: (1) TG1, TG2, TG3 and TG4 are present in saliva, being TG1 and TG4 mainly confined to cellular debris while TG2 and TG3 are present in both soluble and insoluble component of saliva; (2) TG2 isoenzyme is likely to undergo specific proteolysis in the oral cavity by an unknown salivary protease(s) without loss of its activity; (3) TG1 and TG2 are the main active isoenzymes responsible for the salivary transglutaminase activity which increases upon stimulation of saliva.

Future studies will investigate the potential impact of the salivary TGs in celiac disease. Indeed, the early contact of gluten-containing food with salivary TG1 and/or TG2 takes place in the oral cavity. Therefore, the early chemical modifications of TG substrates might potentially and selectively occur during this first step and constitute the initial event which later in the gut will cause the known characteristics of the disease. Our findings could provide some insight into the role of TG2 in the physiopathology of CD which at present remains enigmatic.

Finally, the association of oral malignancies and TG1/TG3 in saliva remains now to be corroborated, but seems to be a plausible hypothesis given the positive association of cancer with low or absent TG1 and TG3 expression in earlier studies (Ohkura et al. 2005; Ta et al. 1990). Assuming an association of TG1 and TG3 activities with these pathological conditions, it will be of special interest to assess salivary transglutaminase activity in clinical groups with severe oral dysplastic tissues and OSCC, in which we would hypothesized decreased salivary TG1 and/or TG3 transamidase activities.

Acknowledgments The authors gratefully acknowledge the financial support of the Marie Curie Action RTN program "Transglutaminase: role in pathogenesis, diagnosis and therapy" (TRACKS, MRTN-CT-2008-36032) and the Marie Curie Industry-Academia partnerships and pathways programme "TRANSCOM" (IAPP-251506) We knowledge Dr. Kiyotaka Hitomi for kindly providing the peptides pepT26(E), and pepK5(E) and Dr. Kim In-Gyu for the interesting discussion on saliva. We also thank all the participants in this study.

Conflict of interest S. El Alaoui and V. Thomas have a financial relationship with the organization that sponsored this research. M. Perez Alea and G. Martin declare to not have financial relationship with the organization that sponsored the research. The authors declare that they have not conflict of interest.

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