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ORIGINAL ARTICLE

Expression pattern of transglutaminases in the early differentiation stage of erupting rat incisor

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Abstract Several studies demonstrated that transglutaminases play a key role in extracellular matrix stabilization needed for cell differentiation. We evaluated transglutaminase expression and activity in the pre-secretory stage of differentiation of the continuously erupting rat incisor. We observed that transglutaminase-mediated incorporation of monodansylcadaverine into protein substrates was specifically located in the apical loop, and along the basement membrane joining mesenchyme and inner dental epithelium in the odontogenic organ. Enzyme activity was associated with mRNAs for transglutaminase 1 and 2. Notably, labelling cells for these isoenzymes were observed in both mesenchymal and epithelial compartments, but not in the basement membrane, in the ameloblast facing pulp anterior region, where ameloblast and odontoblast differentiation begins. These findings demonstrate that transglutaminase 1 and transglutaminase 2 are expressed at a major extent in the pre-secretory stage of regenerating rat incisor, where they probably play complementary roles in cell signalling between mesenchyme and epithelium and extracellular matrix.

Keywords Rat incisor · Differentiation · Transglutaminase 1 · Transglutaminase 2 · Cross-linking activity · Mesenchymal–epithelial interactions

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Introduction

Transglutaminases (TGs) catalyze a calcium-dependent transamidating reaction resulting in the post-translational modification of proteins through the cross-linking of glutamine and lysine residues or the covalent incorporation of polyamines (Folk and Finlayson 1977; Griffin et al. 2002). At present, six TGs have been isolated and characterized as regards structure, function, tissue distribution and substrate specificity (Lorand and Graham 2003; Esposito and Caputo 2005). Among these isoenzymes, tissue transglutaminase or TG2 is the only one, which occurs in an intracellular and extracellular form and is ubiquitous (Thomazy and Fesus 1989; Chen and Mehta 1999). Elevated TG2 expression and enzyme activity inhibit proliferation and accompany cell differentiation in various cell types (Fesus and Piacentini 2002). Notably, other than transamidating activity, TG2 exerts GTPase-related effects on cell signalling between extra- and intra-cellular milieu (Nakaoka et al. 1995).

It has been shown that TG2 is involved in the cross-linking of a variety of mineralization regulatory proteins in the extracellular matrix (ECM) of chondrocytes and osteoblasts (Kaartinen et al. 2002). The protein network in ECM, in turn, appears to be essential for further matrix production, ECM stabilization and progression of differentiation program, ultimately leading to mineralization (Aeschlimann and Thomazy 2000; Nurminskaia and Kaartinen 2006).

In this context, it has been considered that protein cross-linking enzymes participate in ECM maturation and cell adhesion in bone as well as in tooth (Nurminskaya and Kaartinen 2006). In particular, in rat tooth extracts TG activity has been associated with the presence of high-molecular-weight forms of the small integrin-binding

ligand n-linked glycoprotein (SIBLING) proteins, such as dentin matrix protein 1 and bone sialoprotein, containing lysine residues critical for cross-link formation (Kaartinen et al. 2005).

Rat incisor has been extensively studied to characterize cellular and ECM events involved in the odontogenesis, because it continuously regenerates and all stages of differentiation can be found in a single tooth (Smith and Warshawsky 1975). During this process an intricate set of cellular rearrangement is associated with regeneration events sustained by adult stem cells (Harada et al. 1999). Further, the interactions between cells and ECM are determinant in order to promote the synthesis of several proteins, which mediate the interplay between mesenchyme and basement membrane, and between this latter and epithelium (Thesleff et al. 1991; Kieffer-Combeau et al. 2001; Morotomi et al. 2005).

The aim of this study was to evaluate the expression and activity of different TGs, with particular regard to their tissue distribution, in an early stage of differentiation of rat incisor, such as that found in the pre-secretory zone of the apical loop.

Materials and methods

Animals and tissue preparation

Five young Wistar albino rats (Charles River, St. Constant, Canada) weighing 180–200 g were sacrificed under general anaesthesia with chloral hydrate (300 mg/kg intraperitoneally). Animals were then perfused through the left ventricle with a Ringer lactate solution (Sigma, Milan, Italy) followed by 4% formic aldehyde in 0.2 M phosphate buffer pH 7.2–7.4 (PBS) (Sigma). Both mandibles were removed under a stereomicroscope, and fixed in 4% formic aldehyde in 0.2 M PBS for 2 h. Subsequently the two lower incisors were carefully extracted in order to avoid damaging of apical end (odontogenic organ and related pulp), that was subsequently isolated. Specimens were fixed by submersion in 4% formic aldehyde in 0.2 M PBS for 30 min.

After repeated rinsing in PBS, specimens were infiltrated in 12 and 18% sucrose, frozen in liquid nitrogen and longitudinally sectioned using a cryostat. Sections, each 10 μm thick, were placed on gelatinized slides and stored at −20°C until used.

Monodansylcadaverine labelling of tissue slices

TG-mediated incorporation of monodansylcadaverine into cell proteins of tissue slices obtained from rat incisor was used as a tool to characterise the endogenous substrates of TG activity, according to previously described methods (Ientile et al. 2002).

Briefly, after rinsing with PBS, the tissue slices were incubated for 4 h at 37°C in the same buffer containing 0.1 mM monodansylcadaverine (Sigma). For determination of fluorescent substrates incorporated into cell proteins, labelled specimens were briefly rinsed with PBS, then fixed for 5 min at −20°C with methanol. Finally, glass cover slips were twice rinsed with PBS for 5 min and mounted for confocal laser scanning microscopy (CLSM) analysis by a Zeiss 510 microscope (Zeiss Inc., North America).

RT-PCR

Total RNA was isolated from rat incisors using TRIzol (Invitrogen, Milan, Italy). Then, 5 μg of RNA were reverse transcribed with the Thermoscript First Strand cDNA synthesis kit (Invitrogen).

Amplification via PCR was performed in a total volume of 100 μl, containing 5 μl of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 units of *Taq* DNA Polymerase (Celbio, Milan, Italy), and 0.2 μM of specific primers for TG1, TG2, TG3, and TG5 (Primm, Milan, Italy). Beta-actin was also included as internal control for amplification reaction. Primer sequences were deduced by Caccamo et al. (2003), except for those of TG5, which were derived by Candi et al. (2001). PCR reactions were carried out with conditions of one cycle at 95°C (3 min), 25 or 35 cycles each of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and finally one extension cycle of 72°C for 7 min, using a Hybaid PCR Sprint thermocycler (Celbio).

RT-PCR products were separated on 2.5% agarose gel, and visualized by ethidium bromide staining. The identity of cDNAs obtained was confirmed after matching their sequence with known sequences of different TGs.

Finally, the relative amounts of TG transcripts were quantified by densitometric analysis with a AlphaImager 1200 System (Alpha Innotech, San Leandro, CA, USA), after standardization with respect to the amount of beta-actin mRNA.

Immunohistochemistry

Fixed tissue sections were rinsed in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After 1 h of blocking in PBS plus 1% bovine serum albumin (BSA) and 10% goat serum, samples were incubated for 1 h with goat polyclonal antibody against TG1 (N-20) (SantaCruz Biotechnology, DBA, Milan, Italy) (diluted 1:100 in PBS plus

1% BSA), or mouse monoclonal antibody against TG2 (clone CUB 7402, NeoMarkers, Bio-Optica, Milan, Italy) (diluted 1:200 in PBS plus 1% BSA). After rinsing in PBS, sections were incubated for 1 h with FITC-conjugated anti-goat (1:64 in PBS plus 1% BSA) or Texas Red-conjugated anti-mouse (1:50 in PBS plus 1% BSA) secondary antibody (Sigma) to detect TG1 and TG2, respectively. Then, samples were washed in PBS, mounted in PBS/glycerol and examined with confocal laser scanning microscope (LSM 510 Zeiss, Inc).

Negative controls were prepared by incubating samples with either PBS alone or non-immune serum, followed by secondary FITC- or Texas Red-conjugated antibodies. All incubations were performed at room temperature in a humid chamber.

Composite images were made using different software functions. We used a function that overlays the fluorescent signal with light transmission to visualize the morphology of the section. Collected images were digitized at a resolution of 8 bits into an array of 2,048 × 2,048 pixels. Optical sections of fluorescent specimens were obtained using HeNe laser (543 nm) and Argon laser (458 nm) at a 1′2″ scanning speed up to 8 average. The pinhole of the confocal system was closed to a minimum to yield the thinnest possible optical section.

Results

Microscopical observations were carried out in the region of rat incisor apical end, over a 750 μm length area corresponding to the first part of the pre-secretory zone which shows an increasing gradient of cell differentiation from the apical end to the incisal edge (Fig. 1a, b). This region, related to the epithelial compartment, was classified by Smith and Warshawsky (1975) as "odontogenic organ" to be distinguished from the close region indicated as "enamel organ". The odontogenic organ, which arises from epithelium thickening and subsequently budding into underlying mesenchymal pulp, consists of the apical loop,

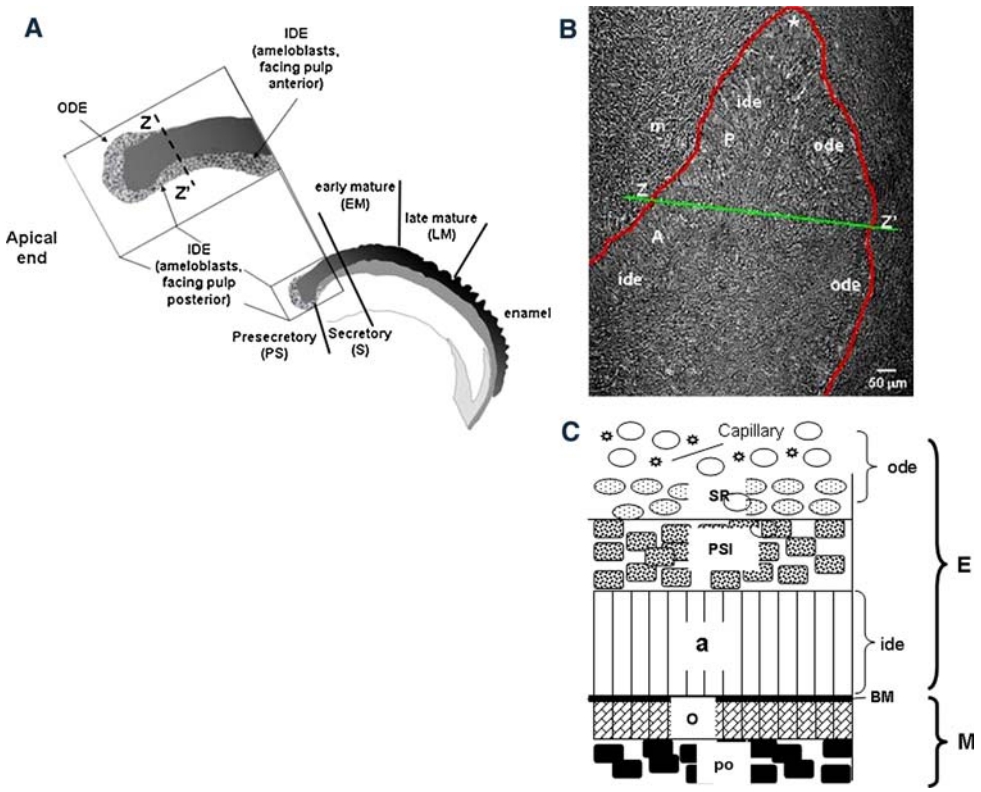

Fig. 1 **a** Schematic diagram of continuously erupting rat incisor showing different maturation stages. Cells precursors of epithelial compartments are present in the outer dental epithelium (*ODE*) and inner dental epithelium (*IDE*). Differentiating ameloblast compartments are also indicated. The line Z–Z′ indicates the site of change from single to multiple layer. **b** Microscopical image of the apical loop (*) of the rat incisor. The inner dental epithelium (*ide*), on the left, and the outer dental epithelium (*ode*), on the right, can easily be distinguished. The line Z–Z′, showing the site of change from single to multiple layer of the outer dental epithelium, divides the ameloblasts facing pulp posterior (*P*), close to the mesenchyme (*m*), from the ameloblasts facing pulp anterior (*A*). **c** Schematic drawing illustrating the organization of odontogenic organ and adjacent mesenchyme. *a* Ameloblasts, *BM* basement membrane, *E* epithelium, *ide* inner dental epithelium, *M* mesenchyme, *ode* outer dental epithelium, *po* pre-odontoblasts, *PSI* provisional stratum intermedium, *SR* stellate reticulum

which is continuous with the ameloblast layer (inner dental epithelium) on the left side and the outer dental epithelium on the right side (Fig. 1a). Cellular layers contained between the inner dental epithelium (IDE) and outer dental epithelium (ODE) are related to provisional stratum intermedium (PSI) and stellate reticulum (SR). A basement membrane (BM) separates the ameloblasts from mesenchymal cells of the pulp (Fig. 1c).

Using monodansylcadaverine as lysine-substrate analogue, we investigated the histochemical profile of TG activity in the rat incisor apical end. An intense fluorescence was observed in the apical loop and the region close to IDE, while was almost absent in the ODE (Fig. 2a). In particular, TG-mediated monodansylcadaverine incorporation into protein substrates seemed mostly localized in correspondence of the BM joining mesenchyme and IDE, and, at a lesser extent, in the BM joining mesenchyme and ODE. This fluorescent signal was not present in the negative control sections and was therefore assumed to represent specific TG activity.

Moving toward the regions corresponding to the "ameloblast region facing pulp posterior" (Fig. 2b)—where ameloblasts start to develop all differentiating morphological features of secretory cells, and mesenchyme condense around the bud—and "ameloblast region facing pulp anterior" (Fig. 2c), an intense fluorescence was observed in the BM between the epithelial cell compartment and the mesenchymal one (Fig. 2b), while a moderately fluorescent signal was present in the intercellular space in both cell compartments (Fig. 2c).

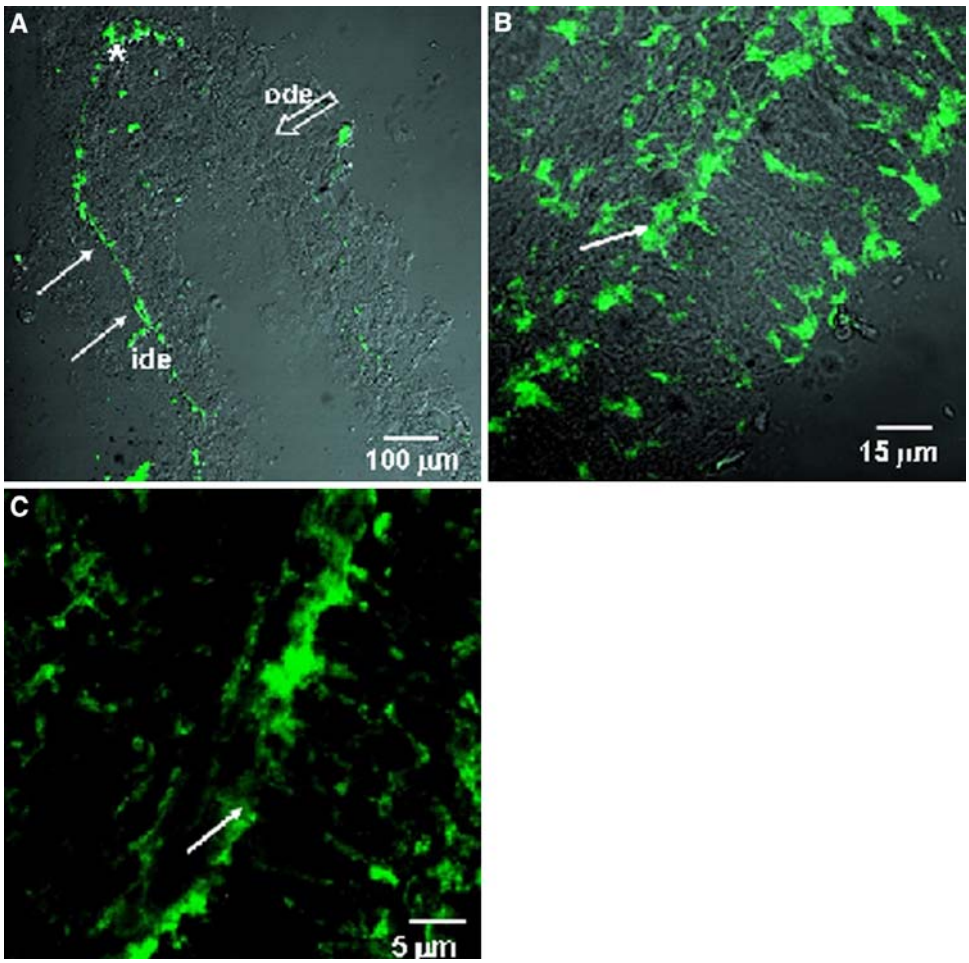

Fig. 2 Analysis of the histochemical profile of TG activity in rat incisor. Microscopical observations were carried out in tissue sections incubated with monodansylcadaverine, a green fluorescent substrate for TG-mediated cross-linking, as described in Materials and methods. **a** The superimposition of fluorescence image with interferential contrast image showed that monodansylcadaverine labelled the basement membrane (*BM*) joining mesenchyme and inner dental epithelium (*arrow*). A less intense fluorescent signal was also present between mesenchyme and outer dental epithelium (*open arrow*). (*) apical loop, inner dental epithelium (ide), outer dental epithelium (ode). **b** Superimposition of fluorescent signal with interferential contrast image of "ameloblast region facing pulp posterior" showing, at a higher magnification, that monodansylcadaverine fluorescence was mostly localized between mesenchyme and inner dental epithelium (*arrow*). **c** A higher magnification of "ameloblast region facing pulp anterior" revealed that a moderate fluorescence was also present in the intercellular space in both epithelial and mesenchymal cell compartment (*arrow*)

We also examined the expression of several known TG proteins in dental apical end. Using specific primer sets for TG1, TG2, TG3, and TG5, we found mRNA transcripts only for TG1 and TG2 (Fig. 3).

Immunohistochemical analyses revealed that both TG1 and TG2 were densely distributed along the apical loop and in the entire odontogenic organ. In this latter, however, the fluorescent signal was more intense in the IDE region than in the ODE. Notably, a sparsely distributed fluorescence was also observed in the thickening mesenchymal region close to the IDE (Fig. 4a, b). A higher magnification allowed to better observe the presence of the two isoenzymes in the cell compartments related to PSI and to SR (Fig. 4c, d). Notably, moving toward the cuspidal region, a dramatic decrease of TG1- or TG2-specific fluorescence was observed in the BM dividing the "ameloblast region facing pulp posterior" from the mesenchyme (Fig. 4e, f).

At the apical limit of the "ameloblast region facing pulp anterior", where ameloblast differentiation begins and adjacent pulp cells are partly differentiated into odontoblasts, labelling cells for TG1 and TG2 were seen densely in both epithelial and mesenchymal compartments (Fig. 4g, h). Notably, the fluorescent signal disappeared from the BM dividing ameloblasts and mesenchymal cells (Fig. 4g, h).

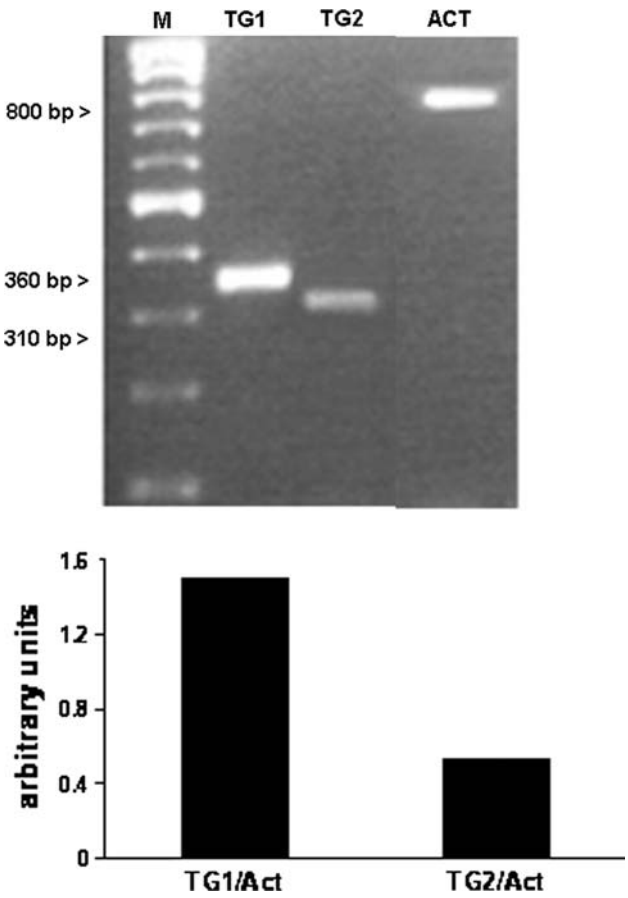

Fig. 3 Analysis of the expression of different TGs in the rat incisor. RT-PCR was carried out using specific primers for TG1, TG2, TG3, TG5, and beta-actin (*ACT*). Then, mRNA levels of TG1 and TG2, which were the only TGs found in tooth samples, were evaluated by densitometric analysis, after standardization with those of beta-actin. *M* molecular weight marker (Ladder 100 bp), *bp* base pair

Discussion

Tooth development is regulated by sequential and reciprocal interactions between mesenchymal and epithelial tissues of the oral environment (Thesleff et al. 1991; Kieffer-Combeau et al. 2001; Morotomi et al. 2005). Signalling factors, released by mesenchymal cells, influence the ectodermic compartment after crossing the BM (Mitsiadis et al. 1995a, b; Kawano et al. 2004). The presence of focal contacts to the secreting pole of future ameloblasts suggests that also the ectoderm, in turn, carries out one modulating function on the underlying mesenchymal cell compartment (Cutroneo et al. 2002).

Our observations in the apical loop of rat incisor underline the presence of TG activity in the odontogenic organ and BM. The distribution of fluorescent monodansylcadaverine in the epithelial and mesenchymal cell compartment demonstrated that both participate in the extracellular pool deposition at BM level. Notably, the mesenchyme condensed in the dental papilla seems to play a role quantitatively more relevant than the epithelium.

The BM layer and BM proteins play an important role in tooth morphogenesis and regeneration. A permanent remodelling of the BM is required to allow for the adequate maintenance of this structure with the progressive and coordinated evolution of both the IDE and mesenchymal pre-odontoblasts/odontoblasts, which, together, contribute to the ECM turnover (Fukumoto and Yamada 2005). Indeed, BM intervene as a substrate and/or the reservoir of active molecules. BM components, i.e. collagen, laminin and nidogen, have been reported as substrates for TG activity (Esposito and Caputo 2005). Further, BM stores high amounts of midkine, a protein secreted by the mesenchyme and mainly involved in the regulation of epithelial–mesenchymal interactions underlying cytodifferentiation in tooth (Mitsiadis et al. 1995b). Notably, midkine dimerization, that is needed for achievement of its full biological activity, is dependent on TG-mediated cross-linking (Kojima et al. 1997).

BM composition changes considerably during ameloblast and odontoblast differentiation, and a regional distribution of BM or BM-associated proteins, such as fibronectin, is observed (Thesleff et al. 1981; Sawada and Nanci 1995; Yoshiba et al. 2000). Interestingly, SIBLING proteins, such as DMP and DPP, which are substrates for TG activity during dentinogenesis (Kaartinen et al. 2005),

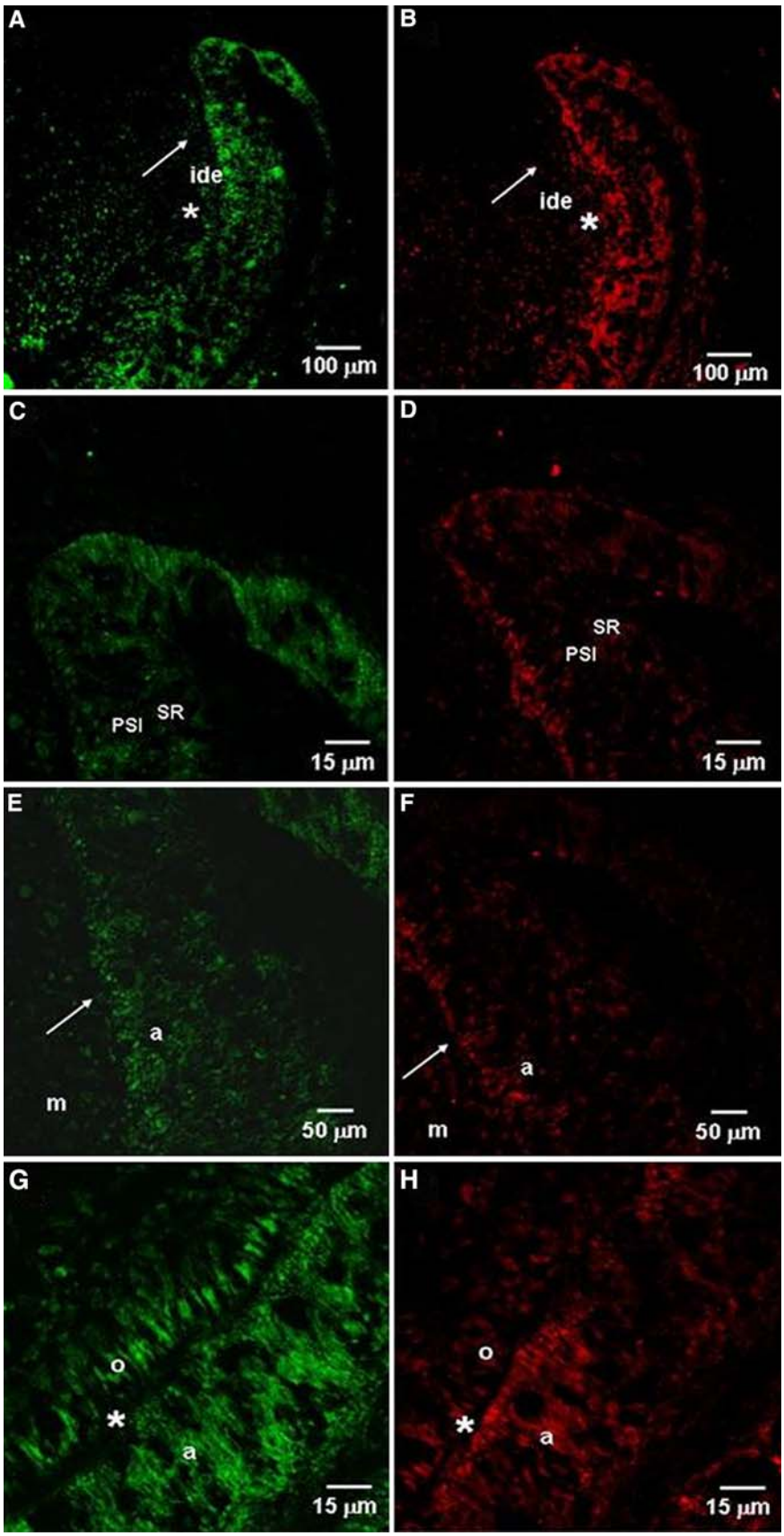

Fig. 4 Histochemical analysis of TG1 and TG2 distribution along the increasing gradient of differentiation in rat incisor apical end. Microscopical observations were carried out after immunostaining with monoclonal antibodies against TG1 (**a, c, e, g**) or TG2 (**b, d, f, h**), followed by FITC- or Texas Red-conjugated secondary antibody, respectively. The presence of TG1 and TG2 was observed along the apical loop, in the entire odontogenic organ and in the mesenchyme thickening region (*) close to the inner dental epithelium (*ide*) (**a, b**). At a higher magnification, labelling cells for both isoenzymes were also observed in the tissue compartments related to "provisional stratum intermedium" (*PSI*) and to "stellate reticulum" (*SR*) (**c, d**). Moving toward the "ameloblast region facing pulp posterior", at a higher magnification, a dramatic decrease of TG1- or TG2-specific fluorescence was observed in the BM (*arrow*) between mesenchyme (*m*) and ameloblasts (*a*) (**e, f**). In the region of "ameloblast facing pulp anterior", where ameloblast (*a*) and odontoblast (*o*) differentiation begins, TG1 (**g**) and TG2 (**h**) were abundant in both mesenchyme and epithelium, while were absent in the BM between the two cell compartments (*)

seem to have an additional role as modulators of the epithelial–mesenchymal signalling during tooth morphogenesis (Alvares et al. 2006; Jadlowiec et al. 2006). Indeed, in early stages of tooth development DPP is present in the epithelial cell layers, whereas in later stages is present mainly in the odontoblasts derived from the mesenchymal layer (Alvares et al. 2006).

This regional distribution of TG substrates in differentiating tooth could explain changes in the distribution of TG1 and TG2, which were first described in this work along the increasing gradient of maturation in the rat incisor.

Even though TG2 mainly localizes in the cytosol in a catalytically latent form, also has the potential to translocate to the nucleus, be externalized, and ultimately co-localize with proteins in the extracellular matrix or on the extracellular side of the plasma membrane (Fesus and Piacentini 2002; Zemskov et al. 2006). Further, a TG2-dependent stimulation of cell adhesion and spreading, not requiring enzyme activity, has been reported, suggesting a novel function of cell surface TG2 as an integrin-associated adhesion co-receptor for fibronectin (Akimov et al. 2000).

Our observations, showing that TG2 was localized in both ameloblast and mesenchymal cell layers, suggest that this isoenzyme, having multiple forms and pleiotropic functions, is involved in the regulation of epithelial–mesenchymal interactions. The presence of TG2 can be required for modification/stabilization of ECM, i.e. through crosslinking of collagen, laminin and nidogen (Esposito and Caputo 2005; Zemskov et al. 2006), and activation of signalling molecules, such as midkine, which favour the mesenchyme–epithelium interplay. Further, TG2 can directly participate in cell–cell signalling by interacting with fibronectin (Akimov et al. 2000), a protein associated to BM, which plays a major role in the modulation of proliferation and differentiation of ameloblasts (Tabata et al. 2003), as also demonstrated by changes in its spatial distribution during tooth morphogenesis (Thesleff et al. 1981; Sawada and Nanci 1995).

Notably, this is the first work describing the presence of TG1, the distribution of which looks widespread at both mesenchymal and epithelial level in rat incisor. TG1 is a unique TG, being the only one membrane-bound among all isoenzymes. However, it also exists as catalytically inactive soluble form in the cytosol, which is severalfold activated by proteolysis. TG1 has been reported to be involved in keratinocyte terminal differentiation by cross-linking proteins beneath plasma membranes (Steinert et al. 1996). It has also been suggested that the formation of covalently cross-linked multimolecular complexes by TG1 is an important mechanism for maintenance of the structural integrity of simple epithelial cells, especially at cadherin-based adherens junctions (Hiiragi et al. 1999).

Given the high amounts and the widespread distribution of TG1 in rat incisor tissues, it is reasonable to speculate that TG1 activity may be dynamically regulated and play a complementary role in cell–cell adhesion and differentiation mediated by TG2. Interestingly, a different substrate specificity has been reported for TG1 and TG2, as well as for other TGs, suggesting a specialized function of each isoenzyme (Esposito and Caputo 2005). In particular, TG1, likewise during the formation of cornified envelope in the epidermis (Esposito and Caputo 2005), could stabilize epithelial tissues in tooth by cross-linking protein substrates such as involucrin and keratin. Indeed, as recently reported, keratin looks widely distributed throughout the dental epithelium in rat incisor (Morotomi et al. 2005).

To summarize, our data extend previous observations on a likely relevant role of TG activity in tooth. The continuously erupting rat incisor is a complex tissue district where mesenchymal and epithelial cells, separated by a basal membrane, proliferate and differentiate in response to reciprocal interactions. In this model, the expression of TG1 and TG2 in different cell compartments seems to suggest a complementary functional role for these two isoenzymes at an early stage of differentiation of rat incisor. However, further work aimed to the characterization of TG substrates in tooth is needed to elucidate this topic.

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