Inactive and Highly Active, Proteolytically Processed Transglutaminase-5 in Epithelial Cells

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Transglutaminases (TGs) are Ca^{2+}-dependent enzymes capable of catalyzing transamidation of glutamine residues to form intermolecular isopeptide bonds. These enzymes are involved in various biological phenomena, including blood coagulation, wound healing, cell death, tissue repair, and terminal differentiation of keratinocytes. Among the TG-family members, TG5 is one of the latest identified enzymes and therefore the less characterized at the functional level. In this work, we reported that TG5 is proteolytically processed in the baculovirus expression system and in mammal epithelial cells. Similar to other members of the TG family—TG1, TG3, and factor XIIIa -, TG5 full-length enzyme has very low enzymatic activity, while the 53-kDa proteolytically processed form is highly active.

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INTRODUCTION

Transglutaminases (TGs: EC 2.3.2.13) constitute a family of Ca^{2+}-dependent enzymes that covalently cross-link proteins by catalyzing the formation of isopeptide bonds between the γ-carboxyamide group of glutamine and the ε-amino group of lysine (Greenberg *et al.*, 1991; Griffin *et al.*, 2002; Lorand and Graham, 2003; Candi *et al.*, 2005; Eckert *et al.*, 2005; Esposito and Caputo, 2005). Resulting cross-linked bonds are covalent, stable, and resistant to proteolysis.

To date, nine members of the family are known: TG1–TG7, factor XIII, and band 4.2. TGs are involved in various biological phenomena, including blood coagulation, wound healing, cell death, tissue repair, terminal differentiation of keratinocytes, signaling, and vesicle trafficking (Lorand *et al.*, 2003; Candi *et al.*, 2005; Eckert *et al.*, 2005).

Among the TG-family members, TG5 is one of the latest identified enzyme together with TG6 and TG7 (Aeschlimann *et al.*, 1998), and therefore the less characterized at the functional level. TG5, similar to TG1 and TG3, is expressed in stratified squamous epithelia such as the upper layers of the epidermis (Candi *et al.*, 2002) and in human hair follicle (Thibaut *et al.*, 2005), and possibly is also responsible for formation of the cornified cell envelope (Candi *et al.*, 2001, 2005; Kalinin *et al.*, 2002). As TG1 and TG3, TG5 cross-links small proline-rich proteins, loricrin, and involucrin in *in vitro* cross-linking experiments, and uses specific glutamine and lysine residues (Candi *et al.*, 2001; Hitomi *et al.*, 2001) in the same protein substrate, suggesting that it acts together with TG1 and TG3 to generate a functional cell envelope.

The low solubility of TG5 expressed both in the baculovirus system and in keratinocytes is not due to membrane binding by lipid modification, but probably to its interaction with vimentin network (Candi *et al.*, 2001). In addition, three different TG5 human splicing isoforms are known, in accordance with TG2 and TG3 (Aeschlimann *et al.*, 1998; Candi *et al.*, 2001; Citron *et al.*, 2001; Zocchi *et al.*, 2007). The enzyme is also able to bind and hydrolyze guanosine triphosphate like TG2 and TG3 (Liu *et al.*, 2002; Ahvazi *et al.*, 2003; Ahvazi *et al.*, 2004a, b; Candi *et al.*, 2004). Native TG5 contains 725 amino acids corresponding to a molecular mass of 81 kDa, both in human and mouse (Aeschlimann *et al.*, 1998); mutations of the human sequence that abolish its activity, are associated to acral peeling skin syndrome (APPS, MIM 609796; Cassidy *et al.*, 2005).

Proteolytic activation in TG-family members is a common feature: factor XIII, TG1, and TG3 are subjected to proteolysis from specific proteases; in all cases, the proteolytic cleavage generates more active enzyme. Blood coagulation factor XIII (FXIII) is a zymogen of tetrameric structure (A2B2) containing two potentially active A-subunits (FXIII-A) and two inhibitory/carrier B-subunits (FXIII-B). Plasma FXIII is transformed into active TG (FXIIIa) by the concerted action of thrombin and Ca^{2+} in the final phase of the coagulation cascade (Muszbek *et al.*, 1996, 1999; Shemirani *et al.*, 2006). TG1 is mostly expressed in the upper spinous and granular layer of stratified squamous epithelia (Thacher and Rice, 1985; Thacher, 1989; Hohl *et al.*, 1998); it has a full-length, membrane-bound form of low specific activity, and two proteolytically processed

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Abbreviations: FXIII, coagulation factor XIII; IMAC, immobilized-metal affinity chromatography; NP-40, Nonidet P40; TG, transglutaminase; TG5(C), transglutaminase-5 C-terminus his/c-myc tagged cDNA; TG5(N), transglutaminase-5 N-terminus his/c-myc tagged cDNA; TX-100, Triton X-100

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forms, mostly cytosolic, with 5- to 10-fold higher specific activities (Kim *et al.*, 1995). During terminal differentiation, part of TG1 is proteolytically processed at Gly^{93} and Gly^{573} into highly active 67/33/10-kDa fragments, while still anchored to membranes (Kim *et al.*, 1995; Steinert *et al.*, 1996a). Among various proteases in differentiating keratinocytes, m-calpain and μ-calpain, calcium-dependent intracellular proteases, as well as the bacterial enzyme, dispase, proteolyze TG1 *in vitro* into 67/33/10-kDa fragments, with specific activity remaining unaltered before and after proteolysis (Hitomi *et al.*, 2000). During keratinocyte differentiation, TG3 is also activated by limited proteolysis of a 77-kDa zymogen into two 47- and 30-kDa fragments, in a region between the catalytic core and the β-barrel-1, located on the 462- to 471-amino-acid sequence range (Ahvazi *et al.*, 2003, 2004a). This process is controlled by cystatin M/E, at least during skin morphogenesis in the neonatal phase. *In vitro*, cathepsin-L (Cheng *et al.*, 2006), like the bacterial enzyme dispase (Kim *et al.*, 1990; Hitomi *et al.*, 1999), is able to proteolyze the zymogen form, resulting in proteolytic activation of TG3.

To further investigate the biochemical properties of TG5, we have expressed the full-length enzyme in the baculovirus system, in keratinocytes, and in human embryonic kidney-293 (HEK-293) cells. In all of these systems, we have found that TG5 is subjected to proteolysis, which activates the enzyme itself.

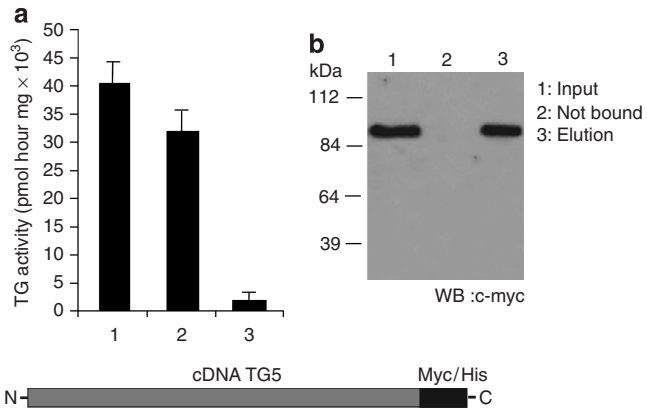

Figure 1. IMAC on active TG5 fractions. SF9 cells were infected with C-terminal his/c-myc TG5 recombinant baculovirus; the pellet was lysed first with 0.1% TX-100 buffer (15 minutes), then with a 1% TX-100/1% NP-40 buffer as described previously (Candi *et al.*, 2001). The TG5 soluble fraction was purified with cobalt IMAC resin as described under Materials and Methods, using his-tag located on protein sequence C-terminal end. TG activity of the soluble and the insoluble fractions one was determined by measuring incorporation of ^{3}H-labeled putrescine into *N,N*-dimethylcasein. TG activity is reported as picomoles of putrescine cross-linked with milligram of total protein per hour. (**a**) Enzymatic activity analysis on unpurified (input, column 1), unbound (NB, column 2), and eluted fraction (E, column 3) revealed that only the first two fractions were active, whereas (**b**) from western blot analysis on the same samples, using anti-c-myc antibody, TG5 was present only in the eluted fraction.

RESULTS

Active TG5 extraction and IMAC purification

About 70% of TG5 expressed both in the baculovirus system and in keratinocytes is insoluble in non-ionic detergents (1% Triton X-100 (TX-100)/Nonidet P40 (NP-40); Candi *et al.*, 2001). Successful total extraction can be obtained only using ionic denaturants such as SDS and urea. The low solubility of TG5 is not due to lipid modification, but probably its interaction with vimentin network (Candi *et al.*, 2001). Active TG5 enzyme was recovered with sequential extractions from the pellet with a 1% TX-100/NP-40 buffer at different extraction times ranging from 1 to 24 hours. The highly enriched semi-purified active TG5 fractions, kept at 4 °C, retain 80% of their activity over 2–3 weeks of storage.

To purify active TG5, immobilized-metal affinity chromatography (IMAC) was performed on 1% TX-100/NP-40-extracted protein, using his-tag located on the protein sequence C-terminal end (transglutaminase-5 C-terminus his/c-myc tagged cDNA (TG5(C)); Figure 1). Surprisingly, following purification, enzymatic activity analysis on both unbound and eluted fractions revealed that only the first fractions was active (Figure 1a), whereas western blot analysis on the same samples, using an anti-c-myc antibody, indicated that TG5 was present only in the eluted fraction (Figure 1b). This experiment suggested that the C-terminal his-tag had been cleaved from the enzyme before purification. To further study this observation, we solubilized TG5 enzyme by sequential extractions from the pellet with a 1% TX-100/NP-40 buffer at different extraction time points (15 minutes, 1, 3, and 24 hours); again, most of the activity was present in soluble fractions upon extraction for 3 and 24 hours (Figure 2a). Western blot analysis of soluble and insoluble C-terminal his-myc TG5 fractions, using a specific anti-c-myc antibody, revealed that full-length TG5 was more abundant in the insoluble fraction, that was the less active (Figure 2b), suggesting that presence of a TG5 C-terminal truncated fragment retains the majority of the enzymatic activity. To purify the active C-terminal truncated TG5 fragment, we prepared another TG5 construct with a his-myc tag at its N-terminus, TG5(N). Active soluble fractions were extracted from the baculovirus system and purified by IMAC, as mentioned above. Activity distribution was similar to C-terminal his-myc sample activity, with the highest values in fractions obtained after 3 and 24 hours of TX-100/NP-40 buffer extraction (Figure 2c), suggesting that his-myc tag position at N- or C-termini did not influence either activity or TG5 solubility. Western blot analysis of active soluble fractions, using anti-c-myc antibody, revealed that TG5 53-kDa fragment increased in active fractions in parallel to the increasing activity (Figure 2d); the full-length protein was present only in the less active insoluble fraction.

TG5 is proteolyzed in two fragments of 53 and 28 kDa in the baculovirus system

The identification of an N-terminal active 53-kDa fragment, co-purifying with the full-length protein, is unquestionably a sign that a proteolytic process allows a protein cleavage at around 2/3 of the enzyme sequence, generating an active 53-kDa N-terminal fragment consisting of the enzyme N-terminus and catalytic domains lacking the