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# Overexpressed transglutaminase 5 triggers cell death

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**Summary.** Transglutaminases are a class of nine different proteins involved in many biological phenomena such as differentiation, tissue repair, endocytosis. Transglutaminase 5 was originally cloned from skin keratinocytes, and a partial biochemical characterization showed its involvment in skin differentiation. Here we demonstrate that transglutaminase 5 is able to induce cell death when intracellularly overexpressed. Transfected cells show enzymatic activity, as demonstrated by fluoresceincadaverine staining. Transfected cells died due to the formation of hypodiploid DNA content, indicating the induction of cell death under these pharmacological conditions. We also show that the primary sequence of transglutaminase 5 contains GTP binding domains which are similar to those in transglutaminase 2. This raises the possibility that transglutaminase 5 is regulated by GTP in a similar fashion to transglutaminase 2.

**Keywords:** Transglutaminase – Cell death – Cross-links – Skin

**Abbreviations:** NP-40, Non-Idet P40; PBS, phosphate buffer saline; PVDF, poly vinylidendifluoride; SDS, sodium dodecyl sulfate; TGase, transglutaminase; MODY, mature onset diabetes of the young; KO, knock out; GFP, green fluorescent protein; FBS, fetal bovine serum; FACS, fluorescent activated cell sorter; PI, propidium iodide

## Introduction

Transglutaminases (TGase; EC 2.3.2.13) are $Ca^{2+}$-dependent enzymes which catalyze intermolecular isopeptide bonds by transamidation of specific glutamine residues (Melino et al., 2000; Lorand and Graham, 2003). The reaction establishes $\varepsilon(\gamma$-glutamyl)lysine isodipeptides and N,N-bis($\gamma$-glutamyl)polyamine linkages, resulting in the post-translational modification of proteins. In mammals, nine distinct types of TGases have been characterized: types 1 through 7, coagulation factor XIIIa, and band 4.2. TGases are involved in various biological phenomena, including blood coagulation, wound healing, cell death, terminal differentiation of keratinocytes, signaling, and vesicle trafficking.

The cross-linking activity of TGase 2 is regulated both by GTP (Achyuthan et al., 1987) and nitric oxide (Melino et al., 1997). It is involved in both intra cellular and extra cellular biological processes, including receptor-mediated endocytosis, cell adhesion, wound healing, and cell death, at least in overexpression (Davies et al., 1980; Haroon et al., 1999; Melino et al., 1994), for review see (Melino and Piacentini, 1998).

Nevertheless, disruption of the mouse TGase 2 gene did not cause a major phenotype (neither in the apoptosis pathway nor in signal transduction), indicating that other enzymes could compensate for the lack of TGase 2 (De Laurenzi and Melino, 2001). More recently, defects have been detected in TGase 2 KO mice, both *in vitro* (Bernassola et al., 2002a; Verderio et al., 2003) and *in vivo* (Freitag et al., 2003; Szondy et al., 2003; Nardacci et al., 2003). More specifically, TGase 2 KO mice develop a glucose deficient metabolism similar to human Mature Onset Diabetes of the Young (MODY); in keeping with this, mutations in TGase 2 have been detected in human MODY patients (Bernassola et al., 2002b). TGase 2 KO mice also demonstrate that the enzyme is involved to a degree in the pathogenesis of Huntington's disease (Mastroberardino et al., 2002).

TGase 5 is a more recently identified member of the TGase family (Aeschlimann et al., 1998), which has not been fully characterized at functional level. TGase 5 is expressed during *in vitro* keratinocyte differentiation, and cross-links specific substrates (Aeschlimann et al., 1998; Candi et al., 2001; Candi et al., 2002b), in parallel with TGase 1 and TGase 3. In addition, it is associated with cytoskeletal elements, colocalizing with the vimentin network (Candi et al., 2002a).

Here we show that TGase 5 is able to induce cell death when over-expressed, similarly to TGase 2. Moreover, the enzyme seems to possess a similar GTP binding pocket to TGase 2, which suggests the possibility that, like TGase 2, its enzymatic activity is regulated by GTP.

## Materials and methods

*Cell culture*

SK-N-Be(2) (human neuroblastoma) and HEK293 (human embryonic kidney) cells were cultured in a 1:1 mixture Ham's F-12:D-Minimal Essential Medium supplemented with 10% heat-inactivated FBS at 37°C in a humified atmosphere of 5% $CO_2$ in air. TGase 5 was HA-tagged in order to monitor the steady state levels of protein induction by Western Blot and laser densitometry.

*Transfection*

The entire coding region of the TGase 5 gene was amplified using primers TGase 5 F1 (5′-AGCTACCATGGCCCAAGGGCTAG-3′, + strand) and TGase 5 R3 (5′-CGTCTGGCGCGTTGTTCCAG-3′, − strand), as described (Candi et al., 2001). Transient transfections of pCDNA3.1-TGase 5 s were performed using Effectene (Qiagen, Crawley, UK) as described by the manufacturer. Briefly, $8 \times 10^5$ keratinocytes were plated in collagen-coated 100 mm dishes one day before transfection. One $\mu$g DNA of each of the four vectors was separately mixed with 16 $\mu$l of enhancer and 50 $\mu$l of Effectene transfection reagent. Transfected cells were kept for 2 days in culture. Transfection efficiencies were monitored by using a cytomegalovirus $\beta$-galactosidase construct (pCDNA3.1; Invitrogen, Groningen, The Netherlands) and were approximately 20%. To assess intracellular enzymatic activity, transfected cells were incubated with 25 mM fluoresceincadaverine (Molecular Probes, Eugene, OG) in KGM medium containing 0.05 mM $Ca^{2+}$ at 37°C, for 3 h. Cells were subsequently washed in PBS, fixed, and then immunostained for TGase 5 using a goat anti-mouse antibody (Alexa Fluor 568, Molecular Probes, Eugene, OG), as described in the confocal microscopy section.

*Western Blot analysis*

Cells were lysed in buffer A (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.5 mg/ml leupeptin, 1 mg/ml aprotinin and 0.5 mM PMSF) for 1 h on ice. The lysate was cleared by centrifugation, and 20 $\mu$g aliquots of cell extract, as determined by the Bradford method, were resolved by electrophoresis in a 12% (w/v) SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to quantitate the steady state levels of protein expression.

*Determination of apoptosis*

To estimate DNA fragmentation, cells were collected at $800 \times g$ for 10 min and fixed with a 1:1 phosphate-buffered saline and methanol-acetone (4:1 (v/v)) solution at −20°C. Hypodiploid sub-G1 events were evaluated by flow cytometry (FACS-Calibur, Beckton-Dickinson, CA, USA) after propidium iodide (PI) staining, as previously described (Melino et al., 1994). For each point 20,000 events were collected, excluding doublets and aggregates by electronic gate.

*Confocal microscopy*

Cells were grown overnight on a glass coverslip and, after the indicated treatments, fixed in 4% paraformaldheyde in PBS (pH 7.4) for 15 min. Confocal images were acquired by using a C1 Confocal Microscope (Nikon) and exciting with 488 nm argon-ion laser line or 542 nm He-Ne laser detecting with the appropriate filter set (515/30 green filter and 595/70 red filter). Images were then processed by using PCM-2000 software (Nikon).

*Computerized homology model*

The three-dimensional model of human TGase 5 was generated using MODELLER, using as a starting point the crystal structure of human TGase 2 (32, PDB id:1KV3). Human TGase 2 and TGase 5 sequences were aligned using LALIGN v.2.0u6 in order to input into MODELLER. The model was optimized using molecular probability density function by conjugate gradient and simulated annealing. Approximately 30 residues of the non-conserved TGase 5 insertion could not be modeled. GDP docked into TGase 5 in the same position and orientation as observed in TGase 2; the GDP and side chains of residues interacting with GDP were refined using MacroModel.

## Results and discussion

### *Transfected transglutaminase 5 is active in vivo*

Human TGase 2 and TGase 5 have 41% sequence identity. The similarities in primary sequence between TGase 2 and TGase 5 lead us to investigate whether these two enzymes could have overlapping effects when over-expressed. To this aim, we transfected SK-N-Be(2) or HEK-293 cell lines with expression vectors bearing TGase 2 or TGase 5 cDNAs.

Figure 1 shows by Western blot the expression of TGase 2 and TGase 5 in transfected SK-N-Be(2) cells in comparison with cells transfected with control vectors (pSG5 and pCDNA3.1, respectively). The induction of the enzyme was significant. Over-expressed enzymes are active in the living cells, as seen in *in vivo* activity measurements by fluoresceincadaverine incorporation, both for cells transfected with TGase 2 and with TGase 5 (data not shown).

This result is relevant considering the fact that at least two transglutaminases, namely clotting factor XIIIa and transglutaminase 1, require proteolytic activation by specific endoproteases able to cleave the amino-terminus. Consequently, these two transglutaminases are not active

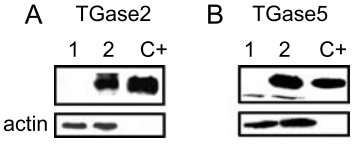


**Fig. 1.** TGase 2 and TGase 5 are transfected in cell lines. Western blot analysis of SN-K-Be(2) transfected with: pSG5 (control, lane 1), pSG5-TGase 2 (lane 2), pCDNA3.1 (control, lane 3), pCDNA3.1-TGase 5 (lane 4), respectively using a specific anti-human TGase 2 antibody (Neomarkers) and anti-c-myc antibody (Santa Cruz) to detect the tagged TGase 5. 20 $\mu$g of total protein was loaded in each lane. The experiment was perfomed twice, a representative blot is shown

when transfected, unless the specific endoprotease is present in the cell. These results demonstrate that TGase 5 does not require such proteolytic activation; alternatively, the specific endoprotease may be constitutively present in both SK-N-Be(2) and HEK-293 cells.

### *Transglutaminase 5 induces cell death*

Next we investigated the induction of cell death. To this end, we co-transfected SK-N-Be(2) and HEK-293 cell lines with expression vectors bearing TGase 2, TGase 5 and GFP cDNA. Cell death was evaluated by analysis of the hypodiploid events by flow cytometry of transfected (GFP positive) cells.

As already described in previous papers (Melino et al., 1994) over-expression of TGase 2 increases the number of hypodiploid events by 4 fold as compared to cells transfected with the control vector (Fig. 2, panel A, top). Similarly, transfection with TGase 5 expression vector (Fig. 2, panel A, bottom) increases hypodiploid events by approximately 4 fold. The same result was obtained transfecting the embryonic cell line HEK293 with TGase 5 expression vector; in this case, the hypodiploid events increased by nearly 3.5 fold as compared to controls (Fig. 2, panel B).

These findings indicate that in over-expression, both TGase 2 and TGase 5 can trigger apoptosis in various cell lines. Clearly, this result must be translated with caution in a physiological *in vivo* setting.

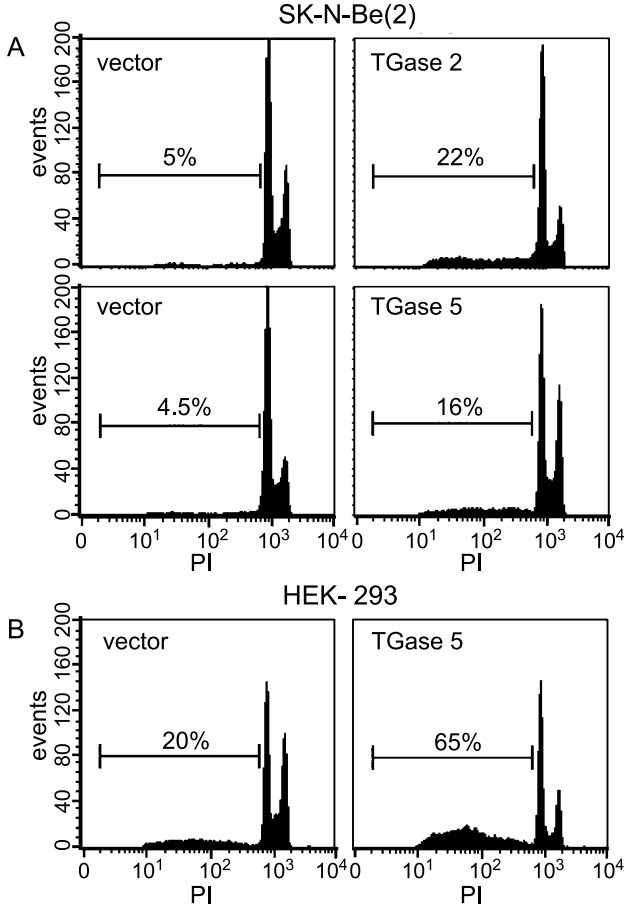


**Fig. 2.** TGase 2 and TGase 5 trigger cell death in transfected cell lines. **A**, *top* Hypodiploid events in SK-N-Be(2) cells transfected with control vector (pSG5) and TGase 2 (pSG5-TGase 2). **A**, *bottom* Hypodiploid events in SK-N-Be(2) cells transfected with control vector (pCDNA3.1) and TGase 5 (pCDNA3.1-TGase 5). **B** Hypodiploid events in HEK-293 cells transfected with control vector (pCDNA3.1) and TGase 5 (pCDNA3.1-TGase 5). TGase 2 and TGase 5 vectors were cotransfected with the GFP-expression vector. The increase of the hypodiploid events is measured only in transfected (GFP positive) cells and is indicated as a percentage. The number of events (10,000) with hypodiploid DNA was measured by flow cytometry (methods are described in the experimental section and in Melino et al., 1994). The x-axis reports the FL2 (DNA content) fluorescence intensity as arbitrary units, and the y-axis reports the number of events measured per channel

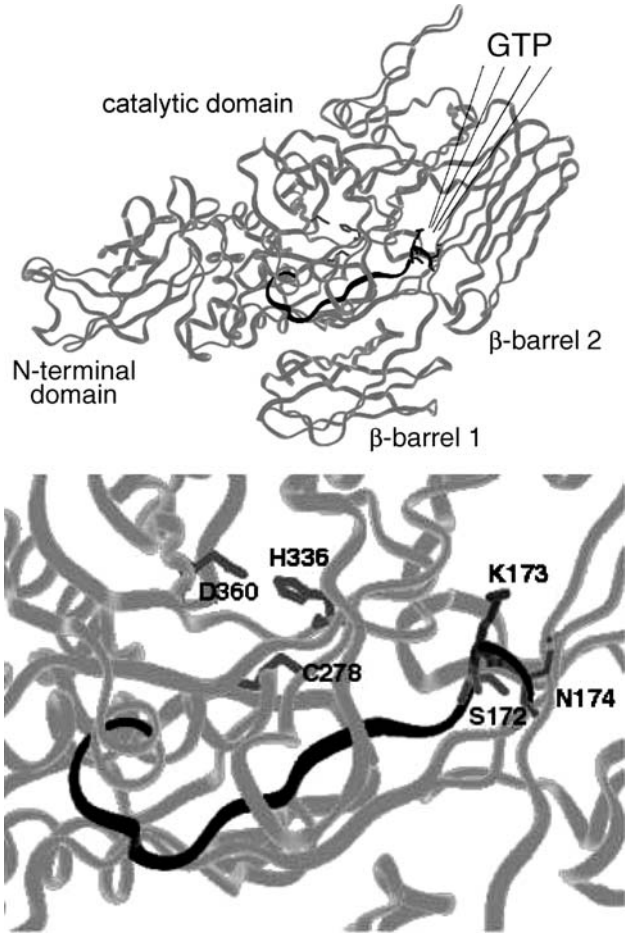


**Fig. 3.** TGase 5 trigger contains residues involved in GTP regulation. Computer homology modeling of TGase 5 based on the crystal structure of TGase 2. The model is shown both at lower magnification (*left panel*) and in a region supposed to interact with GTP (*right panel*). The catalytic residues for cross-linking (D360, H336, C278) are highlighted; the GTP-interacting region is indicated in black; the putative residues involved in the physical interaction with GTP (S172, K173) are indicated

*Transglutaminase 5 contains the residues involved in GTP binding and regulation*

Transglutaminase 2 is regulated by GTP (Achyuthan et al., 1987). This effect is related to a specific sequence including a serine and a lysine (see below in bold underlined). A careful analysis of the primary sequence shows that these residues are conserved in TGase 5:

TGase 5: **YV**MND Y**GFIY QG<u>SK</u>**N
TGase 2: **YVLTQ** Q**GFIY QG<u>SA</u><u>K</u>**

These residues are not conserved in other TGases, with the exception of TGase 3, which contains most of them (but not the relevant K).

Figure 3 reports a computer homology model of TGase 5 based on the crystal structure of TGase 2. The conserved region responsible for the GTP regulation is shown in black, with the crucial S172 and K173 residues. This model implies that TGase 5, similar to TGase 2, is regulated by GTP. This hypothesis awaits an experimental confirmation.

## Conclusion

While TGase 5 has been cloned from keratinocytes (Aeschlimann et al., 1998; Candi et al., 2002a), and it has been shown to cross-link specific epidermal substrates (Candi et al., 2001), the present report suggests that it also has some properties that are reminiscent of TGase 2. In fact, like TGase 2, TGase 5 is able to induce cell death in over-expression, and seems to retain the residues involved in GTP regulation. In conclusion, the regulation of the function of TGase 5 is complex, and clearly different from other epidermal TGases.

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