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Transglutaminase 5 is acetylated at the N-terminal end

A. Rufini[1], **F. Vilbois**[2], **A. Paradisi**[1], **S. Oddi**[1], **R. Tartaglione**[1,3], **A. Leta**[1,3], **G. Bagetta**[3], **P. Guerrieri**[1], **A. Finazzi-Agro'**[1], **G. Melino**[1,4], and **E. Candi**[1]

[1] Biochemistry Laboratory, IDI-IRCCS, University of Rome "Tor Vergata", Rome, Italy
[2] Serono Pharmaceutical Research Institute, Geneva, Switzerland
[3] Department of Pharmaco-biology, University of Calabria, Cosenza, Italy
[4] Medical Research Council, Toxicology Unit, Leicester University, Leicester, United Kingdom

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Summary. Transglutaminases (TGases) are calcium-dependent enzymes that catalyse cross-linking between proteins by acyl transfer reaction; they are involved in many biological processes including coagulation, differentiation, and tissue repair. Transglutaminase 5 was originally cloned from keratinocytes, and a partial biochemical characterisation showed its involvement in skin differentiation, in parallel to TGase 1 and TGase 3. Here, we demonstrate, by electrospray tandem mass spectrometry that TGase 5 is acetylated at the N-terminal end. Moreover, *in situ* measurement of TGase activity shows that endogenous TGase 5 is active upon treatment with phorbol acetate, and the enzyme co-localises with vimentin intermediate filaments.

Keywords: Transglutaminase – Vimentin – Cross-links – Cytoskeleton – Acetylation – Tandem mass spectrometry

Abbreviations: NP-40, Non-Idet P40; PBS, Phosphate buffer saline; PVDF, poly vinylidendifluoride; SDS, sodium dodecyl sulfate; TGase, transglutaminase; MS/MS, tandem mass spectrometry; TPA, 10-O-tetradecanoylphorbol-13-acetate

Introduction

Transglutaminases (TGase; EC 2.3.2.13) are enzymes that catalyze the post-translational cross-linking of protein by acyl transfer reaction (Melino et al., 2000; Lornad and Graham, 2003). In this reaction, the γ-carboxyamide group of glutamine residue and the primary amine of a lysine residue act, respectively, as acyl donor and acceptor substrates to form $\varepsilon(\gamma$-glutamil)lysine bonds. The resulting crosslinked bonds are covalent, stable, and resistant to proteolysis. To date, nine members of the family are known: TGase 1 to TGase 7, factor XIII, and band 4.2. These enzymes are involved in various biological phenomena, including blood coagulation, wound healing, tissue repair, terminal differentiation of keratinocytes, signaling, and vesicle trafficking.

Among the TGase family members, TGase 5 is one of the latest identified enzyme together with TGase 6 and TGase 7 (Aeschlimann et al., 1998), and therefore the less characterised at functional level. TGase 5 is expressed during *in vitro* keratinocyte differentiation, and is able to cross-link specific epidermal substrates (Aeschlimann et al., 1998; Candi et al., 2001; Candi et al., 2002), in parallel with TGase 1 and TGase 3. Beside the epidermis, TGase 5 is expressed in several tissues and is also able to hydrolyse GTP (Candi et al., 2004) *in vitro*. The enzyme has also been found associated with cytoskeletal elements, colocalizing with vimentin net (Candi et al., 2002), which appears to be a substrate for TGase 5 *in vitro* crosslinking experiments (Candi et al., 2001). We have already observed that TGase 5 is insoluble in acqueous solutions (Candi, 2001): about 70% of TGase 5 expressed both in the baculovirus system and in keratinocytes is not solubilized by a combination of non-ionic detergents (1% Triton X-100 and 1% NP-40). Successful extraction can be obtained only using the ionic detergent SDS at concentrations ranging from 0.01 to 1% (w/v) and keeping the sample at room temperature for 1 to 3 h, allowed extraction of active enzymes. However, we failed to identify any lipid modifications by incubating the cells with [^{3}H]myristic and [^{3}H]palmitic acid. Thus, the TGase 5 insolubility is not due to membrane binding, but more probably to TGase 5 interaction to vimentin network (Candi et al., 2001).

In order to understand why TGase 5 is rather insoluble, we investigated other possible post-translational modifications by tandem electro-spray mass spectrometry. There-

fore, we analyzed the TGase 5 primary structure for both recombinant and native enzymes. Furthermore, we studied in more details the interaction of endogenous TGase 5 with cytoskeleton elements.

Materials and methods

Protein sequencing

Recombinant purified TGase 5 was obtained as described previously (Candi et al., 2001). For sequencing by MS/MS, the protein samples were digested with trypsin. All analyses were performed on a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray interface and a nano-electrospray ion source. The instrument was calibrated over the m/z range 50–2400 using horse heart myoglobin and a series of fragment ions obtained by collision-induced dissociation of the peptide Glu-fibrinopeptide B. For nano-electrospray tandem MS analyses, peptide solutions were desalted, concentrated, and eluted into nanospray capillaries (Protana, Odense, Denmark), as described (Wilm et al., 1996). For each sample a peptide mass map was obtained in the single MS mode, followed by in-turn fragmentation of observed peptide ions in the MS/MS mode. Argon was used as the collision gas, and the collision energy was tuned for each peptide to obtain the best possible collision-induced dissociation spectra. The MS/MS spectra were interpreted using the Mascot software (Matrix Science Ltd., London, UK).

Cell cultures

Cryopreserved normal human epidermal keratinocytes (NHEK) were obtained from BioWhittaker (Walkersville, MD) and grown in calf skin collagen (Sigma Chemical, St. Louis, MO)-coated dishes in serum-free keratinocyte medium (KGM; BioWhittaker, Walkersville, MD) at 0.05 mM Ca^{2+}, supplemented with Single-Quots (BioWhittaker, Walkersville, MD), containing: 7.5 (μg/ml) bovine pituitary extract, insulin (0.5 mg/ml) hydrocortisone (0.5 mg/ml), hEGF (0.1 UG/ml). Third passage cells were used for transfection experiments. For treatments with differentiating and proliferating agents, cells were plated and treated at 50% confluence with 10-O-tetradecanoylphorbol-13-acetate (TPA, 10 ng/ml). Control cells were collected before reaching confluence.

Confocal analysis

Normal human epidermal keratinocytes (NHEK) were treated for 1day with TPA (10 ng/ml), then cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 2% NP-40 and 0.2% Triton X-100 for 20 min at room temperature. Cells were then washed with PBS and non-specific binding was blocked by incubating with 5% BSA in PBS for 30 min at room temperature. Indirect immunofluorescence was performed incubating keratinocytes with: anti-TGase 5 antibody (Candi et al., 2002, 1:100 in blocking solution) and anti-vimentin antibody (Sigma Chemicals, St. Louis, MO; 1:50 in blocking solution). Washes were followed by incubation with the appropriate secondary antibodies diluted 1:1,000 in blocking solution for 1 h at room temperature in the dark. The slides were mounted using Prolong Antifade kit (Molecular Probes Inc., Eugene, OR). Fluorescence was then evaluated with a confocal microscope (Nikon Instruments Spa, Eclipse TE200), exciting at 488 nm with an Ar laser and at 542 nm with a He laser. The software used was EZ2000 for PCM2000.

TGase activity measurement in vivo

TGase activities were measured *in vivo* according to Lajemi et al. (Lajemi et al., 1997). Briefly, NHEK were incubated with 25 mM fluo-

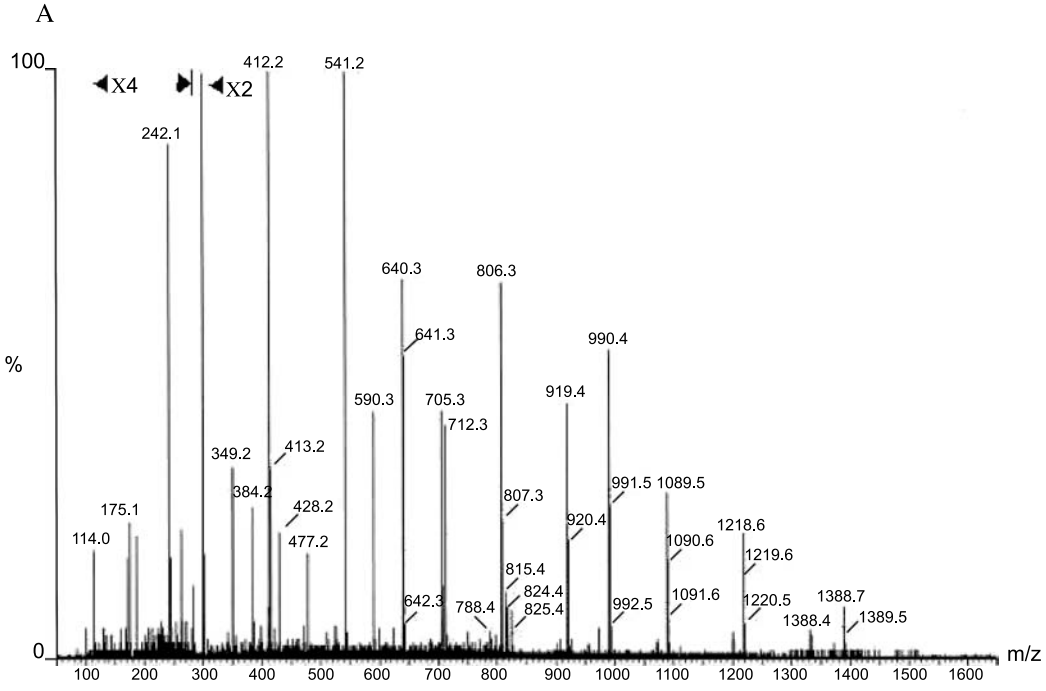

Fig. 1. MS/MS fragmentation of the acetyl-**AQGLEVALTDLQSSR** peptide. **A** Native TGase 5. **B** Recombinant TGase 5. The MS/MS spectra were obtained by fragmentation of the doubly charged precursor ion at m/z 815.30 present in the tryptic digests from both native and recombinant TGase 5. The same fragmentation pattern was obtained from both precursor ions. The sequence specific peptide fragment ions are identified in Table 1

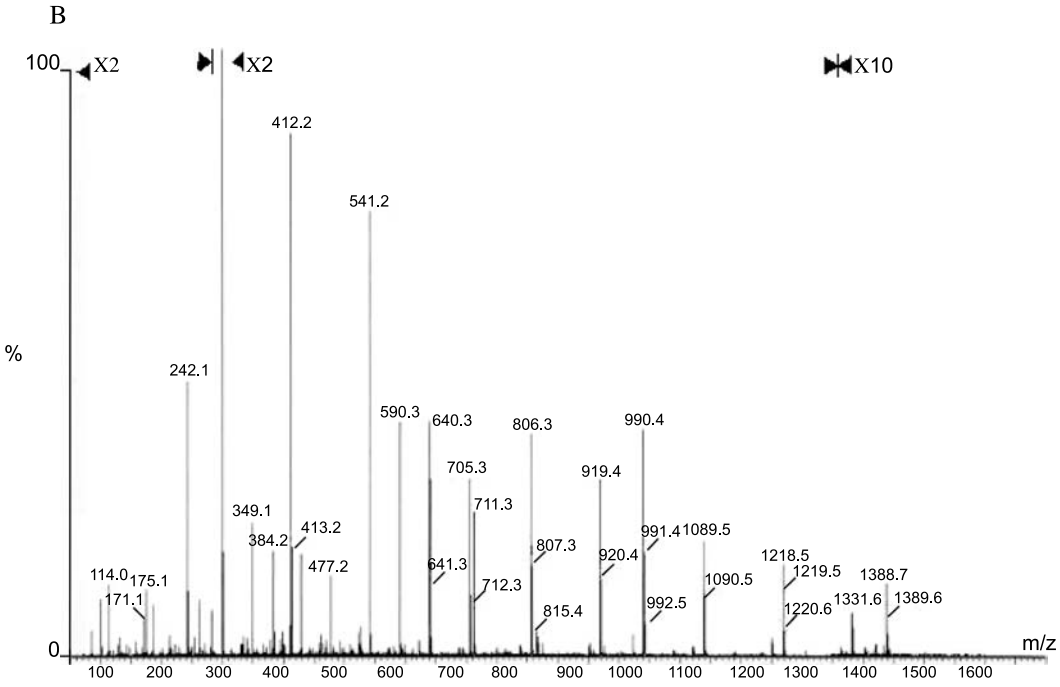

Fig. 1 (*continued*)

resceincadaverine (Molecular Probes, Eugene, OG) in Keratinocytes medium containing 0.05 mM Ca^{2+} at 37°C for 3 hrs. Cells were subsequently washed in PBS, fixed and immunostained for TGase 5 using a goat anti-mouse antibody (Alexa Fluor 568, Molecular Probes, Eugene, OG).

Results

TGase 5 is acetylated at the N-terminal end

In order to analyse the complete primary sequence of the enzyme and identify possible post-translational modifications, we used nano-electrospray MS/MS. Both recombinant and native TGase 5 were fully digested with trypsin, and the resulting peptide mixtures were analysed by MS. A doubly charged peptide ion with an unexpected monoisotopic mass of 1628.84 Da was present in the spectra from both digests. In both cases the amino-acid sequence Acetyl-**AQGLEVALTDLQSSR** corresponding to the N-terminus of TGase 5 was obtained by MS/MS (Fig. 1A, B, Table 1). We never found an unacetylated N-terminal peptide in the analysed samples. All other observed tryptic peptides from TGase 5 were unmodified.

Table 1. Interpretation of the peptide fragment ions observed in the MS/MS spectra of the acetyl-**AQGLEVALTDLQSSR** peptide

#	b	Seq.	y	#
1	114.06	A		15
2	242.11	Q		14
3	299.14	G	1388.74	13
4	412.22	L	1331.72	12
5	541.26	E	1218.63	11
6	640.33	V	1089.59	10
7	711.37	A	990.52	9
8		L	919.48	8
9		T	806.40	7
10		D	705.35	6
11		L	590.33	5
12		Q	477.24	4
13		S	349.18	3
14		S	262.15	2
15		R	175.12	1

Interpretation of MS/MS spectra using Mascot Search Results
Mach query 1 (815.30,2+). Monoisotopic mass of neutral peptide (Mr): 1628.84
Fixed modifications: Carbamidomethyl; variable modifications: N-terminal Acetyl
Ion score: 158

TGase 5 co-localises with vimentin intermediate filament network

The polyclonal rat anti-TGase 5 antibody (Candi et al., 2002) was used for confocal studies on NHEK incubated for one day with TPA (10 ng/ml, w/v). Previous studies have shown that one day treatment of NHEK with TPA and Calcium resulted in a strong induction of TGase 5 mRNA. As shown in Fig. 2A, a filamentous pattern, bearing remarkable similarity to the vimentin intermediate filament

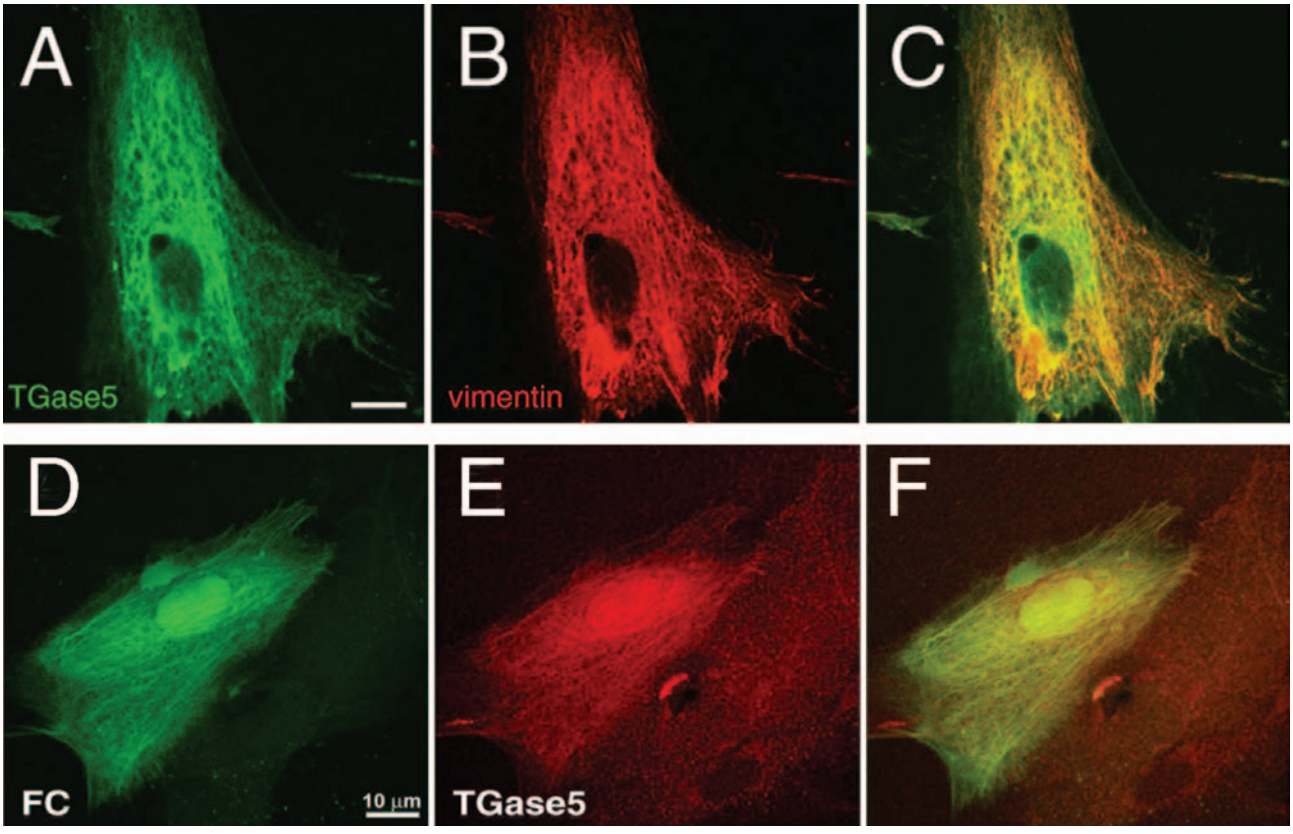

Fig. 2. Cellular localization and activity of TGase 5. Endogenous TGase 5 co-localises with vimentin intermediate filaments. NHEK were treated for 1 day with TPA (10 ng/ml) and stained with anti-TGase 5 antibody (**A**) and anti vimentin antibody (**B**). Cells were optically sectioned using a confocal laser-scanning microscopy, and corresponding images were superimposed to determine the degree of overlap (yellow). Superimposition of TGase 5 (**A**) with vimentin (**B**) shows a remarkable overlap (**C**) of the two staining patterns. To assess whether TGase 5 is active *in situ*, we incorporated fluoresceincadaverine into living cells without additional Ca^{2+} (**D**) and we stained cells with anti-TGase 5 antibody (**E**). Cells were optically sectioned using a confocal laser-scanning microscopy, and corresponding images were superimposed to determine the degree of overlap (yellow). Superimposition of TGase 5 expression (**E**) and activity (**D**) determines a good level of overlap (**F**). Bars correspond to 10 μm

network, was seen in these cells upon immunofluorescence staining with anti-TGase 5 antibody. Double-label immunofluorescence revealed that the antigen reacting with the TGase 5 antibody colocalized precisely with vimentin filaments (Fig. 2B, C). This morphological observations were further supported by the finding that TGase 5 was retained in intermediate filament-enriched cytoskeleton preparations made by using nonionic detergent-containing high ionic strength solution (data not shown).

TGase 5 is active in living cells

We have also performed *in vivo* TGase activity measurements in NHEK after 1 day treatment with TPA. As shown in Fig. 2D, E and F, *in vivo* activity measurements by fluoresceincadaverine incorporation (FC), demonstrated that TGase 5 was active in living cells. TGase activity was localised in the cytosol of the cell along a filamentous pattern (Fig. 2D), suggesting that TGase 5 interacting with vimentin was enzymatically active. It is reasonable that TGase 5 attachment to vimentin network was due to cross-linking activity of TGase itself.

This result seems to be relevant, since at least three transglutaminases, namely clotting factor XIIIa, TGase 1 and TGase 3, require proteolytic activation by specific endoproteases, which cleave their amino-terminus. Thus, these three transglutaminases are not active when overexpressed in cells, unless the specific endoprotease is also present, as is the case for TGase 1 and TGase 3 in differentiating keratinocytes.

Discussion

In this paper we demonstrated by MS/MS, that TGase 5 N-terminal end was always acetylated. Acetylation of

proteins occurs on at different amino-terminal residues (as in the case of TGase 5) or at epsilon-amino group of lysine residues. These post-translational modifications are catalysed by a wide range of acetyltransferases. Amino-terminal acetylation may occur on several eukaryotic proteins and on regulatory peptides, whereas lysine acetylation is observed in a variety of proteins, including histones, transcription factors, nuclear import factors, and alpha-tubulin (for review see Polevoda and Sherman, 2002). Why are proteins acetylated? The biological effect of such post-translational modification is poorly understood. Histone acetylation has been shown to affect nucleosome stability and, generally, is considered to favor gene activation. Many transcription factors such as p53, E2F1 and cJun, are acetylated. In this case, acetylation/deacetylation appears to regulate the ability of these factors to bind the DNA. Likewise, other cytoplasmatic proteins, such as alpha-tubulin, are also known to be acetylated (Piperno et al., 1987). In the latter case the function of this modification is not understood. Acetylated tubulin is observed on stabilised microtubules and over-acetylation of tubulin enhances cell motility (Palazzo et al., 2003). It is also possible, that acetylation could affect the activity of microtubule-associated proteins. It is still very difficult to understand the biological significance of TGase 5 acetylation. We can only speculate that in analogy with microfilaments, acetylation of TGase 5 might be important for regulating the stability of vimentin intermediate filament network. This hypothesis is further supported by the physical association of TGase 5 with intermediate filaments.

Several evidence suggests that TGase can interact with intermediate filaments, and therefore, affect the cytoskeleton of the cell. For example, type II keratin chains keratin 1, 2e, 5, or 6 are crosslinked by TGases to several proteins through a lysine residue located in a conserved region of the V1 subdomain of their head domains (Candi et al., 1998). A highly conserved lysine residue on the head domain of type II keratins is indeed essential for the crosslinking by TGases (Candi et al., 1998). This explains how keratin intermediate filaments are associated with the cell envelope at the periphery of cornified epidermal cells, with relevant medical implications.

Among the TGases family members interacting with intermediate filaments, TGase 2 (also known as tissue transglutaminase, tTG), is involved in both intracellular and extracellular biological processes (Davies et al., 1980; Melino et al., 1994; Haroon et al., 1999). It is constitutively expressed both during development and in adult tissues (reviewed in Melino and Piacentini, 1998). Even though its expression has been correlated with the induction of apoptosis (Nagy, 1997; Thomazy and Davies, 1999) the TGase 2 knock-out (–/–) mice (De Laurenzi and Melino, 2001), do not show a well defined alteration of apoptosis. In addition to its transamidation activity regulated by the GTP-binding activity of the enzyme (Achyuthan et al., 1987), TGase 2 resembles a signal transduction GTP-ase protein (G_h), i.e. a GTP-ase activity coupled to the alpha1-adrenergic receptor that mediates the activation of phospholipase C (Achyuthan et al., 1987). Thus TGase 2 may participate in the signaling transduction pathway as non-conventional G-protein (reviewed in Melino and Piacentini, 1998). TGase 2 also is associated with cytoskeleton components. In fact, biochemical evidence, as well as confocal microscopy analysis demonstrated an association of TGase 2 with the vimentin intermediate filaments network (Trejo-Skalli et al., 1995), beta-tubulin (Piredda et al., 1999) keratin intermediate filaments network (Vijayalakshmi and Gupta, 1994; Zatloukal et al., 1989), and tau protein in the neurofibrillary tangles (Tucholski et al., 1999). Many of these cytoskeleton elements are also TGase substrates *in vitro* (Clement et al., 1998; Tucholski et al., 1999; Selkoe et al., 1982).

In conclusion, several data show that some TGases (TGase 2 and TGase 5) interact with intermediate filaments of the cytoskeleton, but the meaning of this interaction is poorly understood: it is not clear why TGase 2 and/or TGase 5 associate with vimentin/keratin/tubulin filaments, and why TGase 2 and/or TGase 5 use vimentin/keratin/tubulin as crosslinking substrate. The work of Peter Steinert (Candi et al., 1998) has clearly demonstrated a medically relevant mechanism for the assembly of cornified cell structure by permanent covalent binding of the keratin intermediate filament cytoskeleton to the cell envelope by transglutaminase-catalyzed crosslinking (Candi et al., 1998). In these studies Steinert identified the essential role of a conserved lysine residue on the head domains of type II keratins in the supramolecular organization of keratin filaments in cells, whose abnormality results in a very specific skin disease. Thus the biochemical investigation of the interaction of TGases 2 and 5 with intermediate filaments may give important biological and medical informations.

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Authors' address: Eleonora Candi, Biochemistry Laboratory, Istituto Dermopatico Immacolata-IRCCS, c/o Department of Experimental Medicine, University of Rome "Tor Vergata", via Montpellier 1, 00133-Rome, Italy, E-mail: candi@uniroma2.it