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Transglutaminase 5 Cross-links Loricrin, Involucrin, and Small Proline-rich Proteins *in Vitro**

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Eleonora Candi, Sergio Oddi, Alessandro Terrinoni, Andrea Paradisi, Marco Ranalli, Alessandro Finazzi-Agró, and Gerry Melino‡

From the Biochemistry Laboratory, Istituto Dermopatico dell' Immacolata-Istituto di Ricerca e Cura a Carattere Scientifico, Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," via di Tor Vergata 135, 00133 Rome, Italy

Transglutaminases (TGases) are seven enzymes, cross-linking proteins by γ-glutamil-ε-lysine bonds, four of which are expressed in the skin. A new member of the TGase family, TGase 5, has been identified recently, and in the present study we evaluated its role in keratinocyte differentiation *in vitro*. In addition to the previously described isoforms, full-length TGase 5 and Δ3 (deletion of exon 3), we identified two new splicing variants, Δ11 and Δ3Δ11 (deletion of exons 11 or 3, 11). We expressed full-length TGase 5, Δ3, Δ11, and Δ3Δ11 isoforms in the keratinocyte and baculovirus systems. The results indicate that both full-length TGase 5 and Δ11 are active, whereas Δ3 and Δ3Δ11 have very low activity. Expression studies show that full-length TGase 5 is induced during the early stages of keratinocyte differentiation and is differently regulated in comparison with the other epidermal TGases. Kinetic and *in vitro* cross-linking experiments indicate that full-length TGase 5 is very efficient in using specific epidermal substrates (loricrin, involucrin, and SPR3). In keratinocyte expression system, TGase 5 isoforms are retained in an intermediate filament-enriched fraction, suggesting its association with insoluble proteins. Indeed, TGase 5 co-localize with vimentin and it is able to cross-link vimentin *in vitro*.

In all stratified squamous epithelia, including the epidermis, several proteins contribute to the formation of the specialized structure of highly insoluble proteins assembled just beneath the plasma membrane of differentiating keratinocytes, called the cornified cell envelope (CE).[1] CEs are essential for the barrier function of the skin (1–3). The protein composition of CEs varies widely between different epithelia (4). In the skin, an important role is certainly played by loricrin, small proline-rich proteins (SPRs), and involucrin. Loricrin comprises about 75% of the total CE protein mass (3) and is therefore the major component of the CE of epidermal keratinocytes. SPRs serve mainly as cross-bridging proteins by adjoining themselves or other proteins such as loricrin, involucrin, and desmosomal proteins, using multiple adjacent residues of the end domains (4). Involucrin is one of the early structural proteins to be expressed and cross-linked into CE (5). The precise molecular mechanisms leading to CE assembly, and in particular the differential role of all transglutaminases (TGases) expressed, have not been fully elucidated.

TGases (EC 2.3.2.13) are Ca^{2+}-dependent cross-linking enzymes that catalyze an acyl-transfer reaction between the γ-carboxamide group of protein-bound glutamine and various primary amines, most commonly the ε-amino group of lysine residues, thus forming isopeptide bonds between proteins that generate insoluble macromolecular assemblies (6, 7). Four of the seven human TGases (1, 2, 3, 5) are expressed in terminally differentiating epithelia. The recently discovered TGase 5 (originally named X) enzyme awaits characterization (8). TGase 1 is essential for the cross-linking of substrates such as loricrin (9), trichohyalin (10), and SPRs 1 (11), 2 (12), and 3 (13). However, as observed in lamellar ichthyosis, a genetic defect of TGase 1, TGases cannot functionally replace TGase 1 action (14).

TGase 5 has not been fully characterized (8). Accordingly, in this paper we have expressed full-length TGase 5 and its splicing variants in baculovirus system and in keratinocytes and used them to study their biochemical properties. As TGase 5 is induced by calcium treatment of keratinocytes in culture (8), we focused our attention on its role during keratinocyte differentiation and CE assembly *in vitro*.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were of chemical analytical grade. The origin of individual reagents is indicated.

Cloning of TGase 5 by RT-PCR—RNA was extracted using TRIzol (Life Technologies, Inc.) from *in vitro* cultured normal human epidermal keratinocytes (NHEK), HaCat cells, and biopsy from human epidermis, following the manufacturer's instructions. RT-PCR reactions were performed using the RT-PCR One-Step system (Life Technologies, Inc.). 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). The reaction was performed using the following RT-PCR program: 48 °C for 45 min; 94 °C for 2 min; followed by 40 cycles of 94 °C for 45 s, 58 °C for 50 s, 70 °C for 1 min and 40 s; followed by a single step of 10 min at 72 °C. The PCR products were resolved by electrophoresis on an 0.8% agarose gel and stained with ethidium bromide, and the resulting fragments were isolated from the gel and purified using the

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‡ To whom correspondence should be addressed: Biochemistry Laboratory, IDI-IRCCS, University of Rome "Tor Vergata," via di Tor Vergata 135, 00133 Rome, Italy. Tel.: 39-06-7259-6976; Fax: 39-06-20427290; E-mail: gerry.melino@uniroma2.it.

[1] The abbreviations used are: CE, cornified cell envelope; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; IF, intermediate filament; IMAC, immobilized metal ion affinity chromatography; NHEK, normal human epidermal keratinocytes; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; SPR, small proline-rich protein; TGase, transglutaminase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Qiaex II extraction kit (Qiagen, Crawley, UK). About 50 ng of the purified fragments were used for ligation into the PCR2.1 vector and the subsequent transformation of INVa′ *Escherichia coli* cells (TA-Cloning Kit, Invitrogen, Groningen, The Netherlands). Purified DNA of the obtained clones was sequenced with the BigDye Termination Reaction Kit (PerkinElmer Life Sciences) on an ABI-PRISM 377 DNA sequencer (PerkinElmer Life Sciences) according to the manufacturer's recommended protocols.

Detection by RT-PCR in Keratinocytes—For expression studies, a 206-base pair PCR fragment of the TGase 5 gene was amplified using primers TGase 5-F2 (5′-GTGCTGGCTTTGGTGGTGGT-3, +strand) and TGase 5-R4/x (5′-GGGACTGTAGTCTTTGATCTCACTGG-3′, -strand), both at a concentration of 0.4 μM, under the following conditions: standard PCR buffer with 1.5 mM $MgCl_2$ (Life Technologies, Inc.) and 2.5 units of *Taq* polymerase (Life Technologies, Inc.), 500 ng of total RNA, 0.2 μM of each dNTPs, and 0.2 μCi of [^{32}P]αdCTP. The following RT-PCR program was used: a primary step of 20 min at 46 °C followed by a single step of 3 min at 95 °C; 31 cycles consisting of 94 °C for 30 s, 58 °C for 30 s, and 70 °C for 30 s followed by 1 cycle of 72 °C for 3 min. The resulting PCR products were resolved on nondenaturing 7.5% polyacrylamide/TBE (Tris borate-EDTA) gels and revealed by autoradiography.

For the other genes tested, we used the same RT-PCR protocol/program and the following number of cycles and primers: TGase 1, 21 cycles of amplification and primers TG1F-1 (+) (5′-ATGTTGGCATTATCAGTTGTTAGGTTGAGA-3′) and TG1R-1 (−) 5′-AGGTCATCGATGGTTTTGTAGTATTTGCTG-3′); TGase 3, 27 cycles of amplification and primers TG3F-1 (+) (5′-CAGCAGAAATGACCCCAAATACG-3′) and TG3R-1 (−) 5′-AAGGAATCCCCAAAGACCGCAG-3′); β-actin, 16 cycles and primers by Stratagene (catalog no. 302010, La Jolla, CA).

Construction of the Recombinant Transfection Vector and Recombinant Baculoviruses in Insect Cells and Their Expression and Purification—The cDNAs encoding the splicing variants of the TGase 5 gene were cloned into the baculovirus expression vector PVL1392 (PharMingen, San Diego, CA). The fragments containing the cDNA and also those with His-Myc epitope tags were excised from the pCDNA-3.1 vector using the enzymes *Eco*RI and *Pme*I. The vectors were first digested using *Pme*I, filled in using the Klenow fragment of DNA Polymerase-I, and then digested using *Eco*RI. PVL1392 was digested using *Sma*I and *Eco*RI restriction enzymes. This strategy allows the formation of a sticky/blunt site to clone the four variants of TGase 5 cDNA with the correct orientation while keeping the His-Myc epitope of pCDNA-3.1. All of the enzymes were from New England Biolabs (Beverly, MA) and were used according to the manufacturer's recommended protocols.

The cDNAs encoding for TGase 5 and the splicing variants in the pVL1392 vector were under the transcriptional control of the strong baculovirus polyhedrin promoter. The recombinant viruses were obtained by co-transfection of each vector with the modified *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold DNA, PharMingen). BaculoGold DNA carries a lethal deletion and does not code for a viable virus particle by itself. Co-transfection of this DNA with a complementing plasmid construct rescues the lethal deletion of the virus DNA and reconstitutes, by homologous recombination, viable virus particle inside the transfected insect cell (38). Insect cells (Sf9, PharMingen) were grown in Grace's insect medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). In co-transfection experiments, 2×10^6 cells were plated in 60-mm dishes. Two μg of each of the four vectors was mixed separately with 0.5 μg of linearized BaculoGold DNA and incubated for 10 min at room temperature. After incubation, 1 ml of transfection buffer (25 mM HEPES, pH 7.1, 125 mM $CaCl_2$, 140 mM NaCl) was added to each tube and mixed. The DNA transfection buffer mixtures were added to plates that contained 1 ml of regular medium and incubated at 27 °C for 4 h. The medium was then replaced with fresh Grace's insect medium, and the cells were kept at 27 °C for 5 days. The medium containing the recombinant clones was amplified to produce a high titer stock solution, and the titers ($1–8 \times 10^8$ plaque-forming units/ml) assessed by end point dilution. For protein production, cells were maintained at a density of 2×10^6 cells/ml (>95% viability) and infected either as monolayers or in suspension, using a rotary shaker, with $1–4 \times 10^8$ plaque-forming units/ml of culture. The expression of the four TGase 5 enzymes was confirmed by Coomassie Blue staining of total protein and by Western blot using a specific anti-c-Myc tag antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). To extract active TGase 5 enzymes, insect cells were lysed by sonication on ice in a buffer containing 20 mM Tris-HCl, pH 8.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and the protease inhibitor AEBSF (1 mM). The lysates were kept for 15 min on ice and centrifuged at $10,000 \times g$ for 20 min at +4 °C. The cytosol was removed, and the pellet was washed twice for 1 h on ice in the same buffer containing 1% Triton X-100, 1% Nonidet P-40, and 10 mM DTT. The active TGase 5 enzyme was extracted after incubation in the same buffer for 24 h at +4 °C. In some cases, this was followed by an immobilized metal affinity chromatography (IMAC). The conditions for purification were the following: binding of TGase 5 extract in the buffer described above with pre-equilibrated cobalt IMAC resin (Talon Resin, CLONTECH, Palo Alto, CA) for 1 h at room temperature; three washes with the same buffer without detergents; elution with the addition of 100 mM imidazole to the washing buffer. Imidazole did not interfere with TGase activity measurements and was in any case removed it by dialysis.

Construction of the Mammalian Expression Vectors—We reamplified the 3′ portion of TGase 5 cDNA from the base number 1376 to the stop codon using the following primers: TGase 5 F2 (5′-GTGCTGGCTTTGGTGGTGGT-3′) for the + strand; and TGase 5 R1/*Xba*I (5′-CCAGAATTCTAGATGCAAAGTCTAC-3′, full-length and Δ3 variant) and TGase 5R3/Xba-I 5′-CACAGTTCTAGACCTGCTCCGAG-3′, Δ11, Δ3Δ11 variants) for the reverse strand, both containing Xba-I restriction sites that remove the stop codons. The fragments were purified using the methods described above. Fragments and the original cloning vector were *Xba*I- and *Pst*I-digested (New England Biolabs). The generated fragments were then cloned into the vector of origin, allowing the substitution of the 3′ portion of the gene and removal of the stop codons. The entire cDNAs were excised using *Eco*RI/*Xba*I restriction enzymes and inserted into pCDNA 3.1-Myc-Hys vector (Invitrogen) in frame with the epitope. Specific anti-His and anti-c-Myc antibodies for Western blot and immunofluorescence were obtained from Santa Cruz Biotechnology.

Cell Cultures and Transfection—Cryopreserved NHEK were obtained from BioWhittaker (Walkersville, MD) and grown in calf skin collagen (Sigma)-coated dishes in serum-free keratinocyte medium (KGM, BioWhittaker) at 0.05 mM Ca^{2+} supplemented with SingleQuots (BioWhittaker; containing (in μg/ml) 7.5 bovine pituitary extract, 0.5 insulin, 0.5 hydrocortisone, and 5 μM human epidermal growth factor). Third passage cells were used for transfection experiments. Cells at 50% confluence were treated with Ca^{2+} (1.2 mM), 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 10 ng/ml) and retinoic acid (1 μM). Control cells were collected before reaching confluence. Transient transfections of pCDNA3.1-TGase 5s were performed using Effectene (Qiagen) as described by the manufacturer. Briefly, 8×10^5 keratinocytes were plated in collagen-coated 100-mm dishes 1 day before transfection. One μg of DNA of each of the four vectors was mixed separately with 16 ml of enhancer and 50 μl of Effectene transfection reagent. Transfected cells were kept for 2 days in culture. In some cases medium was replaced after 24 h with fresh medium containing 1.2 mM Ca^{2+}. Transfection efficiencies were always monitored by using a cytomegalovirus β-galactosidase construct (pCDNA3.1, Invitrogen) and were approximately to 20%. Cell lysis was performed as described for Sf9 cells. Aliquots of the extracted enzymes were used for TGase assay, column chromatography, and protein quantification using methods described previously. For confocal microscopy, keratinocytes were plated on collagen-coated coverglass, transfected with an equivalent ratio of cell/DNA/lipid reagent, and kept in culture for 2 days.

Solubilization of TGase 5—To solubilize TGase 5 from keratinocytes, we tried the following detergents under the following conditions: deoxycolate (1%), octylglucoside (60 mM), Triton X-100 (1, 2% (v/v)), and CHAPS (10 mM) incubating the extracts for 1 h in ice; SDS (0.05, 0.1, 0.5, 1, and 2% (w/v)) incubating for 1 h at room temperature. Solubilization experiments with urea (1, 2, 3, 5 and 8 M) and DTT (50 mM), increasing the concentrations of NaCl (0.3, 0.5, and 2 N), and using different pH levels (3.6, 5.2, 6.8, and 10.5) were performed by incubating the sample for 15 min at room temperature. All of the experiments (except the one at different pH levels) were done in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM AEBSF. The solubilization of TGase 5 was detected by Western blot, using an anti-tag antibody (anti-c-Myc antibody, Santa Cruz Biotechnology).

Confocal Microscopy—After transfection, cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 2% Nonidet P-40 and 0.2% Triton X-100 for 20 min at room temperature. Cells were then washed with PBS, and nonspecific binding was blocked by incubating the cells with 5% bovine serum albumin in PBS for 30 min at room temperature. Indirect immunofluorescence was performed incubating keratinocytes with: anti-c-Myc antibody (Santa Cruz Biotechnology, 1:1000 in blocking solution), anti-vimentin antibody (Sigma, 1:50 in blocking solution), anti-keratin 14 (Babco, Berkeley, CA, 1:1000 in blocking solution) for 1 h at room temperature. Actin filaments were detected using the phalloidin reagent Alexa Fluor 568. Under the conditions used (1:1000, 1 h at room temperature) to

detect tagged TGase 5, the anti-c-Myc antibody did not detect endogenous c-Myc (data not shown). Washes were followed by incubation with the appropriate secondary antibodies diluted 1:1000 in blocking solution for 1 h at room temperature in the dark. For anti c-Myc, anti-vimentin, and anti-keratin 14, we used goat anti-mouse (Alexa Fluor 488, Molecular Probes Inc., Eugene, OR), donkey anti-goat (Alexa Fluor 568), goat anti-rabbit (Alexa Fluor 568). The slides were mounted using a Prolong Antifade kit (Molecular Probes Inc.). Fluorescence was then evaluated with a confocal microscope (Nikon Instruments Spa, Eclipse TE200), exciting at 488 nm with an Argon laser and at 542 nm with a Helium-Neon laser. The software used was EZ2000 for PCM2000.

Subcellular Fractionation—Transfected keratinocytes were fractionated following the method described previously (28). Briefly, cells were washed twice with PBS and centrifuged at 700 × *g* for 5 min at 4 °C. Cells were gently resuspended in 5 volumes of lysis buffer (10 mM Tris-HCl, pH 7.5, 0.05% Nonidet P-40, 3 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA) and centrifuged at 350 × *g* for 5 min at 4 °C. The cytosols were recovered and kept for Western blot analysis. Pellet containing the crude nuclei was washed once in lysis buffer and twice in wash buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 25 mM NaCl, 1 mM EDTA) by gentle resuspension in 5 volumes of buffer and centrifugation at 350 × *g* for 5 min at 4 °C. For further purification, the nuclei were resuspended in wash buffer, overlaid in wash buffer containing 1 M sucrose, centrifuged at 1200 × *g* for 10 min at 4 °C, and collected in the pellet. All buffers contained the protease inhibitor AEBSF (1 mM). Purified nuclei were resuspended in wash buffer containing 0.5% Triton X-100 and incubated on ice for 30 min to release lipids and Triton X-100-soluble proteins. Nuclei were then centrifuged at 700 × *g* for 5 min at 4 °C. The pellet was resuspended in digestion buffer (10 mM Pipes, pH 6.8, 700 units/ml DNase I, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$) and incubated for 1 h on ice. The digested DNA, chromatin, and associated proteins were eluted by slowly adding NaCl buffer (10 mM Pipes, pH 6.8, 2 M NaCl, 3 mM $MgCl_2$, 1 mM EDTA) with gentle mixing to a final concentration of 1.6 M NaCl. The remaining proteins, containing mainly nuclear matrix proteins and cytoskeleton intermediate filaments, were centrifuged at 5000 × *g* for 20 min at 4 °C and resuspended in a buffer containing 250 mM Tris-HCl, pH 6.8, 2% SDS, 25 mM DTT, 5 mM EDTA, 10% glycerol. The protein concentration of cell fractions was determined by the Lowry method after acid precipitation of the proteins. Samples were electrophoresed on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The cellular fractions were analyzed by Western blot using the following antibodies: anti-vimentin (Sigma), anti-keratin 14 (Babco), and anti-actin (Sigma).

Enzyme Assay—TGase activity was determined by measuring the incorporation of [^{3}H]putrescine (Amersham Pharmacia Biotech) into *N,N′*-dimethylcasein (Sigma) (15). The reaction mixtures contained 100 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM DTT, 10 mM $CaCl_2$, 25 μl of casein (12.5 mg/ml), and 0.2 mM putrescine containing 1 μCi of [^{3}H]putrescine. Different TGases were incubated with the reaction mixture in a final volume of 100 μl at 37 °C. After 10 min of incubation, the reaction was stopped by spotting 25-μl aliquots onto Whatman 3MM filter paper. Unbound [^{3}H]putrescine was removed by washing with large volumes of 15, 10, and 5% trichloroacetic acid and absolute ethanol. Filters were then air-dried, and the radioactivity was measured by liquid scintillation counting. TGase assays were done, for the most part, in triplicate. The experiments for specific activity measurements of TGase 5 were performed following the above protocol, *N,N′*-dimethylcasein was substituted with 2 nmol of specific epidermal substrates (recombinant loricrin, SPR1, SPR2, SPR3, and involucrin, obtained as described previously, (9, 11, 12, 16) and 4 pmol of TGase 5. The amount of the TGase 5 was determined by titration of the active site using [^{14}C]iodoacetamide as described previously (14).

TGases activity was measured *in vivo* following Lajemi *et al.* (17). Briefly, after transfection cells were incubated with 25 mM fluorescein cadaverine (Molecular Probes Inc.) in KGM medium containing 0.05 mM Ca^{2+} at 37 °C, for 3 h. Cells were subsequently washed in PBS, fixed, and immunostained for TGase 5 using a goat anti-mouse antibody (Alexa Fluor 568, Molecular Probes Inc.) as described above under "Confocal Microscopy."

Cross-linking of Recombinant Human Loricrin and SPR3—Recombinant loricrin and SPR3 were obtained as described previously (9, 13). TGase 1 and TGase 3 enzymes were also obtained as described in a previous paper (9), and full-length TGase 5 enzyme was extracted and purified from infected Sf9 cells using Triton X-100 and Nonidet P-40 detergents. For *in vitro* cross-linking studies using loricrin as a complete TGase 5 substrate, the purified unlabeled or ^{35}S-labeled loricrin was equilibrated by dialysis into a buffer containing 50 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.02 mM AEBSF, pH 7.5. The reactions were adjusted to 10 mM $CaCl_2$ and 5 mM DTT (for control sample) to initiate the reactions at 37 °C. In cross-linking experiments, 45 μg (1.7 nmol) of ^{35}S-labeled loricrin was utilized in a 160-μl reaction volume and 38 μg (2.2 nmol) of SPR3 in a reaction volume of 200 μl. To standardize the reactions for comparison of the two substrates, the same amount of enzymatic activity was used for each substrate. These activities were measured by [^{3}H]putrescine incorporation into casein (15), and we used the amount of TGase 1, TGase 3, and TGase 5 that incorporates 1000 dpm/h (95 pmol/h) into the casein/μg of substrate. Aliquots were withdrawn at selected times, and the reactions were stopped by the addition of 10 mM EDTA. For double cross-linking experiments, we cross-linked loricrin and SPR3 to completion with TGase 1 and TGase 3, and then we added TGase 5 and *vice versa.* The cross-linked products were separated on 10% polyacrylamide gels and analyzed by autoradiography for loricrin and by Western blot using an antibody that recognizes the C terminus of SPR3 (kindly given by Dr. Kartasova). To use vimentin as TGase 5 substrate we incubated 1.8 μg of vimentin (from bovine lens, Sigma) with the amine donor 0.8 mM 5-(biotinamido)pentylamine (Pierce) in a buffer containing 50 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$, and 5 mM DTT. The reaction started by adding an amount of TGase 5 that incorporated 1000 dpm/h (95 pmol/h) into the casein for 2 h at 37 °C. Controls were performed without $CaCl_2$ and with 10 mM EDTA, and with $CaCl_2$ and without vimentin. Before starting the reaction, the solution containing vimentin was dialyzed sequentially against 5 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.02 mM AEBSF, and 170 mM KCl to remove urea and to achieve filaments reassemble. The products labeled with 5-(biotinamido)pentylamine were visualized with streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech).

Kinetic Studies of Putrescine Incorporation into Recombinant Human Loricrin, Involucrin, and SPR3—Kinetic reactions were carried out using full-length TGase 5 obtained as described previously. Six concentrations of unlabeled loricrin (0.8, 1.2, 1.5, 2.3, 3.5, and 7 μM), two different concentrations of [^{3}H]putrescine (104 and 208 μM; Amersham Pharmacia Biotech, specific activity, 39 Ci/mmol), seven concentrations of SPR3 (0.7, 1, 1.5, 2, 3, 4, and 6 μM), three concentrations of [^{3}H]putrescine (90, 180, and 300 μM), seven concentrations of involucrin (0.5, 1, 1.5, 2, 3, 5, and 7 μM), three concentrations of [^{3}H]putrescine (100, 200, and 300 μM), and an appropriate amount of enzyme were used in a final volume of 100 μl for 10 min at 37 °C. For each enzyme, the amount of activity utilized was standardized at 80 pmol/min putrescine incorporation into casein as described above. The reactions were stopped by spotting 35 μl of the initial mixture on filter paper, washing the filter sequentially for 10 min in 15, 10, and 5% cold trichloroacetic acid, and washing it finally 100% ethanol. Filters were dried, and the radioactivity was counted. These reaction conditions utilized a large molar excess of putrescine to achieve linearity of reaction kinetics (15) so as to favor TGase attachment of only one amine group of the putrescine to loricrin rather than TGase cross-linking of the substrates by putrescine or cross-linking of substrates to themselves. Indeed, control experiments were performed for two concentrations of loricrin (1.2 and 7 μM), involucrin (0.5 and 7 μM) and SPR3 (1.5 and 6 μM) with two concentrations of putrescine (respectively, 104 and 208 μM for loricrin, 180 and 300 μM for SPR3, 200 and 300 μM for involucrin), and no detectable oligomerization of substrates was observed by Western blotting and autoradiography. The data for the initial velocity of [^{3}H]putrescine incorporation into substrates conformed to a modified double displacement mechanism as described previously for TGase catalyzed reactions (18). Kinetic constants were calculated as described previously (13–15).

RESULTS AND DISCUSSION

Identification of Two New Splicing Variants, Expression in Baculovirus System and NHEK—In addition to the two described isoforms of TGase 5, we detected, by RT-PCR of mRNA from human keratinocytes (Fig. 1*A*) and HaCat cells (Fig. 1*B*), two additional isoenzymes generated by a new alternative splicing lacking exon III or XI (Fig. 1, *A–C*). According to TGase 2 exon/intron organization, we named these splicing variants Δ3 (8), Δ11, and Δ3Δ11. The TGase 5 isoforms with deleted exon XI (Δ11 and Δ3Δ11) have a shift in the reading frame leading to the translation of 25 new amino acids followed by a premature stop codon (Fig. 1*C, gray boxes*); therefore, exons 11, 12, and 13 are missing in the C terminus of the Δ11 and Δ3Δ11 variants.

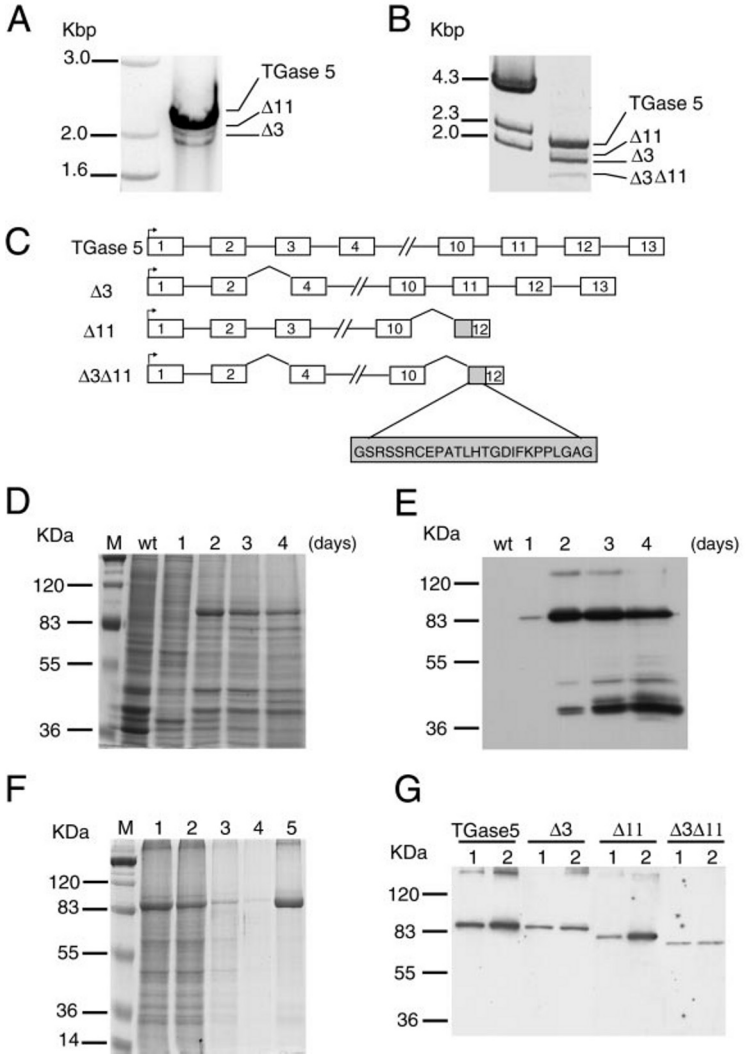

FIG. 1. **Detection of TGase 5 splicing variants, expression and purification in baculovirus and keratinocytes.** *A*, TGase 5 and the splicing variants Δ3 and Δ11 detected by RT-PCR using mRNA isolated from normal human skin. *B*, TGase 5 and the splicing variants Δ3 and Δ11, and the combination of the two forms, Δ3Δ11, detected by RT-PCR using mRNA isolated from HaCat. cDNA sizes of full-length TGase 5, Δ3, Δ11, and Δ3Δ11 detected and sequenced are, respectively, 2169, 1933, 1807, and 1562 base pairs. Details for the RT-PCR conditions and the primers used are found under "Experimental Procedures." *C*, schematic representation of TGase 5 splicing variants. Spliced-out exons are indicated with respect to the full-length form of TGase 5. Exon/intron boundary was designed by comparison with TGase 2. The *gray boxes* indicate that a shift is present in the reading frame that generates the addition of 25 new amino acids and a premature stop. The sequence of the additional 25 new amino acids at the C-terminal end of Δ11 and Δ3Δ11 variants is shown below. Because of the frameshift, the Δ11 and Δ3Δ11 variants were also missing exons 12 and 13. *D*, time-course of the baculovirus-expressed full-length TGase 5. The enzyme is detected by Coomassie Blue staining after 2 days of infection as a band of 84 KDa. *E*, Western blot of TGase 5 time-course using an anti-tag antibody. The *lower bands* are due to degradation that normally occurs in baculovirus-infected Sf9 cells. *F*, purification of TGase 5 by IMAC. The enzyme was extracted as described under "Experimental Procedures." *Lane 1* represents the material loaded on the column, *lanes 2–4* are the washes, and *lane 5* is the eluant containing pure TGase 5. *G*, expression of TGase 5 and its isoforms in NHEK. Cells were transiently transfected with the four TGase 5 isoforms and kept for 1 day in proliferating (0.05 mM Ca^{2+}, *lane 1*) and differentiating (1.2 mM Ca^{2+}, *lane 2*) conditions. Equal amounts of total protein (20 μg) were loaded and Western blot was performed using anti-tag antibody.

To investigate the biochemical features of TGase 5 isoforms, we cloned the cDNAs coding for TGase 5 and its splicing variants in expression vector suitable for generating recombinant baculoviruses and for transfection in normal human epidermal keratinocytes. The C-terminal ends of these cDNAs were modified to add a polyhistidine and c-Myc tag. All of the isoforms were expressed in the baculovirus system. Fig. 1*D* shows that maximum production of full-length TGase 5 was achieved after 2 days of infection with the recombinant virus, as also confirmed by the corresponding Western blot analysis using a specific anti-c-Myc antibody (Fig. 1*E*). TGase 5 had an apparent molecular size of 84 KDa, and after 2 days of infection other bands appeared on the Western blot indicating ongoing degradation due to the effect of the infection of recombinant baculoviruses on insect cells. In fact, these lower molecular mass bands were not detected in keratinocyte expressed TGase 5 (Fig. 1*G*). Moreover, TGase 5 underwent autocatalytic cross-linking to itself as shown by the band of high molecular mass (about 200 kDa; Fig. 1, *E* and *G*). This phenomenon has been described previously for TGase 2 and factor XIIIa (19). The recombinant enzymes, obtained both in the baculovirus system and in keratinocytes, were purified to homogeneity by IMAC utilizing the polyhistidine C-terminal tag (Fig. 1*F*, *lane 5*). To test the stability and the expression of TGase 5 and its isoforms, we transiently transfected them in NHEK. Cells were grown in proliferating and differentiating conditions (0.05 and 1.2 mM Ca^{2+}, respectively) for 72 h to test whether TGase 5 is proteolytically processed in high calcium, as already described for TGase 1 (20). Western blot analysis revealed a band of 84 KDa for full-length TGase 5 enzyme (Fig. 1*G*, *lanes 1* and *2*)

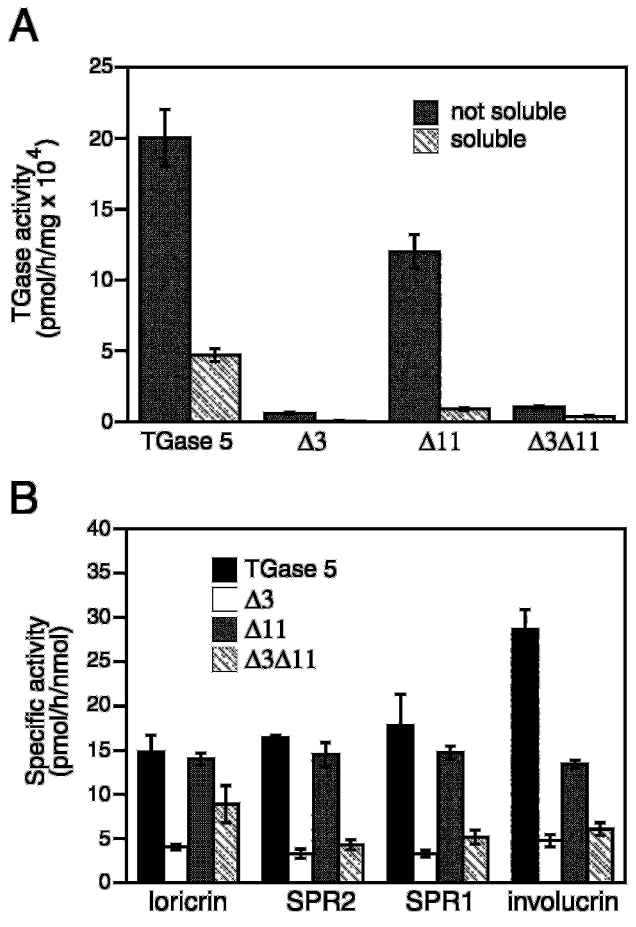

FIG. 2. **Activity measurements of the recombinant enzymes.** *A*, activity measurements of TGase 5 and its splicing variants using *N,N′*-dimethylcasein and [^{3}H]putrescine as substrates. Infected Sf9 cells were lysed, and activity was measured in the soluble and not soluble fractions. *B*, specific activity measurements for TGase 5 and its splicing variants using as specific substrates recombinant loricrin, SPR1, SPR2, and involucrin. The amount of substrates used (2 nmol) was evaluated using the Lowry method, and the enzymes were measured by titration of the active site using [^{14}C]iodoacetamide (14). All of the data reported in this figure are an average of 5–8 separate experiments.

and other bands of lower molecular mass corresponding to the splicing variants. There were no substantial differences among proliferating and differentiating conditions, indicating that, unlike TGase 1, TGase 5 and its isoforms were not post-translationally activated by proteolytic processing.

Enzymatic Activity of TGase 5 Isoforms—To measure TGase activity, infected Sf9 cells were lysed by sonication in a buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM DTT, and a protease inhibitor mixture) containing 0.1% Triton X-100. Most of the enzymatic activities of the TGase 5 isoforms were detected in the Triton X-100-insoluble fraction (Fig. 2*A*). However, the active enzymes were obtained if we further extracted these insoluble fractions in the same buffer containing 1% Triton X-100 and 1% Nonidet P-40 (data not shown) for 24 h at +4 °C. These enzymes, further purified by IMAC and kept at −20 °C, where they retain 80% of their activity over 2–3 weeks of storage, were used for cross-linking and kinetic studies. The recombinant full-length TGase 5 enzyme generated with the baculovirus system is active as indicated by the incorporation of radioactive putrescine into *N,N′*-dimethylcasein (Fig. 2*A*). No activity essentially was observed in the variants Δ3 and Δ3Δ11 (Fig. 2*A*). However, the new splicing variant that we have described, Δ11, is active and retained 60% of the activity of the full-length enzyme (Fig. 2*A*) as found by assaying the enzymatic activity using *N,N′*-dimethylcasein as acyl donor and radiolabeled putrescine as acceptor substrate. To test whether the observed decrease in activity of Δ11 variant also occurs with other substrates, we measured the specific activity for all of the recombinant isoenzymes utilizing, as acyl donor, specific recombinant epidermal substrates such us loricrin, SPR1, SPR2, and involucrin. As shown in Fig. 2*B*, full-length TGase 5 and the Δ11 variant have similar specific activities utilizing loricrin, SPR1, and SPR2 as substrates, whereas full-length TGase 5 has 2-fold higher specific activity in comparison with Δ11 variant when involucrin is used as substrate. The Δ3 and Δ3Δ11 splicing variants are considerably less active.

Solubilization Properties of TGase 5—About 70% of TGase 5 expressed both in the baculovirus system and in keratinocytes is not solubilized by the combination of nonionic detergents (1% Triton X-100 and 1% Nonidet P-40); therefore, we tried different approaches to understanding the reason for such insolubility. First, we checked the solubility features of the enzyme using several detergents at different concentrations and conditions. In Fig. 3 we show representative experiments summarizing the results obtained. TGase 5 could not be extracted by different detergents (deoxycolate, octylglucoside, Triton X-100, CHAPS) (Fig. 3*A*). Successful extraction was obtained only using the ionic detergent SDS (0.1, 0.5, and 1% (w/v)) keeping the sample at room temperature for 1 h (Fig. 3*B*). Changing the extraction conditions to 0.01 and 0.05% (w/v) SDS for 3 h at room temperature allowed extraction of active enzymes. However, SDS-extracted enzymes quickly lost their activities; therefore, these solubilized enzymes were not used for enzymatic studies. We failed to identify any lipid modifications by metabolic labeling of cells with [^{3}H]myristic and [^{3}H]palmitic acids, indicating that, unlike TGase 1, TGase 5 insolubility is not due to lipid attachment. Moreover, there were also no lipid modifications detected at the N-terminal end by in tandem electrospray mass spectrometry (data not shown). Because of the involvement of an ionic detergent in TGase 5 solubilization, we explored the possibility that TGase 5 was associated with insoluble protein through hydrophobic interactions and electrostatic charges. Therefore, we treated samples at different concentrations of ionic strength (NaCl 0.3, 0.5, and 2 N) and at different pH levels (3.6, 5.2, 6.8, 10.5), but we still could not solubilize the enzyme (not shown). We then explored the possibility of a role for disulfide bridges in keeping TGase 5 insoluble and found that the presence of DTT (50 and 100 mM) has very little effect in solubilizing TGase 5 (not shown). Using denaturing agents such as urea (Fig. 3*C*), we observed that 5 M urea is able to solubilize about 90% of the enzyme. DTT alone is not able to achieve the same solubilization of TGase 5 as can be obtained in the presence of urea (Fig. 5*C*). These observations suggest that the insolubility of TGase 5 is due to the strong interaction of this enzyme with insoluble proteins that only denaturing agents (such as urea) can destabilize. Similar solubility properties have been observed in TGase 5 expressed in Sf9 insect cells and in NHEK, both for full-length enzyme and its splicing variants.

Subcellular Localization by Centrifugation and Confocal Microscopy—Keratinocytes were separated by centrifugation into cytosolic and nuclear fractions. The nuclear fraction was washed in a sucrose gradient and further separated into the Triton X-100 fraction (containing lipids and soluble proteins), the chromatin fraction (containing histones and nucleus-associated proteins), and the insoluble fraction (containing cytoskeleton intermediate filaments (IF) and nuclear matrix) (21). We tested the obtained fractions by immunoblotting for vimentin and keratin 14 (insoluble fraction) and α-actin (cytosol) (Fig. 4, *B–D*, respectively). TGase 5 immunoreactivity

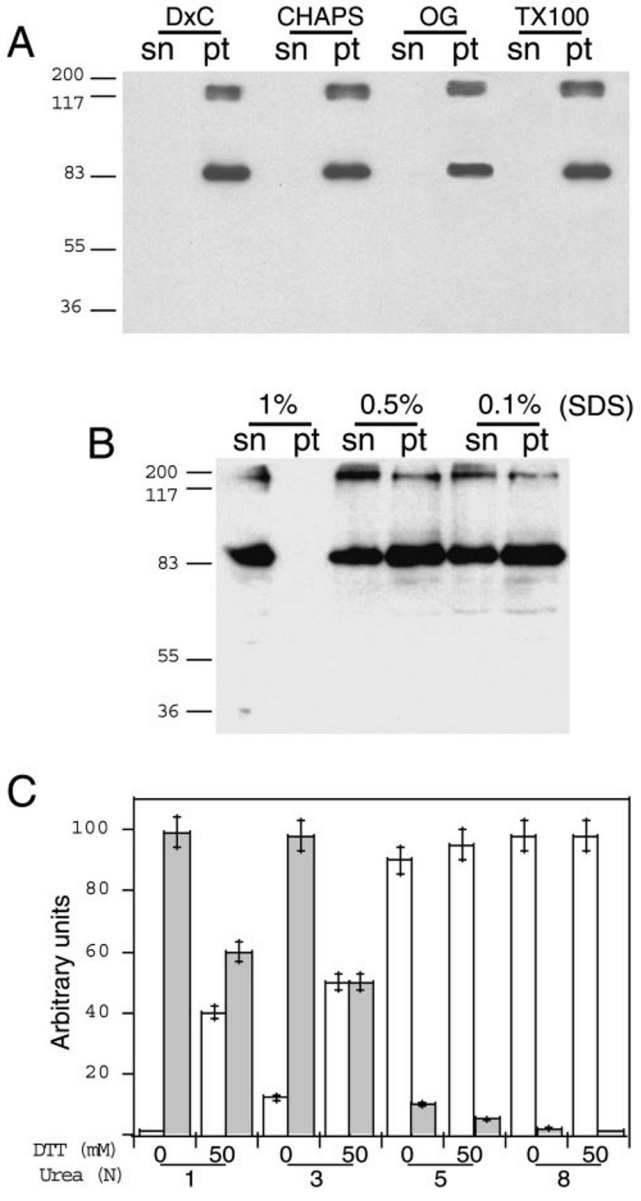

FIG. 3. **Solubilization properties of TGase 5.** *A*, NHEK were lysed, and different detergents were tested at the following concentrations: deoxycholate (*DxC*, 1%, v/v), CHAPS (10 mM), octylglucoside (*OG*, 60 mM), Triton X-100 (*TX100*, 1% v/v); the extracts were incubated for 1 h in ice. *B*, SDS extraction of TGase 5 obtained by incubating cell extracts for 1 h at room temperature. *sn*, supernatant; *pt*, pellet. *C*, solubilization experiments of TGase 5 with urea (1, 2, 3, 5, and 8 M) in the presence or absence of DTT (50 mM). Samples were incubated for 15 min at room temperature. *White bars* indicate solubilized TGase 5. In every experiment shown in this figure NHEK were used, and the solubilization of TGase 5 was detected by Western blot using anti-tag antibody. The figure shows representative results of 4–6 different experiments.

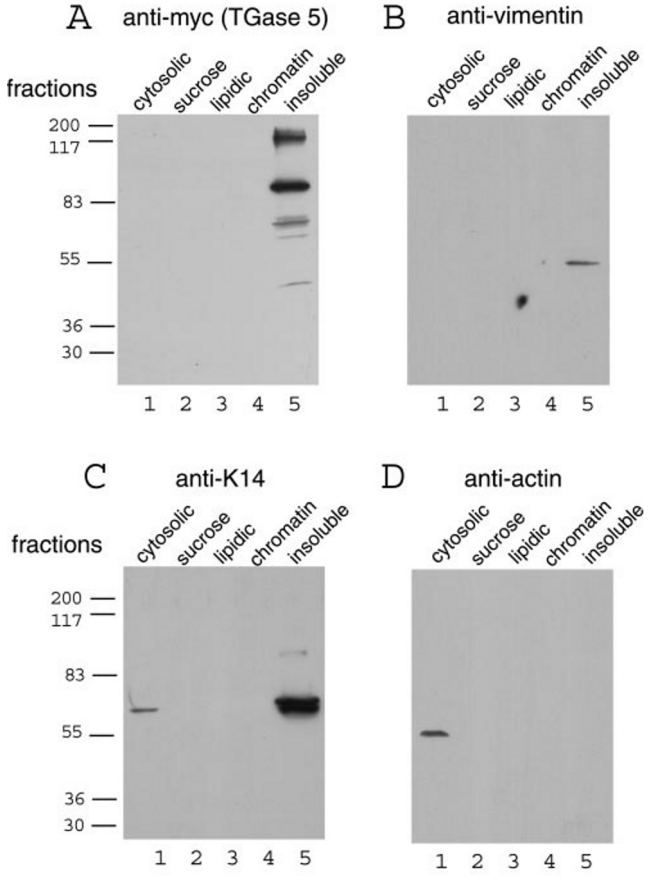

FIG. 4. **Subcellular fractionation of TGase 5.** Transfected NHEK were subfractionated by centrifugation in the presence of high ionic strength detergents as described under "Experimental Procedures." *Lane 1* corresponds to the cytosolic fraction, *lane 2* to the sucrose gradient fraction, *lane 3* to the Triton X-100 fraction (containing lipids and soluble proteins), *lane 4* to the chromatin fraction (containing histones and nuclear associated proteins), and *lane 5* to the insoluble fraction (containing nuclear matrix and cytoskeleton). *A*, Western blot for TGase 5 using anti-tag antibody. *B*, Western blot for vimentin. *C*, Western blot for keratin 14 (*K14*). *D*, Western blot for α-actin. The figure shows representative results of 3–5 experiments.

was found in the cytoskeleton and nuclear matrix fraction (Fig. 4*A*, *lane 5*) together with the intermediate filaments vimentin and keratin 14 (Fig. 4, *B* and *C*, *lane 5*). The additional lower molecular mass bands that appear in Fig. 4*A, lane 5*, are probably due to degradation of the sample during cellular fractionation, even though protease inhibitors were added to the buffer.

To verify co-localization of TGase 5 with vimentin and keratin 14, and also to study the cellular localization of *in vitro* expressed TGase 5, we performed confocal microscopy in normal keratinocytes. *In situ* measurement of TGase activity, using as the primary amine the fluorescent substrate fluorescein cadaverine (Fig. 5*B, green*), revealed that the perinuclear transfected TGase 5 (Fig. 5*A, red*) is active (Fig. 5*C, yellow* staining) and therefore able to incorporate fluorescein cadaverine in living cells. We then transfected NHEK with TGase 5 and performed indirect immunofluorescence using anti-c-Myc antibody to detect TGase 5, anti-vimentin antibody, anti-keratin14 antibody, and the reagent phalloidin to detect actin. TGase 5 was detected in the cytosol as granular deposition (Fig. 5, *A, D*, and *G*), although its expression was mainly localized perinuclearly. Double staining with anti-TGase 5 (Fig. 5*D, green*) and anti-vimentin (Fig. 5*E, red*) antibodies showed a co-localization of these two molecules (Fig. 5*F*, appearance of *yellow*) or with another molecule that co-localizes with vimentin. Comparison of the vimentin network between transfected (Fig. 5*E*) and nontransfected cells (Fig. 5*O*) showed a modification of the network, where TGase 5 is accumulated perinuclearly. Because TGases are able to use vimentin as substrate *in vivo* (22), it is tempting to speculate that the clumping of the vimentin network in the proximity of TGase 5 is associated with cross-linking of vimentin with itself or with other intermediary molecules. *In vitro* experiments using vimentin filaments as acyl donor and 5-(biotinamido)pentylamine as acceptor substrate resulted in the incorporation of 5-(biotinamido)pentylamine into vimentin, indicating that *in vitro* vimentin is at least able to provide the acyl donors during

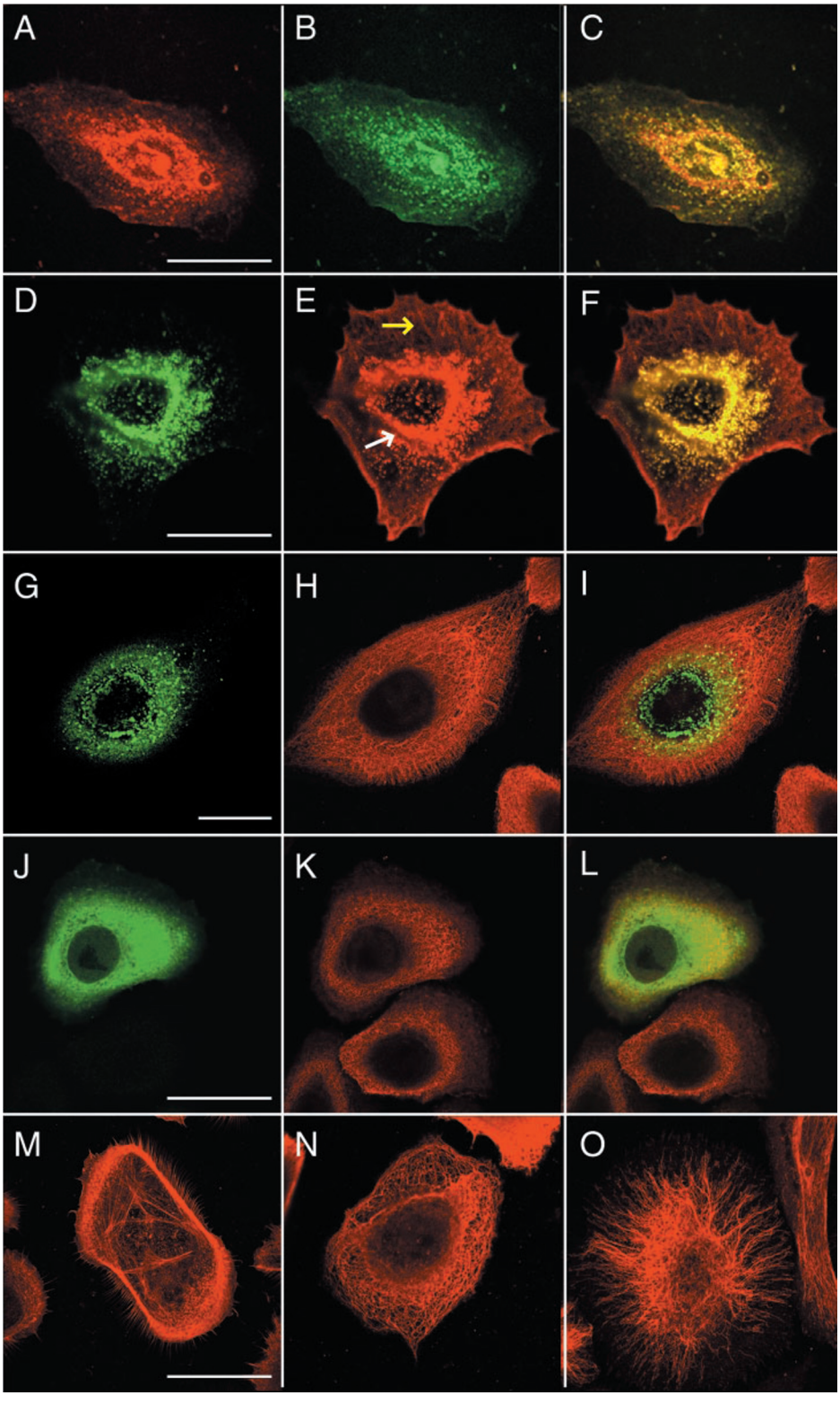

FIG. 5. **Cellular localization of TGase 5.** TGase 5 was transfected in NHEK, cells were stained with anti-c-Myc antibody (*A, red*) to detect TGase 5. To assess whether TGase 5 is active, we incorporated living cells with fluorescein cadaverine without additional Ca^{2+} (17) (*B, green*). Cells were optically sectioned using confocal laser-scanning microscopy, and corresponding images were superimposed to determine the degree of overlap (appearance of *yellow*). Superimposition of TGase 5 and the activity determines a good level of overlap (*C, yellow*). To assess the potential co-localization of TGase 5 with vimentin network, NHEK were stained to detect TGase 5 (*D, green*), and vimentin (*E, red*). Superimposition of TGase 5 (*D*) with vimentin (*E*) shows a remarkable overlap (*C*) of the two staining patterns. Clumps of vimentin filaments are indicated by the *white arrow*, and vimentin filaments are indicated by the *yellow arrow*. Cells were also stained with anti-keratin 14 antibody (*H, red*). Superimposition of TGase 5 (*G, green*) and keratin 14 staining does not show overlap (*I*). TGase 5 expression does not interfere with keratin network as shown by the comparison among transfected (*H*) and nontransfected cells (*N*). TGase 2 transfected in cells (*J*) stays in the cytosol and does not accumulate perinuclearly as TGase 5 (*A, D*, and *G*). TGase 2 accumulation does not disturb keratin 14 network (*K*). Superimposition of TGase 2 (*J*) and keratin 14 (*K*) shows little or not overlap (*L*). As control for NHEK culture conditions, we include the immunofluorescence for the following cytoskeleton elements in nontransfected cells: actin filaments (*M*), keratin 14 (*N*), and vimentin (*O*). As can be observed, these cytoskeleton components seem normal, indicating that we used optimal culture conditions. *Bars* correspond to 10 μm. The *panels* show representative results of 4–5 experiments.

TGase 5-catalyzed reaction (Fig. 7*E*).

To check that TGase 5 co-localization with the vimentin IF network in cultured keratinocytes is specific, we studied its cellular localization with keratin 14, which, together with keratin 5, is the more abundant cytoskeleton element in proliferating keratinocytes. Co-localization is not observed among TGase 5 and keratin 14/5 cytoskeletons (Fig. 5, *G–I*). Moreover, in this case, TGase 5 does not disturb the keratin 14/5 filament network, as we could see by comparison the network in transfected (Fig. 5*H*) and nontransfected cells (Fig. 5*N*). On the other hand, if we transfected another TGase in culture keratinocytes, we did not observe the same TGase 5 behavior. Indeed, TGase 2 enzyme overexpressed in NHEK (Fig. 5*J*) stays in the cytosol and does not interfere with the keratin 14 network (Fig. 5, *J–L*) or vimentin (not shown).

Expression Studies of TGase 5 during in Vitro Keratinocyte Differentiation—The ability of the TGases expressed in epidermis to cross-link the same substrates, although with different efficiencies (9, 11–13), makes it important to understand the mechanisms that control and modulate the expression of these

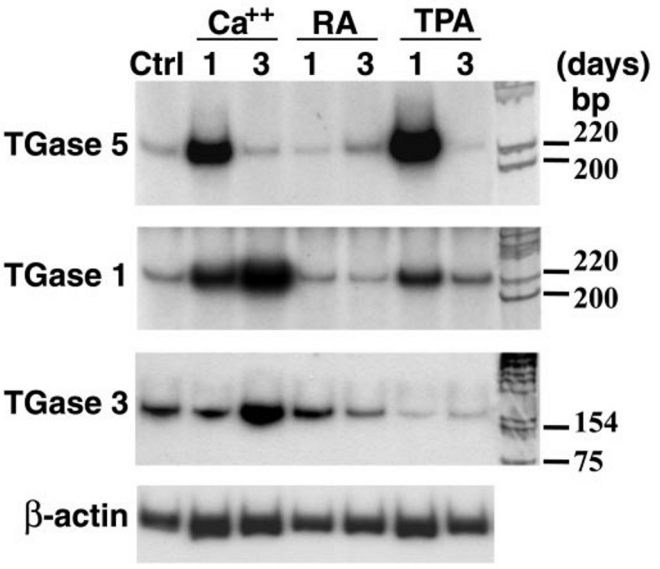

FIG. 6. **Expression of TGases in NHEK.** NHEK were kept in culture and treated for 1 and 3 days with 1.2 mM Ca^{2+}, 1 μM retinoic acid, and 10 ng/ml TPA. Total RNA was extracted, and RT-PCR was performed as described under "Experimental Procedures." Control (*Ctrl*) represents NHEK collected at 60% confluence to avoid differentiation. The size of the amplified portions are indicated by the markers on the *right*. RT-PCR conditions and primers used are indicated under "Experimental Procedures." The *panels* show representative results of 3–5 different experiments.

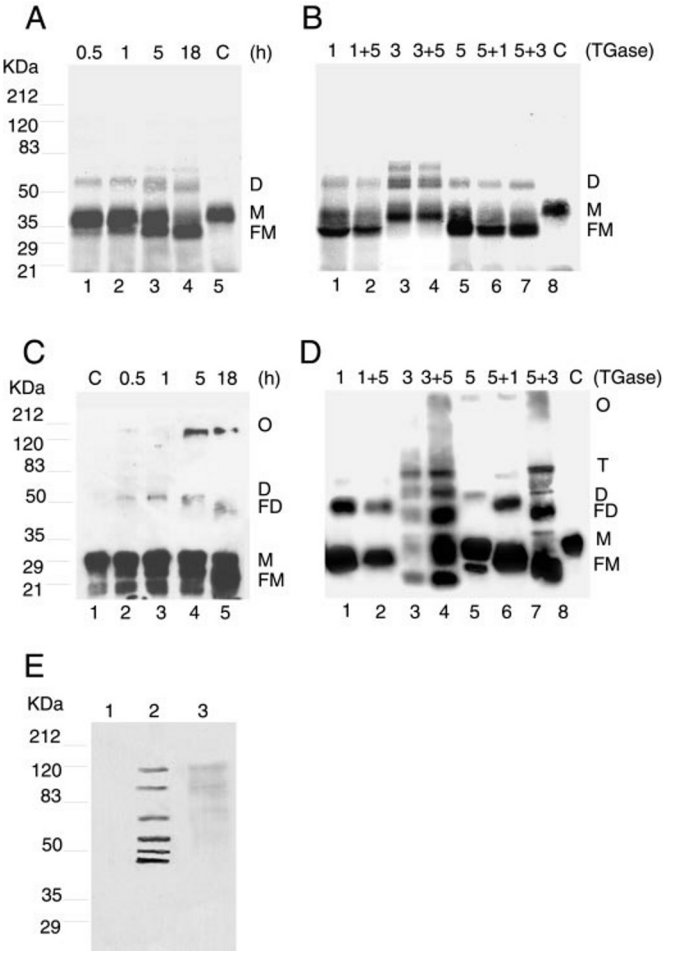

FIG. 7. **TGase 5 cross-links loricrin and SPR3.** Equal amounts of activity of TGase 5, TGase 1, and TGase 3 were measured as described under "Experimental Procedures" and used in this experiment. The SDS-gels were examined by autoradiography for loricrin (*panels A* and *B*) and by Western blot for SPR3 (*panels C* and *D*). Times of reaction for loricrin (*A*) and SPR3 (*C*) cross-linking with TGase 5 were 0.5, 1, 5, and 18 h. Controls (*C*) represent reactions carried out for 18 h in the presence of EDTA instead of Ca^{2+}. In double cross-linking experiments for loricrin (*B*) and SPR3 (*D*), both substrates were reacted for 18 h with one enzyme followed by another 18-h reaction with another enzyme. We used the following order: TGase 1 (*lane 1*) followed by TGase 5 (*lane 2*); TGase 3 (*lane 3*) followed by TGase 5 (*lane 4*); TGase 5 (*lane 5*) followed by TGase 1 (*lane 6*); and TGase 5 followed by TGase 3 (*lane 7*). Controls represent reactions carried on for 36 h in the presence of EDTA instead of Ca^{2+}. On the *right*, the products of cross-linking reactions are indicated: *M*, monomer; *D*, dimer; *T*, trimer; *FM*, fast monomer; *FD*, fast dimer. TGase 5 is able to use vimentin as substrate (*E*). TGase 5 incorporates the acceptor substrate 5-(biotinamido)pentylamine into vimentin as shown by the multiple bands appearing in *panel E*, *lane 2*. *Lane 1* is a control containing TGase 5, vimentin, 5-(biotinamido)pentylamine, and EDTA instead of Ca^{2+}; *lane 3* is another control containing TGase 5,5-(biotinamido)pentylamine and Ca^{2+}. Molecular size markers are indicated on the *left*. The *panels* show representative results of 3–4 experiments.

TGase genes in the epidermis. TGase 2 is restricted to the proliferative or basal layer. Its expression is induced by retinoids but is inhibited by Ca^{2+} and by the phobol ester TPA (23). TGase 1 is expressed all over the epidermis but is more abundant in the upper (spinous and granular) layers (24). The TGase 1 transcript is negatively regulated by retinoids and is induced by TPA and Ca^{2+} (23, 25). TGase 3 is present only in the granular layer. It is poorly expressed *in vitro* (26), but proTGase 3 mRNA is induced by Ca^{2+} treatment (27). To study the expression pattern of full-length TGase 5 in comparison with TGase 1 and TGase 3, we treated NHEK with differentiating agents (Ca^{2+}, 1.2 mM; TPA, 10 ng/ml) or with retinoic acid (1 μM) (23, 25, 26, 28–30). By RT-PCR of total mRNAs, we detected an accumulation of TGase 5 within 24 h of treatment of NHEK with Ca^{2+} and TPA, whereas retinoic acid did not significantly alter full-length TGase 5 expression (Fig. 6). This Ca^{2+}- and TPA-induced accumulation decreased with time and after 3 days of treatment was back to the control level. On the contrary, mRNAs for both TGase 1 and TGase 3 continued to accumulate during keratinocyte differentiation induced by Ca^{2+} throughout the 3 days of treatment. Although we do not have information on the relative protein level of expression of TGase 1, TGase 3, and TGase 5 both in cultured keratinocytes and in the epidermis, these results suggested that TGase 5 is differently expressed and regulated during keratinocyte differentiation *in vitro*.

In Vitro Cross-linking of Specific Epidermal Substrates by TGase 5—Many data have documented that loricrin is the major substrate for TGases in epidermis. In previous studies (9) we have shown that it is cross-linked by TGase 1, TGase 3, and to a minor extent by TGase 2, generating different types of cross-linking. Therefore, we decided to use ^{35}S-labeled recombinant loricrin for *in vitro* cross-linking experiments and to resolve the cross-linking products by SDS-polyacrylamide gel electrophoresis and autoradiography. TGase 5 is also able to utilize loricrin as complete substrate (Fig. 7*A*) in the sense that it provides both glutamine and lysine for cross-linking. The enzyme was able to complete the reaction in 18 h, and further incubation time or the addition of more enzyme did not add more isopeptide bonds. TGase 5 uses loricrin to form both interchain (see dimer appearance, Fig. 7*D*) and intrachain cross-linking (see the fast monomer, *FM*), although no high molecular mass oligomerization is observed as for TGase 1 (9). Loricrin cross-linking products catalyzed by the action of TGase 1 (Fig. 7*B*, *lane 1*) and TGase 5 (Fig. 7*B*, *lane 5*) are more similar then loricrin cross-linking products formed by TGase 3 (Fig. 7*B*, *lane 3*). *In vitro* double cross-linking experiments performed utilizing the epidermal TGases (TGase 1 and TGase 3) and TGase 5 added to the reaction mix in a different tem-

poral order (Fig. 7*B*) revealed that once each of the three enzyme has performed its action, the other cannot use loricrin as substrate any further, probably because of physical impediment of the cross-links already formed. In fact, if we cross-link loricrin to completion with TGase 1 (Fig. 7*B*, *lane 1*) and with TGase 3 (Fig. 7*B*, *lane 3*) and then add TGase 5 (Fig. 7*B*, *lanes 2* and *4*), the cross-linking pattern does not change significantly. The same phenomena are observed if we perform cross-linking reactions first using TGase 5 (Fig. 7*B*, *lane 5*) and then adding TGase 1 and TGase 3 (Fig. 7*B*, *lanes 6* and *7*).

We also performed cross-linking and double cross-linking experiments using recombinant SPR3 as substrates. Resolving the cross-linking reactions by SDS-polyacrylamide gel electrophoresis and Western blotting showed that TGase 5, like loricrin, utilizes recombinant SPR3 as complete substrate (Fig. 7*C*). The majority of SPR3 is utilized by forming intrachain cross-linking that generates a fast monomer (see *FM* in Fig. 7*C*, *lanes 2–5*) and a fast dimer (see *FD* in Fig. 7*C*, *lane 5*). TGase 5 also forms interchain cross-links that result in dimer and oligomer formation (see *D* and *O* in Fig. 7*C*). We have already described that TGase 1 and TGase 3 utilize different residues in SPR3 (13). Most likely, there is a well defined temporal order of cross-linking of SPR3 *in vivo*, first TGase 3 followed by TGase 1. At this point, as with loricrin, we wanted to assess the relationship between TGase 5 and the other TGases in the cross-linking pattern of oligomerization. We therefore performed double cross-linking experiments of TGase 1, TGase 3, and TGase 5. *In vitro* double cross-linking experiments suggested that TGase 5 uses different glutamine and lysine residues of SPR3 in comparison with TGase 3 and TGase 1. Indeed, if we cross-link SPR3 to completion with TGase 1 (Fig. 7*D*, *lane 1*) and with TGase 3 (Fig. 7*D*, *lane 3*) and then add TGase 5 (Fig. 7*D*, *lanes 2* and *4*), the cross-linking patterns do not change. Interestingly, if we perform cross-linking reactions using first TGase 5 (Fig. 7*D*, *lane 5*) and then adding TGase 1 and TGase 3, we do observe a significant change in the cross-linking pattern (Fig. 7*D*, *lanes 6* and *7*). TGase 1, added after the action of TGase 5, is able to add more intrachain cross-links both at the level of monomer and dimer, therefore forming a fast monomer and fast dimer (Fig. 7*D*, *lane 6*). TGase 3, as well, adds considerably more isopeptide bonds to SPR3. In fact, in addition to the formation of intrachain cross-linking at the monomer level, we have a complex set of interchain and intrachain cross-links that generate dimers, trimers, and high molecular weight oligomers. This observation suggests that TGase 5 cannot perform its catalytic action on SPR3 after the action of TGase 1 and TGase 3, probably because of the physical impediment of the cross-links already formed. The results obtained in the double cross-linking experiments performed both with loricrin and SPR3, suggest therefore that TGase 5, as compared with other TGases, has a specific action toward different substrates.

Kinetics of TGase 5 in Cross-linking Recombinant Loricrin, Involucrin, and SPR3—To obtain quantitative information on cross-linking of specific epidermal substrates, kinetic constants were determined. We decided to evaluate the efficiency of TGase 5 in cross-linking recombinant loricrin, involucrin, and SPR3. By using high concentrations of putrescine, we suppressed the possibility of putrescine oligomerization to loricrin, involucrin, and SPR3. Fig. 8*A* shows that loricrin stays as a putrescine-labeled monomer after 20 min of incubation with the radiolabeled polyamine as revealed by Western blot (Fig. 8*B*). The same results were obtained with SPR3 and involucrin (not shown). The two tested substrates have already been shown to be complete substrates for epidermal TGases (9, 13), donating both the glutamines and the lysines used for cross-linking. Moreover, the estimates of kinetics values are also complicated by the fact that several Gln in the same protein can be used (see the case of loricrin and TGase (3, 9)). Thus the data obtained represent "average data" for the multiple Gln residues used. Comparative kinetic data of TGase 5 in cross-linking loricrin, SPR3, and involucrin are shown in Table I. TGase 5 uses all three of the specific substrates tested in a similar way as shown by the kinetic efficiency values (Kcat/K_m). K_m values also indicate that TGase 5 has a high affinity for these substrates. Comparison of these values with previous K_m data for TGase 1, TGase 3 and TGase 2 shows that TGase 5 has about a 2.5-fold higher affinity than TGase 1 and TGase 2 (respectively, 18.7 and 18.1 μM) for SPR3 and 1.3-fold higher affinity than TGase 3 (10.5 μM). For loricrin, TGase 5 has about a 3.5-fold higher affinity than TGase 1 and TGase 2 (respectively, 16.9 and 16.0 μM) and a similar affinity to TGase 3 (5.0 μM). Finally, for involucrin, we estimated that TGase 5 has about a 30-fold higher affinity than for full-length TGase 1 (114 μM), whereas TGase 5 affinity for involucrin is similar to TGase 1 when the latter is included in lipid vesicles (2.3 μM (31)). These findings show that TGase 5 has an efficiency similar to TGase 1 in cross-linking involucrin during keratinocyte differentiation.

Conclusion—TGase 5 is a novel enzyme that has not been fully characterized, and so far the only functional information available is that, similar to TGase 1 and TGase 3, it is induced

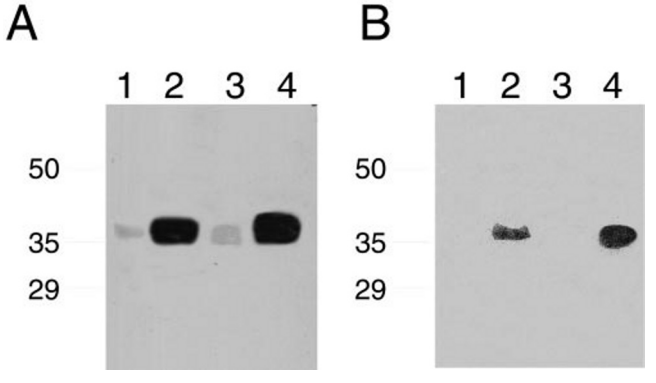

FIG. 8. **TGase 5 affinity for loricrin.** The full-length baculovirus TGase 5 was used for the present experiments. Control experiments for kinetic conditions (*A* and *B*) were performed by incubating loricrin (1.2 μM, *lanes 1* and *3*; 7 μM, *lanes 2* and *4*) with two concentrations of putrescine (respectively, 104 μM, *lanes 1* and *2*, and 208 μM, *lanes 3* and *4*) for 20 min in the presence of Ca^{2+}. No detectable oligomerization of the substrate was observed by Western blotting (*A*) and autoradiography (*B*), indicating that under these conditions only putrescine is used as acceptor substrate and not loricrin endogenous lysines. [^{3}H]putrescine-labeled loricrin is detectable after 10 days of autoradiography only for the higher concentration (*lanes 2* and *4*) of substrate.

TABLE I
Kinetic parameters of cross-linking of recombinant loricrin, involucrin, and SPR3 by TGase 5

	K_m	K_{cat}	K_{cat}/K_m	V_{max}
	μM	min^{-1}	$min^{-1} \cdot \mu M^{-1}$	$pmol \cdot min^{-1}$
SPR3	7.7 ± 2.6	0.26 ± 0.07	0.035 ± 0.01	2.6 ± 0.7
Loricrin	4.4 ± 1.3	0.21 ± 0.10	0.054 ± 0.02	2.1 ± 1.0
Involucrin	3.7 ± 1.4	0.14 ± 0.06	0.038 ± 0.01	1.4 ± 0.6

by calcium treatment of cultured keratinocytes (8). Therefore, we focused our attention on the possible biochemical role that it may play during keratinocyte differentiation and CE assembly *in vitro*. First, TGase 5 transcript is induced by differentiating agents in normal keratinocytes, whereas treatment with retinoic acid has no (or possibly inhibitory) effects. Second, evaluation of the kinetic constants for TGase 5 in cross-linking the specific epidermal substrates loricrin, involucrin, and SPRs demonstrated that this enzyme uses the three specific substrates tested in a similar way and with high affinity as shown by the kinetic efficiency (Kcat/K_m) and K_m values. A comparison of the K_m values for these substrates with previous data measured for TGase 1, TGase 3 and TGase 2 (9, 13, 16) indicated that TGase 5 enzyme has comparable affinity with loricrin, involucrin, and SPR3. Moreover, the fact that in the double cross-linking experiments performed both with loricrin and SPR3, TGase 5 had a specific action toward these epidermal substrates as compared with TGase 1 and TGase 3, and that TGase 5 is differently regulated during *in vitro* keratinocyte differentiation, being expressed at highest level within 24 h, led us to hypothesize that TGase 5 may play a role in keratinocytes differentiation in parallel to TGase 1 and TGase 3.

However, other data make the interpretation more complex. In cultured keratinocytes, over-expressed TGase 5 is detected in the cytosol as a granular deposition, where it is mainly localized perinuclearly. It is co-localized with the vimentin IF network or with an insoluble IF-associated protein that interacts with vimentin. This observation is supported by the findings that TGase 5 is retained in the IF-enriched fraction obtained by cellular fractionation, using nonionic detergent containing high ionic strength solution, both in NHEK and Sf9 cells, and also by the solubility features of the enzyme. The only data available on the association between the TGases and cytoskeleton concern the detection of a TGase-like antigen tightly bound to the vimentin IF network in fibroblasts (22, 33), which could possibly be due to TGase 5. In addition, many cytoskeleton elements and proteins associated with the IF network are substrates for TGases (34–36), including vimentin (37); and here we report that TGase 5 is able to use, at least *in vitro*, vimentin as acyl donor substrate.

The fact that the vimentin network is expressed only in cultured keratinocytes and not in the epidermis (32) suggests that, beside its possible involvement in keratinocyte differentiation, TGase 5 could have an additional role in cells normally expressing vimentin where TGase 5 is also present. TGase 5 has, in fact, also been detected in other cell lines (dermal fibroblast, osteosarcoma, eritroleukemia (8)) and in other tissues (data not shown).

In conclusion, the data reported here suggest that TGase 5 may be a very complex enzyme at the functional level and that its main role could be related to its association with cytoskeleton components both in keratinocytes and in other tissues.

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