*J. Biochem.* **125**, 1048-1054 (1999)

# Characterization of Recombinant Mouse Epidermal-Type Transglutaminase (TGase 3): Regulation of Its Activity by Proteolysis and Guanine Nucleotides

**Kiyotaka Hitomi,*,[1] Shinya Kanehiro,* Koji Ikura,† and Masatoshi Maki***

**Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601; and* †*Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Kyoto 606-8585*

Received January 27, 1999; accepted March 9, 1999

**Epidermal-type TGase (TGase 3) is involved in the formation of the cornified cell envelope by cross-linking a variety of structural proteins in the epidermis. Unknown proteases activate this enzyme from the zymogen form by limited proteolysis during epidermal differentiation. It has been difficult to isolate sufficient quantities of native enzymes from tissues for biochemical studies of the properties of TGase 3. In this paper, we circumvented these problems by expressing recombinant full-length mouse TGase 3 in a baculovirus system, and purifying it to homogeneity by successive chromatography and HPLC. Treatment of the purified recombinant protein with dispase, a bacterial protease known to activate zymogens, produced activated TGase 3. The migration of TGase 3 zymogen in SDS-polyacrylamide gel electrophoresis was anomalous when the proTGase 3 was pre-incubated with calcium ion. GTP inhibited the enzymatic activity of recombinant TGase 3. Calpain, a calcium-dependent neutral protease, was a candidate protease, but had no effect on the activation of TGase 3 zymogen.**

**Key words: baculovirus, calcium, calpain, guanine nucleotide, transglutaminase.**

Transglutaminases [EC 2.3.2.13] are $Ca^{2+}$-dependent enzymes that catalyze the post-translational modification of proteins by transamidating the available glutamine residues. This reaction results in the formation of $\varepsilon$-($\gamma$-glutamyl)lysine cross-links of isopeptide bonds either within or between polypeptides. TGases constitute a large protein family and are distributed in various tissues and cells (*1, 2*).

In the epidermis, four TGases have been reported to exist in different amounts. Two of these are TGase 1 (TGase K) and TGase 3 (TGase E), which are involved in the formation and assembly of the cornified cell envelope by cross-linking various structural proteins in terminally differentiated keratinocytes (*3*). The third enzyme, TGase 2 (TGase C), stabilizes the dermoepidermal junction in the basal cell layer (*4*). Recently, a fourth novel member (TGase X) was discovered in human keratinocytes, in experiments taking advantage of homologous sequences in the TGase family using reverse transcription-PCR (*5*).

TGase 3 is expressed in the last stage of terminal differentiation of the epidermis (*6, 7*) and the inner root sheath and the medullary layers in hair follicles (*8*), but is not expressed in cultured keratinocytes (*9*). TGase 3 accounts for more than 75% of the total TGase activity in the epidermis, although there is far less TGase 3 than TGase 1 and TGase 2 (*10*). Both TGase 1 and TGase 3 are expressed in differentiated epidermal cells, but they seem to have different functions. For example, TGase 3 primarily catalyzes the intrachain cross-linking of the structural protein loricrin, whereas TGase 1 forms larger oligomeric complexes by interchain cross-links (*11*). TGase 3 cannot compensate for cell envelop formation in patients with lamellar ichthyosis, an autosomal recessive disorder of cornification, where normal TGase 1 activity is absent (*12-14*). Although both enzymes are also expressed in hair follicles, TGase 3, rather than TGase 1, preferentially cross-links the major structural proteins such as trichohyalin and keratin intermediate filaments (*15*).

TGase 3 is activated from its protype by limited proteolysis as in the case of TGase 1 (*16*) and Factor XIII (*17, 18*) . Native proTGase 3 from guinea pig skin was purified and partially characterized by Kim *et al.* (*10*). The cDNAs for human and mouse TGase 3 were cloned by PCR using degenerated primers based on the peptide sequences of the guinea pig enzyme (*9*). Both proteins contain 692 amino acids with a molecular mass about 77 kDa based on the deduced amino acid sequence. The proTGase 3, synthesized as a 77 kDa zymogen, is proteolysed into a 50 kDa component containing the catalytic domain and a 27 kDa molecule upon activation by dispase (bacterial protease), thrombin or trypsin. The proteolysis mechanisms and the enzymes responsible for the *in vivo* activation of TGase 3 are not understood. Among various intracellular proteases, calpain ($Ca^{2+}$-dependent neutral protease) had been a candidate because calcium ions dominate in keratinocyte differentia-

[1] To whom correspondence should be addressed. Tel: +81-52-789-5541, Fax: +81-52-789-5542, E-mail: hitomi@agr.nagoya-u.ac.jp

Abbreviations: TGase, transglutaminase; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; CBZ-Gln-Gly, carbobenzoxy-*l*-glutaminylglycine; CBB, Coomassie Brilliant Blue.

tion (*19*).

Although the mechanisms regulating the activation of the TGase 3 zymogen (proTGase 3) during epidermal differentiation have drawn considerable attention, there is little information available regarding the biochemical properties of human and mouse TGase 3. Since too little TGase 3 is expressed in the skin to allow studies of its biochemical properties and activation mechanisms, it is useful to obtain a recombinant enzyme to investigate the structure, the effects of activating proteases, and the mechanisms of proteolytic activation. The functional expression of proTGase 3 in *Escherichia coli*, however, failed because the recombinant proteins were recovered as inclusion bodies. Therefore, we decided to express recombinant proTGase 3 in a baculovirus-insect cell system. In this system, recombinant baculoviruses are used as vectors to express heterologous genes under the forceful polyhedrin promoter in insect cells, providing correctly folded, modified proteins in large amounts (*20*).

In this study, recombinant proTGase 3 was expressed successfully and purified to homogeneity in amounts sufficient to enable us to obtain novel results. Unexpected intramolecular cross-linking was observed when proTGase 3 was incubated with high concentrations of $Ca^{2+}$, and inhibitory effects of GTP on its enzymatic activity were also discovered. Furthermore, the effects of calpain on the proteolysis and activation of proTGase 3 were examined.

## MATERIALS AND METHODS

*Materials*—Growth medium and its supplement for insect cells were purchased from GIBCO BRL. Dispase (Grade 1) was from Boehringer Mannheim. Carbobenzoxy-l-glutaminylglycine (CBZ-Gln-Gly), the substrate for the TGase assay, was from Peptide Institute (Osaka). $\mu$- and m-Calpains were from Nacalai Tesque (Kyoto). Other reagent grade chemicals were obtained from Sigma, Nacalai Tesque and Wako Pure Chemicals (Osaka).

*Preparation of Recombinant Baculoviruses for the Expression of Wild-Type Mouse ProTGase 3 and Its Active-Site Mutant*—cDNA fragments for mouse proTGase 3 were obtained by a reverse transcription-PCR method using several oligonucleotide primers based on the cDNA sequence previously reported (*9*). A full-length cDNA was constructed in pBluescript SK vector (Stratagene). Mutant proTGase 3 cDNA containing the active site Cys-273 (TGC) replaced by Ser-273 (AGC) was prepared by PCR using a Quick-change Site-Directed mutagenesis kit (Stratagene). Two primers, 5′-CCAGTCCAATTTGGCCAGAGCTGGGTCTTTGC-3′ and 5′-GCAAAGACCCAGCTCTGGCCAAATTGGACTGG-3′ (complementary), were used for the reaction. The nucleotide sequences of the whole wild-type proTGase 3 cDNA and the mutated region of the active site-mutated cDNA were confirmed by an automated fluorescent sequencer ABI PRISM 310 (PE Applied Biosystems). Both cDNAs were cloned into the baculovirus transfer vector pBacPAK9 from Clontech Lab (BacPAKTM Baculovirus expression system) by insertion at the *Xba*I and *Pst*I sites.

Sf9 cells, a clonal isolation of *Spodoptera frugiperda*, were grown at 27°C in IPL-41 medium supplemented with tryptose-phosphate broth, 5% (v/v) fetal bovine serum (Biowhittaker, MD, USA), 50 mg/ml streptomycin, 50 units/ml penicillin, and 250 ng/ml fungizon (GIBCO BRL), either in monolayer or spinner flasks. The constructed plasmid was transfected together with a linearized BacPAK6 virus DNA (Clontech Lab) into Sf9 cells with liposome (Lipofectin, GIBCO BRL). After plaque purification, a single clone of each recombinant virus was amplified and used for protein expression (*20*). For preliminary analysis, Sf9 monolayer cells ($5\times10^5$/35 mm dish) were infected with recombinant baculovirus and incubated for 2 days. For protein expression and purification, cells were grown in a spinner flask (100 ml) to a density of $1\times10^6$ cells/ml and then incubated with a high-titer virus stock at a multiplicity of infection of 2-5. The cells were collected 2 days after infection.

A sample of total cell lysates was prepared by TCA (trichloroacetic acid) fixation. Briefly, the cells were washed and suspended in PBS to which TCA was added at a final concentration of 5%. The TCA-precipitates were dissolved by Urea/DTT/Triton and mixed with SDS-dye buffer.

*Purification of Recombinant Mouse ProTGase 3*—All purification steps except HPLC (SMART system) were performed at 4°C. The infected Sf9 cells ($5\text{-}10\times10^7$) were harvested by centrifugation, washed twice with PBS containing 1 mM EDTA, and lysed with 10 ml of hypotonic buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1 $\mu$M pepstatin, 20 $\mu$g/ml leupeptin]. The cell lysates were centrifuged at $12{,}000\times g$ for 20 min and then at $100{,}000\times g$ for 30 min. The supernatant was dialyzed against buffer A [20 mM Tris/HCl (pH 8.0), 1 mM EDTA, 5 mM 2-mercaptoethanol] and applied to a column containing 5 ml of DEAE-Sephacel (Pharmacia) anion exchange resin, previously equilibrated with buffer A. After washing with 5 column volumes of buffer A, the bound proteins were eluted in a stepwise fashion with buffer A containing 50, 150, and 250 mM NaCl. The fractions eluted with buffer A containing 50 mM NaCl were dialyzed against buffer B [20 mM Tris-acetate (pH 6.0), 1 mM EDTA, 5 mM 2-mercaptoethanol] and applied to a column containing 0.5 ml of heparin-Sepharose CL6B (Pharmacia) equilibrated with buffer B. Stepwise elutions with buffer B containing 50, 150, and 250 mM NaCl were performed after washing the column with 5 volumes of buffer B. The fractions eluted with buffer B containing 150 mM NaCl were collected. The pooled fractions were dialyzed against buffer A, loaded onto MonoQ anion exchange column (SMART-system, Amersham-Pharmacia), and eluted with a linear gradient of 0-200 mM NaCl in buffer A. The fractions (~30 mM NaCl) containing proTGase 3 were pooled and loaded onto a size fractionating column (SMART-system Superdex 200) equilibrated with buffer C [20 mM Tris/HCl (pH 7.0), 50 mM NaCl, 0.5 mM EDTA, 1 mM 2-mercaptoethanol]. The peak fractions for proTGase 3 were collected and used for further experiments.

*Activation by Limited Proteolysis with Dispase*—Activation of the purified recombinant proTGase 3 by proteolysis was conducted with dispase as described by Kim *et al.* (*10*). Incubation was performed at 30°C for 20 min at a protease level of 0.06 unit/ml.

*Measurement of TGase Activity*—A modification of the previously published assay method was used (*21*). This method is based on the measurement of the peptide-bound

gamma-glutamyl hydroxamate formed from carbobenzoxy-*l*-glutaminylglycine (CBZ-Gln-Gly) in the presence of hydroxylamine and the enzyme. Assays were performed at 37°C in 0.2 ml reaction mixtures containing 150 mM Tris/HCl (pH 7.5), 2 mM $CaCl_2$, 8 mM DTT, 30 mM CBZ-Gln-Gly (neutralized to pH 7), and 100 mM hydroxylamine. The reactions were terminated by the addition of an equal volume of a mixture prepared from equal portions of 15% TCA, 2.5 N HCl, and 5% ferric chloride in 0.1 N HCl. The absorbance of the stopped solution was measured at 525 nm. An OD value at 525 nm of 0.29 corresponded to 1 mM of product formed in the reaction mixture.

*Mobility-Shift on SDS-PAGE by Incubation with Calcium Ion*—In most cases, the recombinant purified proTGase 3 was incubated for 5–10 min at 30°C in a solution of 50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, and the indicated final concentration of $CaCl_2$. In the case of varying pH, 50 mM sodium acetate (pH 5.0), sodium phosphate (pH 6.0) and Tris/HCl (pH 7.0–9.0) were used. The incubated solutions were mixed with SDS-dye buffer and heated in boiling water. Samples were analyzed on 7.5% SDS-PAGE and stained by Coomassie Brilliant Blue (CBB).

*Effects of Nucleotides on TGase Activity*—Each nucleotide (disodium salt) was dissolved in pure water and neutralized by the addition of NaOH. Nucleotide solutions had no effect on the color development of the reaction between ferric ion and CBZ-gamma-glutamyl hydroxamate (reaction products in the TGase assay) in the reaction mixture. Each nucleotide solution was pre-incubated with activated TGase 3 for 10 min at 37°C and the enzymatic activity was measured as described above.

*Digestion of ProTGase 3 with Calpain*—The purified proTGase 3 was digested with porcine erythrocyte $\mu$-calpain or kidney m-calpain. Reaction mixtures containing 20 mM Tris/HCl (pH 7.0), 5 mM cysteine, and 2 mM $CaCl_2$ were incubated for 30 min at 30°C, and analyzed for TGase activity.

## RESULTS

*Expression and Purification of Full-Length Mouse ProTGase 3 in Baculovirus-Infected Sf9 Cells* Since attempts to express the proTGase 3 protein in bacteria resulted in the formation of insoluble inclusion bodies, a baculovirus expression system was used to circumvent this problem. Insect cells were infected with recombinant baculovirus encoding full-length mouse proTGase 3 cDNA under the control of the polyhedrin promoter. The maximum expression level was observed from 36 to 48 h post-infection at a multiplicity of infection of 2–5.

Although large amounts of the 77-kDa recombinant proTGase 3 protein were expressed in infected Sf9 cells, less than one-third of the proTGase 3 produced was recovered in the soluble fraction (Fig. 1, lanes 1 and 2). For purification, the suspension-cultured cells were collected and lysed with hypotonic buffer containing protease inhibitors. ProTGase 3 was purified from the lysate by DEAE-Sephacel chromatography and heparin-Sepharose affinity chromatography based on the content of the 77-kDa band. To obtain highly purified proTGase 3, contaminating proteins in the fraction were excluded by anion ion-exchange chromatography and size fractionation chromatography (SMART system). The molecular mass estimated with Superdex 200 was about 85 kDa, suggesting that the

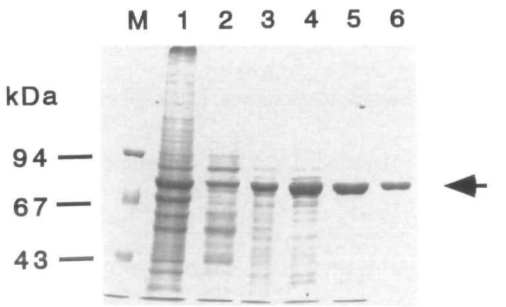


Fig. 1. **Purification of mouse proTGase 3 expressed in Sf9 cells.** Approximately 1–4 $\mu$g of proteins at each step from baculovirus-infected Sf9 cells (lanes 1–6) was separated by 7.5% SDS-PAGE and the gel was stained with CBB. Molecular mass markers (Lane M), total cellular extract (lane 1), soluble fraction (applied sample for DEAE chromatography) (lane 2), the DEAE-Sephacel chromatography eluate (lane 3), the heparin-Sepharose affinity chromatography eluate (lane 4), the peak fraction from anion-exchange chromatography (Mono Q) (lane 5), the peak fraction from size separation (Superdex 200) (lane 6). The arrow indicates the 77 kDa mouse proTGase 3.

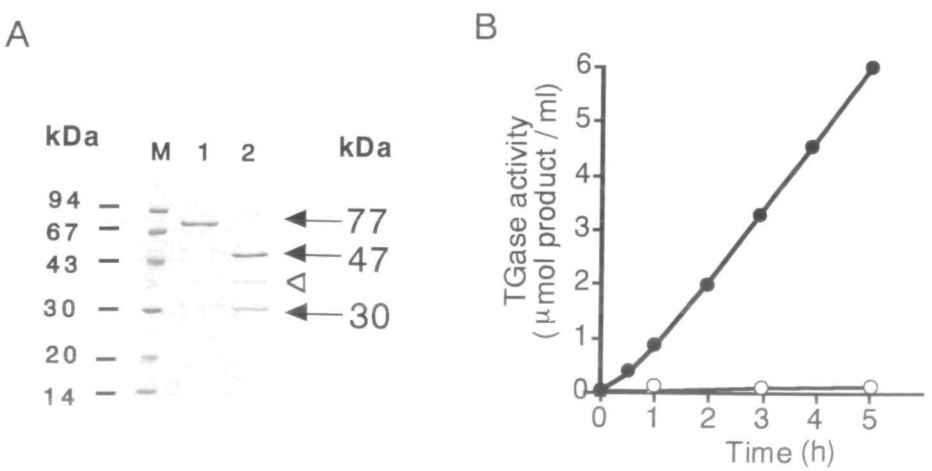


Fig. 2. **Activation of recombinant purified proTGase 3.** (A) 12.5% SDS-PAGE analysis of approximately 2 $\mu$g of purified proTGase before (lane 1) and after (lane 2) digestion with 3 milliunits of dispase in a solution containing 50 mM Tris/HCl (pH 7.0) for 20 min at 30 C. The processed bands (47 and 30 kDa) are indicated by arrows. Lane M, molecular mass markers. The open arrowhead indicates the point where dispase was added to the reaction mixture. (B) The enzymatic activity of proTGase 3 before (open circles) and after (closed circles) proteolysis by dispase was assayed as described in "MATERIALS AND METHODS." The reaction was continued up to 5 h. TGase activity is expressed as $\mu$mol of products ml of the reaction.

purified proTGase 3 was in a monomeric form. The SDS-PAGE profile of the total protein at each step of purification is shown in Fig. 1. After these purification steps, approximately 70 $\mu$g of proTGase 3 was obtained from 100 ml of Sf9 cells suspension culture ($2\times10^8$).

*Activation of Recombinant Mouse ProTGase 3*—To confirm that the purified recombinant proTGase 3 had enzymatic activity, proteolytic activation was performed by protease digestion according to the previous report by Kim *et al.* (*10*). The ability of dispase, a neutral bacterial protease that has been used to activate guinea pig proTGase 3, to activate the recombinant proTGase 3 was examined. As shown in Fig. 2A, the 77-kDa proTGase 3 was proteolysed into 47- and 30-kDa molecules by dispase treatment. The smaller fragment migrated to a slightly larger position than the corresponding guinea pig TGase 3 fragment. The TGase activity of both the digested and undigested proTGase 3 preparations was measured using CBZ-Gln-Gly and hydroxylamine as substrates. The proteolysed TGase 3 had apparent transamidating activity, whereas no enzymatic activity was observed for the untreated preparation (Fig. 2B). These results were also confirmed by the observation of cross-linking of bovine caseins and the incorporation of radiolabeled polyamine into dimethyl casein (data not shown).

*Incubation of ProTGase 3 with a High Concentration of $Ca^{2+}$ Affects Its Mobility on SDS-PAGE Mediated by TGase Activity*—During the course of purification, we observed that treatment with a high concentration of $Ca^{2+}$ caused a mobility-shift of the proTGase 3 on SDS-PAGE (Fig. 3, A and B). The 77-kDa molecule migrated slightly faster when incubated in a solution containing $Ca^{2+}$ at 30°C for 5 min. The rate of migration depended on the pH of the solution during incubation (Fig. 3A) and on the $Ca^{2+}$ concentration (Fig. 3B). Furthermore, the mobility-shift was almost completely inhibited by the presence of *N*-ethyl maleimide, a sulfhydryl alkylating reagent (data not shown). These results raised the possibility that the TGase activity of recombinant proTGase 3 might be responsible for the aberrant migration, because the enzyme reacts at a

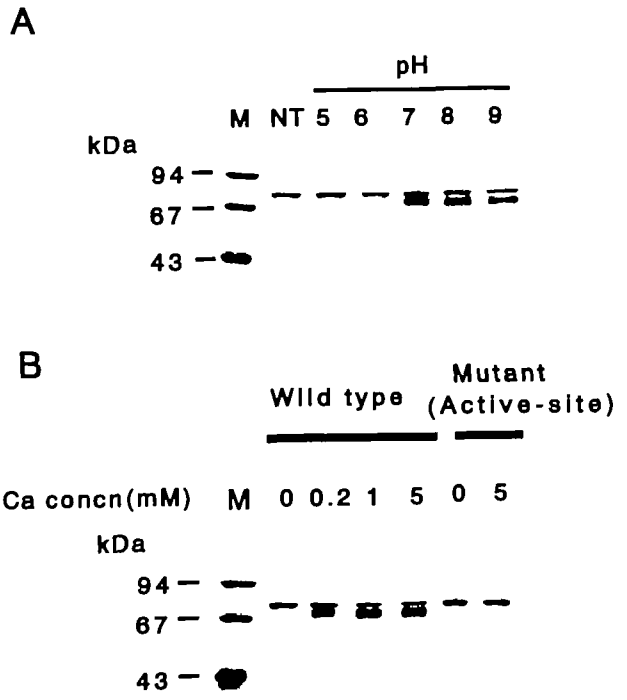


Fig. 3. **Mobility-shift of proTGase 3 in the presence of high $Ca^{2+}$ concentrations.** Purified proTGase 3 was incubated with $Ca^{2+}$ for 10 min at 30°C and then with an equal volume of SDS-buffer and boiling for 7.5% SDS-PAGE analysis. M: molecular mass marker. (A) Incubation in a solution containing buffer [50 mM sodium acetate (pH 5), 50 mM sodium phosphate (pH 6), or 50 mM Tris/HCl (pH 7, 8, 9)] at a final concentration of 1 mM of $CaCl_2$. NT means untreated proTGase 3. (B) Incubation at a final concentration of 0, 0.2, 1, or 5 mM of $CaCl_2$. The purified active-site mutant of proTGase 3 was incubated simultaneously under fixed $Ca^{2+}$ concentration (5 mM).

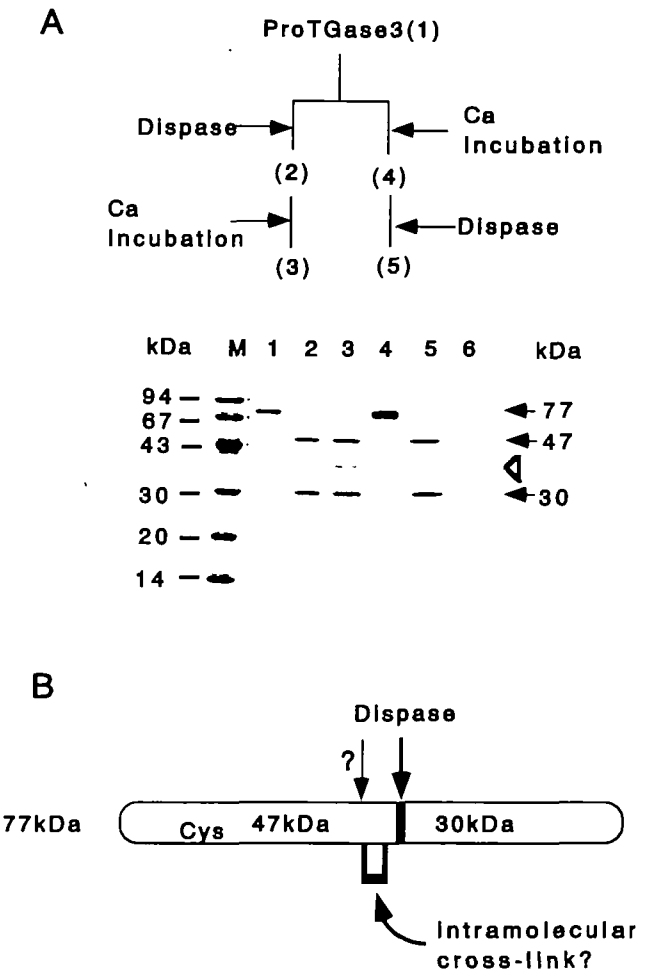


Fig. 4. **Dispase-treatment and $Ca^{2+}$-dependent mobility-shift of proTGase 3.** (A) Scheme of the reaction for dispase treatment and incubation with high $Ca^{2+}$ concentrations (above). The reacted solutions were separated by 12.5% SDS-PAGE (below). Molecular mass markers (lane M), purified proTGase 3 (lane 1), dispase-treated TGase 3 (lane 2), dispase-treatment followed by incubation with $CaCl_2$ (lane 3), incubation with $CaCl_2$ (lane 4), incubation with $CaCl_2$, followed by digestion with dispase (lane 5). Incubation in $CaCl_2$ solution at a final concentration of 5 mM and treatment with dispase were performed at 30°C for 10 min and 20 min, respectively. In lane 6, only the dispase solution was applied. The open arrowhead indicates the dispase as described in the legend to Fig. 2A. (B) Predicted reaction model of the TGase 3 zymogen treated with $Ca^{2+}$ and dispase. Cys means the active site of TGase 3. The bold arrow indicate the putative cleavage site of proTGase 3. The parallel arrow indicates possible attacked site by dispase.

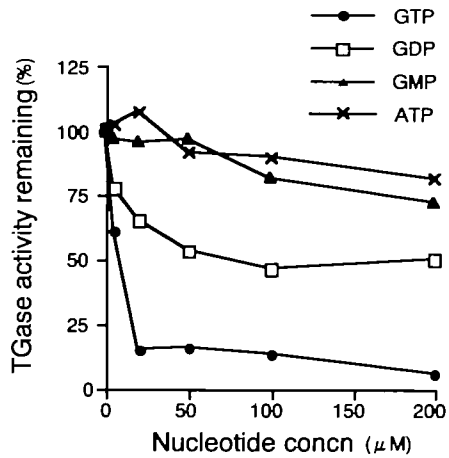


Fig. 5. **Effects of GTP, GDP, GMP, and ATP on mouse TGase 3 activity.** Increasing concentrations of GTP (●), GDP (□), GMP (▲), and ATP (×) were preincubated for 10 min with activated mouse recombinant TGase 3 (2 μg) before the TGase reaction for 30 min as described under the "MATERIALS AND METHODS." The remaining enzymatic activities were calculated based on the amount of products without nucleotide.

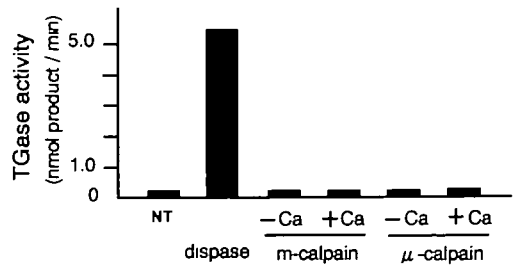


Fig. 6. **Lack of proteolytic activation of proTGase 3 by m- and μ-calpains.** Recombinant proTGase 3 (2 μg) was digested with 3 milliunits of dispase, or 1 μg of m- or μ-calpain in the presence or absence of 2 mM $CaCl_2$. All digestive reactions were performed for 30 min at 30°C. The TGase activities of the reacted solutions were measured as described in "MATERIALS AND METHODS" by incubation with substrates for 60 min. NT means the activity of proTGase 3 without protease treatment. The TGase activity is expressed as nmol of products/ml.

higher pH and has an active site cysteine residue. To verify that the $Ca^{2+}$ dependent mobility-shift was attributed to the enzymatic activity, an active-site mutant of proTGase 3 was prepared in the baculovirus system and compared with the wild type. The mobility of the mutant proTGase 3 was not affected by the presence of high $Ca^{2+}$ concentrations (Fig. 3B). These results suggest that intrinsic TGase activity was responsible for the aberrant mobility caused by $Ca^{2+}$.

Furthermore, successive reactions with protease and $Ca^{2+}$ were performed in order to compare the sizes of the fragments obtained (Fig. 4A, above). The sizes of the two dispase-treated proTGase 3 fragments were not affected by incubation with $Ca^{2+}$ (Fig. 4A, lanes 2 and 3). When $Ca^{2+}$-treated proTGase 3 containing aberrant bands (Fig. 4A, lane 4) was digested with dispase, the fragments migrated to positions corresponding to the 47- and 30-kDa dispase-treated fragments and no aberrations were observed (Fig. 4A, lane 5). These results suggest that the aberrant migration was caused by a structural change in the zymogen, and not by proteolytic processing in either the N-terminal or C-terminal regions.

*Inhibition of TGase 3 Activity by Nucleotides*—Tissue-type TGase (TGase 2) has recently been reported to be a GTP-binding protein and its enzymatic activity is specifically blocked by the presence of GTP. This led us to examine the effect of GTP on the enzymatic activity of TGase 3. As shown in Fig. 5, both GTP and GDP were found to cause concentration-dependent inhibition of TGase 3 activity at 25-200 μM. The concentrations of GTP and GDP required for 50% inhibition were 10 and 50 μM, respectively. Higher levels of ATP and GMP were necessary to inhibit the enzymatic activity. Unlike TGase 2, increasing concentrations of $Ca^{2+}$ did not block the inhibition of GTP (data not shown). These results were reproduced by the incorporation of radiolabeled polyamine into dimethyl casein (data not shown).

*Inability of μ- and m-Calpains to Activate ProTGase 3*—The protease responsible for the activation of the TGase 3 zymogen *in vivo* remains unknown. Since the cellular $Ca^{2+}$ concentration controls epidermal differentiation *in vitro*, the ability of calpain, a $Ca^{2+}$-dependent neutral protease, to activate the zymogen was examined. Ubiquitous mammalian calpain exists as two isozymes with different $Ca^{2+}$ sensitivities: μ-calpain is activated at micromolar levels of $Ca^{2+}$ and m-calpain is activated at millimolar levels of $Ca^{2+}$. Recombinant TGase 3 was incubated with μ- and m-calpain in the presence of $Ca^{2+}$ for 30 min at 30°C with sufficient amounts of calpain to completely digest the casein. The TGase activities of the reacted solution were measured for transamidating activity (Fig. 6). The size of the 77-kDa proTGase 3 protein remained unaltered following incubation with μ- or m-calpain (data not shown) and no increase in enzymatic activity was observed. These results suggest that calpains are not involved in the proteolytic activation of TGase 3 *in vivo*.

## DISCUSSION

Since proTGase 3 expressed in *E. coli* was harvested as an insoluble fraction and denaturation/renaturation procedures to recover the enzymatic activity have not yet been established, the baculovirus expression system was used to produce the recombinant mouse zymogen form of TGase 3. Generally, this system has major advantages for the expression of recombinant mammalian proteins compared to bacterial expression vectors, because of the efficient production of a soluble form. Even in the baculovirus system, however, most of the recombinant proTGase 3 was not recovered in the soluble fraction, suggesting that the protein is likely insoluble. Since the expression levels in infected-Sf9 cells were significantly high, sufficient purified protein was readily obtained from the soluble fractions by two successive chromatography and HPLC steps (Fig. 1). The 47- and 30-kDa fragments were obtained upon proteolysis with dispase (Fig. 2), as was reported for the native enzyme from guinea pig. Furthermore, the digested proTGase 3 had enzymatic activity, implying that the recombinant proteins were correctly folded (Fig. 2B). The observed size of the smaller fragment (30 kDa protein) was slightly larger than the one reported (27 kDa) by Kim *et al.* (*10*), probably due to differences in the primary structures

of guinea pig and mouse TGase 3.

The aberrant migration of proTGase 3 on SDS-PAGE after incubation with high concentrations of $Ca^{2+}$ was an unexpected phenomenon. Incubation induced the mobility-shift without intermolecular aggregation (Fig. 3). Treatment of TGase 2 (tissue-type guinea pig TGase) under the same conditions produced larger molecules by intermolecular cross-linking, but did not result in a mobility-shift (data not shown). It is unlikely that the mobility-shift was the result of proteolytic degradation by contaminating proteases or processing by proTGase 3 itself for the following reasons: (i) Protease inhibitors of serine and cysteine proteases as well as calpastatin (a protein that inhibits calpain) could not prevent the aberrant mobility (data not shown). (ii) The recombinant protein of a similarly purified active-site mutant of proTGase 3 was not affected by $Ca^{2+}$ treatment (Fig. 3B). (iii) The N-terminal amino acid residue of the shifted band was blocked (probably acetylated as observed in the case of TGase 2) in the untreated proTGase 3. This indicates that the N-terminal of the shifted band remained unaltered. (iv) The C-terminal structure was also unchanged. After the TGase 3 zymogen was treated with dispase, the sizes of the fragments obtained (47 and 30 kDa) remained identical, even following incubation with $Ca^{2+}$. On the other hand, the proTGase 3 pre-incubated with $Ca^{2+}$ was proteolysed into 47 and 30 kDa fragments as in the case of proTGase 3 not treated with $Ca^{2+}$ (Fig. 4A).

Therefore, the mobility-shift was assumed to result from intramolecular cross-linking leading to structural changes in the TGase 3 zymogen (Fig. 4B). However, it is unclear why the intramolecularly cross-linked zymogen split into fragments with sizes similar to those derived from the untreated zymogen. Although the dispase cleavage site of mouse TGase 3 is presumed to be at Ser-471 (bold arrow in Fig. 4B), as is the reported cleavage site in guinea pig TGase 3 (*9*), other sites in the immediate vicinity might be also cleaved, thereby removing the intramolecular cross-linked region. Identifying the cross-linking sites would clarify the mechanism for generating heterogenous zymogens by $Ca^{2+}$-treatment.

Kim *et al.* reported that raising the concentration of $Ca^{2+}$ to 100 mM in the absence of a reductant increased the TGase 3 activity of the zymogen (*10*); however, our results were not reproducible. The $Ca^{2+}$-activated minor TGase activity of the zymogen might act on the enzyme molecule itself, but this would not be detected by our assay system. Although the physiological consequences of this phenomenon remain unknown, unmasked TGase might participate in transamidation upon the entry of $Ca^{2+}$ into a cell or its release from intracellular stores.

The inhibition of TGase 3 enzymatic activity by GTP was also an unexpected result. Inhibitory regulation of activity by GTP appears to be a characteristic feature of TGase 2, whereas the activities of TGase 1 and Factor XIII are reported to be unaffected by GTP (*22*). GTP acts as a noncompetitive inhibitor of TGase 2 activity, and is also hydrolyzed to GDP by TGase 2 (*23*). In our experiments, the effective inhibitory concentration of the nucleotide was similar to that of TGase 2. Furthermore, weak inhibition by GMP and ATP was observed, as in the case of TGase 2 (*22, 24*). This inhibition was not the result of the chelating effect of GTP on $Ca^{2+}$-dependent enzymatic activity, because ATP showed weak inhibition at the same concentration. Typical GTP-binding motifs are not seen in the primary sequence of proTGase 3 as in the case of TGase 2 (*25*). The following characteristics of inhibition, however, differ from those of TGase 2. First, TGase 3 did not show GTPase activity. Neither proTGase 3 nor TGase 3 showed GTPase activity; both failed to hydrolyze $^{32}$P-GTP (Hitomi, K., unpublished observation). Second, the inhibition was not affected by the concentration of $Ca^{2+}$, whereas higher $Ca^{2+}$ concentrations blocked the inhibition of TGase 2 activity by GTP, although it has been reported that local concentrations of GTP and $Ca^{2+}$ regulate intracellular TGase 2 activity (*26, 27*). Intracellular GTP might regulate TGase 3 activity by mechanisms distinct from those for TGase 2. Recently, *in situ* analysis showed that the presence of GTP prevented the $Ca^{2+}$-dependent proteolysis of TGase 2 (*28*). The effects of GTP on the degradation of proTGase 3 by various proteases are under investigation.

The proteases responsible for the *in vivo* activation of proTGase 3 remain unknown. In keratinocyte culture, increasing $Ca^{2+}$ concentration induces the onset of terminal differentiation (*29*). Calpain represents a major class of non-lysosomal intracellular proteases that function in a $Ca^{2+}$-dependent fashion (*30*) and has shown to be activated in cultured keratinocytes upon $Ca^{2+}$ treatment (*31*). Therefore, calpain has been proposed as a candidate for the activation of proTGase 3. Ando *et al.* reported that the calpain treatment enhanced the enzymatic activity of partially purified TGase from porcine skin, although the TGase fraction used in their paper could not be ascribed to TGase 1 or TGase 3 (*19*). As shown in Fig. 6, the digestion of recombinant proTGase 3 with either calpain did not activate proTGase 3. The predicted structural features of TGase 3 based on the amino acid sequence of TGase 3 suggest that the 47- and 30-kDa molecules are separated by a flexible hinge region (*9*). This region is considered to be highly sensitive to proteases, because activation *in vitro* can be induced by several proteases including dispase, thrombin, trypsin, and proteinase K (*10*). The failure of calpain to activate proTGase 3 suggests that proTGase 3 might be cleaved by other specific intracellular proteases. In the epidermis and cultured keratinocytes, various proteases have been demonstrated to participate in differentiation and skin repair (*32-34*). Serine protease and calpain convert filaggrin, the intermediate filament-associated protein of the cornified epithelia, from its latent form by proteolysis (*31, 35*). Caspases are also candidates because epidermal differentiation involves the apoptosis of keratinocytes. Since we have produced enough recombinant proTGase 3 protein to search for and test proteases responsible for the activation, work in this area is in progress.

We thank Yuko Horio and Takamasa Toyoda for technical assistance, and Mr. Shigemichi Nishikawa (Wakenyaku, Co., Ltd.) for providing us with insect cells and technical advice.

## REFERENCES

1. Greenberg, C.S., Birckbichler, P.J., and Rice, R.H. (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* **5**, 3071-3077
2. Aeschlimann, D. and Paulsson, M. (1994) Transglutaminase: protein cross-linking enzymes in tissues and body fluids. *Thrombosis Haemostasis* **71**, 402-415

3. Rice, R.H., Rong, X., and Chakravarty, R. (1990) Proteolytic release of keratinocyte transglutaminase. *Biochem. J.* **265**, 351-357
4. Lichti, U., Ben, T., and Yuspa, S.H. (1985) Retinoic acid-induced transglutaminase in mouse epidermal cells is distinct from epidermal transglutaminase. *J. Biol. Chem.* **260**, 1422-1426
5. Aeschlimann, D., Koeller, M.K., Allen-Hoffmann, B.L., and Mosher, D.F. (1998) Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. Detection and identification of transglutaminase gene products based on reverse transcription-polymerase chain reaction with degenerate primers. *J. Biol. Chem.* **273**, 3452-3460
6. Kim, S.-Y., Chung, S.-I., and Steinert, P.M. (1995) Highly active soluble processed forms of the transglutaminase 1 enzyme in epidermal keratinocytes. *J. Biol. Chem.* **270**, 18026-18035
7. Lee, J-H., Jang, S-I., Yang, J-M., Markova, N.G., and Steinert, P.M. (1996) The proximal promoter of the human transglutaminase 3 gene. Stratified squamous epithelial-specific expression in cultured cells is mediated by binding of sp1 and ets transcription factors to a proximal promoter element. *J. Biol. Chem.* **271**, 4561-4568
8. Lee, S.-C., Kim, I.-G., Marekov, L.N., O'Keefe, E.J., Parry, D.A., and Steinert, P.M. (1993) The structure of human trichohyalin. Potential multiple roles as a functional EF-hand-like calcium-binding protein, a cornified cell envelope precursor, and as an intermediate filament-associated protein. *J. Biol. Chem.* **268**, 12164-12176
9. Kim, I.-G., Gorman, J.J., Park, S.-C., Chung, S.-I., and Steinert, P.M. (1993) The deduced sequence of the novel protransglutaminase E (TGase 3) of human and mouse. *J. Biol. Chem.* **268**, 12682-12690
10. Kim, H.C., Lewis, M.S., Gorman, J.J., Park, S.-C., Girard, J.E., Folk, J.E., and Chung, S.-I. (1990) Protransglutaminase E from guinea pig skin. Isolation and partial characterization. *J. Biol. Chem.* **265**, 21971-21978
11. Candi, E., Melino, G., Mei, G., Tarcsa, E., Chung, S.-I., Marekov, L.N., and Steinert, P.M. (1995) Biochemical, structual and transglutaminase substrate properties of human loricrin the major epidermal cornified cell envelope protein. *J. Biol. Chem.* **270**, 26382-26390
12. Jeon, S., Djian, P., and Green, H. (1998) Inability of keratinocytes lacking their specific transglutaminase to form cross-linked envelope: Absence of envelops as a simple diagnostic test for lamellar ichthyosis. *Proc. Natl. Acad. Sci. USA* **95**, 687-690
13. Matsuki, M., Yamashita, F., Ishida-Yamamoto, A., Yamada, K., Kinoshita, C., Fushiki, Ueda, E., Morishita, Y., Tabata, K., Yasuno, H., Hashida, M., Iizuka, H., Ikawa, M., Okabe, M., Kondoh, G., Kinoshita, T., Takeda, J., and Yamanishi, K. (1998) Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1. *Proc. Natl. Acad. Sci. USA* **95**, 1044-1049
14. Candi, E., Melino, G., Lahm, A., Ceci, R., Rossi, A., Kim, I.-G., Ciani, B., and Steinert, P.M. (1998) Transglutaminase 1 mutation in lamellar ichthyosis. Loss of activity due to failure of activation by proteolytic processing. *J. Biol. Chem.* **273**, 13693-13702
15. Tarcsa, E., Marekov, L.N., Andreoli, J., Idler, W.W., Candi, E., Chung, S.-I., and Steinert, P.M. (1997) The fate of trichohyalin. Sequential post-translational modification by peptidyl-arginine deiminase and transglutaminase. *J. Biol. Chem.* **272**, 27893-27901
16. Kim, S.-Y., Chung, S.-I., and Steinert, P.M. (1995) Highly active soluble processed forms of transglutaminase 1 enzyme in epidermal keratinocytes. *J. Biol. Chem.* **270**, 18026-18035
17. Takahashi, N., Takahashi, Y., and Putnam, F.W. (1986) Primary structure of blood coagulation factor XIIIa (fibrinoligase). *Proc. Natl. Acad. Sci. USA* **83**, 8019- 8023
18. Ichinose, A., Bottenus, R.E., and Davie, E.W. (1990) Structure of transglutaminase. *J. Biol. Chem.* **265**, 13411-13414
19. Ando, Y., Imamura, S., Murachi, T., and Kannagi, R. (1988) Calpain activates two transglutaminase from porcine skin. *Arch. Dermatol. Res.* **280**, 380-384
20. Richardson, C.D. (1995) *Baculovirus Expression Protocols*, Human Press, Totowa, New Jersey
21. Folk, J.E. (1970) Transglutaminase (Gunea pig liver). *Methods Enzymol.* **174**, 889-895
22. Achyuthan, K.E. and Greenberg, C.S. (1987) Identification of a guanosine riphosphate-binding site on guinea pig liver transglutaminse. Role of GTP and calcium ions in modulating activity. *J. Biol. Chem.* **262**, 1901-1906
23. Lee, K.N., Birckbichler, P.J., and Patterson, M.K. (1989) GTP hydrosis by guinea pig liver transglutaminse. *Biochem. Biophys. Res. Commun.* **162**, 1370-1375
24. Bergamini, C.M., Signorini, M., and Poltronieri, L. (1987) Inhibition of erythrocyte transglutaminase by GTP. *Biochim. Biophys. Acta* **916**, 149-151
25. Takeuchi, Y., Birckbichler, P.J., Patterson Jr., M.K., and Lee, K.N. (1992) Putative nucleotide binding sites of guinea pig liver transglutaminase. *FEBS Lett.* **307**, 177-180
26. Bergamini, C.M. (1988) GTP modulates calcium binding and cation-induced conformational changes in erythrocyte transglutaminase. *FEBS Lett.* **239**, 255-258
27. Smethurst, P.A. and Griffin, M. (1996) Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by $Ca^{2+}$ and nucleotides. *Biochem. J.* **313**, 803-808
28. Zhang, J., Lesort, M., Guttmann, R.P., and Johnson, G.V.W. (1998) Modulation of the *in situ* activity of tissue transglutaminase by calcium and GTP. *J. Biol. Chem.* **273**, 2288-2295
29. Hennings, H., Michael, D., Cheng, C., Steinert, P.M., Holbrook, K., and Yuspa, S.H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**, 245-254
30. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) Structure and physiological function of calpains. *Biochem. J.* **328**, 721-732
31. Yamazaki, M., Ishidoh, K., Suga, Y., Saido, C.H., Kawashima, S., Suzuki, K., Kominami, E., and Ogawa, H. (1997) Cytoplasmic processing of human profilaggrin by active mu-calpain. *Biochem. Biophys. Res. Commun.* **235**, 652-656
32. Skytt, A., Stromqvist, M., and Egelrud, T. (1995) Primary substrate specificity of recombinant human stratum corneum. *Biochem. Biophys. Res. Commun.* **211**, 586-589
33. Zepter, K., Haffner, A., Soohoo, L.F., De Luca, D., and Tang, H.P. (1997) Induction of biologically active IL-1 beta-converting enzyme and mature IL-1. *J. Immunol.* **159**, 6203-6208
34. Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J.P., and Rio, M.C. (1997) Expression of matrix metalloproteinases during rat skin wound healing. *J. Cell. Biol.* **137**, 67-77
35. Resing, K.A., Thulin, C., Whiting, K., Al-Alawi, N., and Mostad, S. (1995) Characterization of profilaggrin endoproteinase 1. A regulated cytoplasmic endoproteinase of epidermis. *J. Biol. Chem.* **270**, 28193-28198