To whom correspondence should be addressed. Fax: +81-52-789-5542; E-mail: hitomi@agr.nagoya-u.ac.jp

**Note**

**Immunological Detection of Proteolytically Activated Epidermal-type Transglutaminase (TGase 3) Using Cleavage-site-specific Antibody**

Kiyotaka Hitomi, Naoki Ikeda, and Masatoshi Maki

Department of Applied Molecular Biological Sciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Received July 11, 2003; Accepted July 30, 2003

Transglutaminase 3 (TGase 3), involved in the cross-linking of structural proteins in the epidermis, is activated by limited proteolysis of zymogen into two fragments during keratinocyte differentiation. Using recombinant TGase 3, the N-terminus sequence of the proteolyzed fragment was analyzed. Antibody against the synthetic peptide corresponding to the cleavage site specifically detected the fragment in the mouse forestomach extract.

Key words: transglutaminase; proteolysis; forestomach; keratinocyte

Transglutaminase (TGase) is an enzyme that catalyzes the Ca^{2+} dependent cross-linking reaction between a γ-carboxyamide group of glutamine and an ε-amino group of lysine or other primary amine.1,2) To date, eight human TGase isozymes (Factor XIII, TGases 1–7) making up a large protein family have been found.3) Among these isozymes, TGase 3 is involved in the formation of the cornified envelope in skin keratinocytes, where TGase functions to cross-link structural proteins such as involucrin, loricrin, and small proline-rich proteins during epidermal terminal differentiation.4) TGase 3 activation requires cleavage of a 77-kDa zymogen by an unknown protease to release two fragments of 47 and 30 kDa which then associate non-covalently to form the active enzyme.5) Although proteases responsible for the proteolytic activation remain unknown, dispase, a protease from *Bacillus polymyxa*, is able to proteolyze the zymogen resulting in activation in vitro.6,7) Dispase has been used for a tissue disaggregation procedure and accidentally found to cleave specifically zymogen of TGase 3. It has been difficult to biochemically clarify the activation mechanisms of TGase 3 because the amounts of the proteolyzed TGase 3 are quite low in the epidermis.

In order to know the proteolytic activation mechanisms, it is crucial to determine the cleavage site of TGase 3 zymogen. The cleavage sites of human and mouse TGase 3 have been predicted based on the data for purified guinea pig protein, but not directly identified.5) This study consists of three parts: (i) determination of the cleavage site, (ii) production of antibody specific for the site and verification of its specificity, and (iii) examination of cross-reactivity with an in vivo fragment.

First, recombinant proteins for human and mouse TGase 3 were purified from baculovirus-infected insect cells and then digested with dispase (Fig. 1A, left). The N-terminal amino acid sequences of 30-kDa fragments were determined by automated Edman degradation. As shown in Fig. 1B, the cleavage sites are situated before Phe-465 both in human and mouse TGase 3.

Next, we generated an antibody that specifically recognized the cleavage site. Based on the determined amino acid sequence, a peptide (Phe-Gly-Ala-Thr-Ser) corresponding to the N-terminal amino acid sequence of the mouse 30-kDa fragment with an additional Cys residue at the C-terminus was synthesized. Then, the Cys residue of the peptide was covalently cross-linked with keyhole limpet hemocyanin (KLH) using *m*-maleimidobenzoil-N-hydroxysuccinimide ester, and used as the immunogen to raise antibody in rabbits. The antibody was affinity-puriﬁed from antisera using a peptide-immobilized column. Further, in order to remove antibody that cross-reacted with TGase 3 zymogen, the antibody solution was passed through a gel immobilizing the acetylated peptide (Ac-Phe-Gly-Ala-Thr-Ser). The puriﬁed antibody (anti-FGATS) predominantly reacted with the 30-kDa fragment, suggesting that it distinguishes the newly exposed N-terminal cleavage site generated by dispase-digestion from the sequence in the zymogen. (Fig. 1A, right).

Then, in order to conﬁrm the in vivo cleavage site, we examined whether the anti-FGATS antibody recognized the 30-kDa fragment in the mouse epithelial tissue. In mouse forestomach, where TGase 3 is expressed as in the case of skin and hair follicle, a

---

† To whom correspondence should be addressed. Fax: +81-52-789-5542; E-mail: hitomi@agr.nagoya-u.ac.jp

Abbreviations: TGase, transglutaminase; CBB, Coomassie Brilliant Blue; KLH, keyhole limpet hemocyanin
Fig. 1. Limited Proteolysis of TGase 3.
A, Recombinant mouse TGase 3, purified from baculovirus-infected insect cells, was digested with dispase. One μg of each protein before (lane 1) and after (lane 2) digestion was subjected to 10% SDS-PAGE, followed by CBB staining (left) and Western blotting using anti-FGATS antibody (right). The white, gray, and black arrows indicate the zymogen form, 47-kDa, and 30-kDa fragment, respectively.

B, Cleavage sites of recombinant proteins for human and mouse TGase 3 proteolyzed by dispase. The amino acid sequence of the N-terminal sites for the 30-kDa fragments of both human and mouse TGase 3, generated by digestion with dispase, are indicated. The arrows before F indicate the cleavage site. The arrows in the dotted line indicate the cleavage sites that had been predicted based on the sequence of purified guinea pig TGase 3. The amino acid sequence in the box corresponds to the synthesized peptide.

Fig. 2. Western Blotting Analysis of the Proteolyzed TGase 3 in the Mouse Forestomach Extract.
Mouse forestomach extract was prepared and TGase 3 was partially purified. The proteins were analyzed by Western blotting using polyclonal antibodies against the zymogen form of mouse TGase 3 (A) and anti-FGATS peptide (B), as described in the legend to Fig. 1B. lane 1, crude extract; lane 2, 0.15 M NaCl elution fraction on DEAE-chromatography (20 mM Tris-HCl, pH 8.0).

A polyclonal antibody raised against TGase 3 zymogen detected both the zymogen and the proteolyzed forms (Fig. 2A). However, probably due to instability, the amount of 30-kDa fragment was much lower than that of the 47-kDa fragment. In order to enrich these molecules in the extract, DEAE anion-exchange chromatography was performed. As shown in lane 2 of Fig. 2A, 30-kDa fragment was detected in the partially purified fraction. When the same sample was analyzed using anti-FGATS antibody, the 30-kDa fragment was apparently detected (Fig. 2B).

In the partially purified sample (lane 2), the band was clearer than that in the crude extract (lane 1). These results suggest that the in vivo cleavage site is at the N-terminal side of Phe-465.

In contrast to the estimated in vivo cleavage site by the immunological reaction with anti-FGATS antibody, Steinert et al. had predicted the putative cleavage site would be between Ser-469 and Ser-470 (arrows in dotted line in Fig. 1B) both in human and mouse TGase 3. To test the possibility of a secondary cleavage site, the antibody specific to the Ser-Arg-Asn-Pro-Glu peptide was prepared in the same manner as that for producing anti-FGATS antibody. This antibody recognizes specifically the N-terminus of the Ser-residue. However, no immunological-reaction was observed for the 30-kDa fragment both in the dispase-digested recombinant TGase 3 and also in the forestomach extract (data not shown).

In our recent report, human TGase 3 appeared to be located in the cytoplasm in the cultured keratinocytes and outer layer of the epidermis by immunohistochemical analysis using monoclonal antibody. The anti-FGATS antibody obtained in this study was not suitable for such analysis because of some cross-reactivity with the zymogen form and also because of the low amounts of activated TGase 3. The anti-FGATS antibody will be useful to monitor the degree of proteolytic activation and to search for the activating proteases by Western blotting.
Acknowledgment

We greatly appreciate the help of Dr. Ryu-ichi Moriyama for protein sequencing. Thanks are also to Dr. Hideki Shibata for fruitful discussion. This work was supported by a Grant-in Aid for Scientific Research No. 14560063 from the Ministry of Education, Science, Sports, and Culture of Japan (to K.H.).

References