Biochemical and Biophysical Research Communications 478 (2016) 343–348

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Analysis on transglutaminase 1 and its substrates using specific substrate peptide in cultured keratinocytes

Miki Yamane, Kayoko Sugimura, Hiroko Kawasaki, Hideki Tatsukawa, Kiyotaka Hitomi*

Graduate School of Pharmaceutical Sciences, Nagoya University, Chikusa, Furoh-cho, Nagoya, 464-8601, Japan

ARTICLE INFO

Article history:
Received 7 July 2016
Accepted 9 July 2016
Available online 11 July 2016

Keywords:
Transglutaminase
Keratinocyte
Epidermis

ABSTRACT

Transglutaminase (TGase) catalyzes protein cross-linking reactions essential for several biological processes. In differentiating keratinocytes, TG1 (keratinocyte-type) is crucial for the cross-linking of substrate proteins required for the complete formation of the cornified envelop, a proteinaceous supermolecule located in the outermost layer of the epidermis. TG1 expressions and its substrate were induced in cultured keratinocytes at differentiation-stage specific manner. In the cultured keratinocytes, we used the TG1-specific substrate peptide, which enables the specific detection of enzymatic activity to investigate its induction patterns. As a further application of the substrate peptide, several substrate candidates of TG1 that may be essential for cornified envelope formation were identified and characterized.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Transglutaminase (TGase: E.C. 2.3.2.11) catalyzes the cross-linking reaction of identical or different substrate proteins by covalent isopeptide-bond formation between glutamine and lysine residues [1,2]. In addition to this catalytic reaction, primary amine is incorporated resulting in the attachment of amine to the glutamine-donor substrate protein. In these reactions, calcium ions are essential as a trigger of enzyme activation.

TGase comprises a protein family consisting of eight isozymes, each of which shows unique distribution and substrate specificity. The TGase family plays multiple physiological roles: Factor XIII, a coagulation factor, cross-links fibrin molecules into a polymer. TG1, TG3 and TG5 are expressed in the skin epidermis and contribute to keratinization [3,4]. TG2, which is ubiquitously expressing, is involved in multiple functions by cross-linking various functional molecules such as transcription factors, extracellular matrix proteins and signal transduction molecules [2]. TG4, TG6 and TG7 have been less investigated regarding their functions because of their lower levels of expression compared to those of other TGases.

The skin barrier in the epidermis is completed by the formation of proteinaceous supermolecule, the cornified envelop (CE), beneath the terminally differentiated keratinocytes [3–5]. During the differentiation of keratinocytes, the activities of TGases are responsible for the irreversible cross-linking of several structural proteins. These constitutive structural proteins have thus far been identified by purification of the CE components and analysis by protein digestion and sequencing [6]. By this approach, predominant proteins such as loricrin, involucrin and small proline-rich proteins (SPRs) have been identified and they are shown to be prominent TGase substrates in the form of recombinant protein *in vitro* [7–9]. Other substrate proteins constituting CE have also been identified, but not all of the members have yet been determined.

To date, we have established highly reactive substrate peptides that work as glutamine-donors specifically to each TGase isozyme [10–12]. These 12-amino acids sequences are functional substrate by specifically incorporated into lysine-donor substrates in the presence of each isozyme. For example, fluorescence-labeled peptides can detect *in situ* enzymatic activity in non-fixated tissue sections, where the area containing active TGase shows a significant signal [13]. These substrate peptides can also be used for *in vitro* activity detection and the identification of lysine-donor substrates [14].

Among the substrate peptide sequences, K5 was identified as being specific for TG1 activity [11]. The peptide form, pepK5, appeared to be effective at detecting *in situ* TG1 activity in the skin

Abbreviations: bio-Cd, 5-(biotinamido)pentylamine; CE, cornified envelop; DTT, dithiothreitol; GAPDH, glyceroaldehyde 3-phosphate dehydrogenase; PBS, phosphate–buffered saline; TBS, tris–buffered saline; TGase, transglutaminase.

* Corresponding author. Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, 464-8601, Japan.
E-mail address: hitomi@ps.nagoya-u.ac.jp (K. Hitomi).

http://dx.doi.org/10.1016/j.bbrc.2016.07.051
0006-291X/© 2016 Elsevier Inc. All rights reserved.

epidermis in adult whole sections and developing of fetal mice [13,15]. This specific and simple detection system provides a method of diagnosing human skin diseases [16].

In this study, the expression of substrate and *in vitro* TG1 activity were analyzed in the differentiating cultured keratinocytes. Furthermore, the candidates identified using the pepK5 peptide as novel TG1 substrates were investigated for possible reactivity as lysine-donor substrates. These established detection and substrate identification systems should provide clues to reveal the novel members involved in CE formation, which determine the “destiny” of barrier functions in the skin epidermis.

2. Materials and methods

2.1. Peptides

Both the substrate peptide pepK5 (YEQHKLPSSWPF) and its mutant peptide (pepK5QN: YENHKLPSSWPF) for negative control were synthesized by Biosynthesis (Lewisville, TX), with attachment of biotin at the *N*-terminus.

2.2. Antibodies

Antibody against the recombinant protein for human TG1 was prepared from rabbit serum immunized the recombinant enzyme and then purified by affinity-chromatography using TG1-immobilized gel [17]. Polyclonal antibodies against involucrin and GAPDH (glyceroaldehyde 3-phosphate dehydrogenase) were purchased from Santa Cruz Biotechnology (Dallas, TX) and Millipore (Merck Millipore, Darmstadt, Germany), respectively.

2.3. Culture of primary keratinocytes

Human neonate keratinocyte cells were purchased from (Toyobo, Osaka, Japan) and cultured in the specified serum-free medium (Epilife, Lifetechnologies, Carlsbad, CA) containing several growth factors such as EGF, bovine pituitary extract, hydrocortisone, and insulin. For differentiation, cells were grown to semi-confluent, and then the medium was replaced into differentiation medium containing higher calcium ion.

2.4. Reverse transcription PCR

Total RNA was prepared from differentiating keratinocytes and used for transcription by reverse-transcriptase (TAKARA Bio, Kyoto, Japan). Then, cDNAs as a template, PCR was performed using specific primers (TG1; 5’-GTGTGGACTTGCTGAGCTC-3’ and 5’-GATTCTGCCCACTGGCCTTG-3’, involucrin; 5’-CCTGGAGAAGCAGGAGGCAC-3’ and 5’-ATTTATGTTTGGGTGGCCAC-3’, GAPDH; 5’-ACTGGCATGGCCTTCCGTGT-3’ and 5’-CCTGCTTCACCACCTTCTTG-3’) and analyzed the amplified products by 2% agarose gel electrophoresis.

2.5. Immunoblotting

Cells were harvested and sonicated in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol, 0.1% Triton-X100 and protease inhibitor cocktail (Merck Millipore). After centrifugation, the supernatant and precipitated fractions were obtained by treatment with SDS-containing buffer and boiling.

For immunoblotting, the samples were subjected to SDS-PAGE and then polyvinylidene difluoride (PDVF) membrane (Merck Millipore). After blocking the membrane with PBS containing 5% skimmilk, the membrane was reacted with primary antibody. The membrane was reacted with the secondary antibody conjugated peroxidase and then the signals were developed using the chemiluminescent reagent (Thermo Scientific, Rockford, IL).

2.6. Detection of in vitro TG1 activity

For detection of *in vitro* activity, the biotin-labeled pepK5 was added to the supernatant fraction from the cellular extract at a final concentration of 100 μM and incubated for at 37 °C for 30 min [11]. The reaction product was subjected to western blotting and then the membrane was reacted with the streptavidin-conjugated peroxidase following treatment of chemiluminescence reagent. As a negative control, the mutant peptide, pepK5QN, was used.

2.7. Identification of substrate candidates

The cellular extract was prepared as in the same procedure of *in vitro* detection of activity. After incubation, to exclude the non-specific binding, the extract was incubated in the presence of 50 μM biotinylated pepK5QN for 10 min, and then applied to streptavidin-sepharose gel (Supplemental Figure, left). The through fraction was reacted with 50 μM pepK5 for 30 min and then applied to streptavidin-sepharose gel and washed by PBS buffer (Supplemental Figure, right). The eluted fraction was separated by 12.5% SDS-PAGE following Coomassie Brilliant Blue staining.

The target proteins excised from the stained gel were treated with trypsin in the presence of 0.01% Max surfactant (Promega, Madison, WI) at 50 °C for 1 h. Then, the trypsinized peptides were fractionated by a Dina nano-HPLC with a reverse-phase chromatography using C18 column (KYA Technologies, Tokyo, Japan). Each fraction was mixed with α-cyano-4-hydroxycinnamic acid and spotted on MALDI plate. MALDI-TOF mass spectrometry was performed by a 5800 Proteomics Analyzer (ABSCIEX, Tokyo, Japan). MS and MS/MS data for each peptide were analyzed by Protein pilot (ABSCIEX).

2.8. Expression and analysis of recombinant proteins for possible substrates

To obtain full-length cDNAs for human SPR1, kallikrein-10, α-crystallin B, and galectin-7, reverse-transcription PCR was carried out. Each cDNA was cloned into the expression vector, pET24dHis, which enables to attach the hexahistidine-tag upon expression in *E. coli.* [10]. Then these cloned expression vectors were used for transformation of BL21(DE3)pLysS. Each recombinant protein was purified by metal-ion affinity chromatography using TALON gel (TAKARA).

For each purified protein, the incorporation level of biotin-pepK5 (20 μM) was evaluated in the reaction mixture containing TBS buffer containing 5 mM CaCl2 and 1 mM DTT in the presence of recombinant TG1 at 2 ng/ml (Zedira, Darmstadt, Germany). The reaction products were subjected to SDS-PAGE and western blotting. Streptavidin-peroxidase was used for detection of biotin-incorporated proteins by development of chemiluminescent reagent.

3. Results

3.1. Expression patterns of TG1 and involucrin in differentiating keratinocytes

To confirm that the differentiation of keratinocytes was induced in the culture, the expression of involucrin, a major substrate, as well as TG1 was investigated at both the transcriptional and the protein levels. For the analysis of mRNA level, the

Download English Version:

https://daneshyari.com/en/article/10747891

Download Persian Version:

https://daneshyari.com/article/10747891

Daneshyari.com