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Title

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

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Keywords: transglutaminase; keratinocyte; epidermis

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.

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 was 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.

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-labelled 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 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 ^{o}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

chemiluminesence 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 ^{o}C for 1 hr. 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 $CaCl_2$ 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 reverse-transcription PCR products were analyzed using mRNAs from the cultured cells at various differentiation period. As shown in Fig. 1A, the expression of both mRNAs was induced upon differentiation during the days 1-14 although slight expression for involucrin was observed in the undifferentiated cells (day 0). Regarding the

protein levels analyzed by immunoblotting, both were also induced in a differentiation dependent manner (Fig. 1B). As for the expression pattern of TG1, since this enzyme is mainly attached to the plasma membrane, most proteins were harvested as insoluble fractions.

3.2 In vitro activity of TG1 in the differentiating keratinocytes

In the cell lysates from the differentiating cultured cells on the indicated days after the initiation of differentiation, proteins that incorporated biotin-labeled pepK5 via endogenous TG1 activity were detected (Fig. 2). As a negative control, the reaction product using the mutant peptide (pepK5QN) was paralleled on day 14. When comparing the reacted protein patterns, several proteins with different molecular sizes were detected and mostly increased as possible glutamine-acceptor substrates, depending on the differentiating period. The levels of some protein decreased, probably due to further cross-linking to other proteins resulting in the formation of proteins with a higher molecular mass and/or insoluble proteins. These results suggested that the level of active TG1 increased and catalyzed several substrates in differentiating keratinocytes.

3.3 Purification and identification of substrates for TG1

In the cell lysates prepared from cultured cells on day 0 (proliferating) and day 14 (differentiating), we attempted to identify possible TG1-catalysed cross-linking substrate proteins. The lysine-donor substrates that specifically incorporated biotin-labeled pepK5 via endogenous TG1 were purified with avidin-affinity chromatography and peptide mass fingerprinting (supplemental Figure).

The SDS-PAGE analysis result shown in Fig 3A indicates the patterns of

eluted proteins from avidin-immobilized gel. Several different proteins were affinity-purified in the eluate between fractions obtained from day 0 and 14 samples (Fig. 3A). The eluted fractions (Fig. 3B) were subjected paralleled and excised. The protein bands analyzed for mass spectrometry were marked by numbers to clearly refer to the proteins contained in each band.

The identified candidate substrates from the analyzed bands are listed in Table 1. Among these proteins, in addition to the several known substrates (underlined), αB-crystallin, galectin-7 and kallikrein-10 were newly identified as possible substrates.

3.4 Analyses of substrate candidate proteins

To confirm the reactivity of the possible substrates that we newly identified, α-crystallin B, galectin-7, and kallikrein-10, each of these recombinant proteins was expressed in bacteria and then purified close to homogeneity by metal-ion affinity chromatography. Either pepK5 or pepK5QN was co-incubated with each protein in the presence of TG1. Reactions were also performed with SPR1 as a positive control. As shown in Fig. 4, apparent time-dependent incorporation of the biotin-labeled peptide was observed for kallikrein-10 and α-crystallin B whereas there was no incorporation in the case of pepK5QN. As for galectin-7, however, less reaction product was shown probably due to the low numbers of lysine residues (3 residues in 136 amino acid residues: 11 of 89 in SPR, 9 of 276 in kallikrein-10, and 10 of 175 in α-crystallin B). These results indicated that our identified substrates react as lysine-donor substrates by the catalytic reaction of TG1.

4. Discussion

Cornified envelope (CE) consists of several structural proteins that are covalently cross-linked by TGases. In the established model, TG1 beneath the membrane and in the cytoplasm plays a major role by cross-linking several kinds of substrate protein including involucrin, loricrin, and SPRs, and may contribute to the inclusion of unknown substrates into CE [3-5]. However, there is less information available on the possible substrates other than these well-investigated proteins, most of which were directly identified as proteins of CE purified from the epidermis.

Generally, to analyze the enzymatic activities of TGases, the labeled primary amine (e.g. biotin-labeled pentylamine) has been used to evaluate the amounts of incorporation into glutamine-donor substrates. However, using this system, it is impossible to discriminate the isozyme-specific activity of TGases. In our study, taking advantage of a specific substrate peptide (pepK5) for TG1, we first investigated the variation of *in vitro* activity of TG1 during the differentiation of keratinocytes (Fig. 2). Since this system could detect the enzymatic activity in an isozyme-specific manner, this revealed the precise pattern of TG1 enzymatic activity. At the early stage of differentiation, the activity was enhanced and maintained at a similar level. In addition, this level is consistent with the expression pattern at the translational and transcriptional levels (Fig. 1). According to the proposed mechanism for CE formation, TG1 plays a wide range of roles, to attach platforms at the plasma membrane by cross-linking involucrin with ceramide and to fix the pre-crosslinked proteins that have previously been created in the cytoplasm. When considering the various substrates cross-linked by the enzyme, this pattern of TG1 activity seems reasonable for their functions.

Next, we attempted to excavate the novel substrate proteins using pepK5 as specific bait (Fig. 3). This allows the purification and identification of isozyme-specific substrates that incorporated biotin-peptide. Here, upon the identifications of TG1-specific substrates by this system, purified proteins in the differentiating and undifferentiating keratinocytes were precisely compared, and we obtained several novel substrate candidates as well as various known substrates such as SPRs, annexins, involucrin, epiplakin, desmoplakin, hornein, and S100 proteins [3, 4, 5, 18, 19]. This result indicated that our system was successfully on identifying the substrates in keratinocytes.

Among the proteins, three proteins were selected as novel potential glutamine-acceptor substrates: α-crystalline B, galectin-7, and kalliklein-10. Although these selected proteins have not been previously proposed as CE components, they are preferentially cross-linked by TG1, suggesting that they take important positions upon CE formation. The α-crystallin B, a chaperone protein found in lens, was recognized as the preferred substrate for TG2 and part of its sequence (TVQQEL) has been used as a prominent substrate peptide [20, 21]. Crystallin was unexpectedly found as favored substrate in cultured keratinocytes, but it might be a common substrate in epithelial tissues for barrier function. Since this protein was harvested in the avidin-purification in a form with a much greater molecular size in addition to its original molecular mass (16 kDa), the partner substrate as a lysine-acceptor would be necessary to clarify its role. Galectin-7, one member of the glycosylation-binding protein family, is purified as a monomer protein. Because this protein was involved in keratinocyte differentiation in a previous study, this modification might contribute to its function [22]. Kallikrein-10 is a serine protease but its target substrates

remained unknown. The kallikreins are an essential protease family that play important roles in the degradation of profillagrin or corneodesmosome in epidermis [23]. It was unexpected that only kalliklein-10 was identified as a favored TG1-substrate candidate despite the fact that several functional kallikrein members were expressed in the keratinocytes. Although there is a little information on the substrate of this protease, modification by TG1 might provide a clue to clarify the significance.

In this identification method, the selected proteins were limited to the glutamine-acceptor substrates. Because the specificity of the lysine-donor substrates shows less tendency for each isozyme in the enzymatic reaction, it is currently difficult to obtain lysine-acceptor substrate peptides. Accordingly, identification of the counterpart (lysine-acceptor) substrate is essential for subsequent steps using the obtained lysine-donor substrates. The procedures introduced in this study, however, can be applied to the any tissues upon not only detection of the enzymatic activity and but also identification of possible substrates. Regarding the candidates identified in this study, investigation of the localization of the products in the CE fraction of the epidermis and also search for counterpart substrates are ongoing.

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Figure legends

Fig. 1 Expression patterns of TG1 and involucrin during differentiation of the keratinocytes

Products of reverse-transcription PCR from cDNA were subjected to 2% agarose gel electrophoresis (A). The lysates from the cells were analyzed by immunoblotting using each antibody (B). GAPDH was used as a negative control in each case.

Fig. 2 Time-dependent increase in *in vitro* activity of TG1

TG1 *in vitro* activities in differentiating keratinocytes were analyzed by the incorporation of biotin-labeled pepK5 into cellular lysate. The reaction products were subjected to 12.5 % SDS-PAGE following western blotting. The peptide-incorporating proteins were detected by streptavidin-peroxidase and chemiluminescent reagent. QN indicates the reaction products containing pepK5QN instead of pepK5 as a negative control.

Fig. 3 Purification of possible substrate proteins in proliferating and differentiating keratinocytes

The cell lysates from days 0 and 14 were pretreated with pepK5QN and streptavidin-immobilized gel absorption. The flow-through fractions were reacted with pepK5 and re-subjected streptavidin-gel chromatography. A, T, and W represent the applied, flow-through, and washing fractions, respectively. The proteins bound to streptavidin gel were eluted by adding SDS-containing buffer (eluate; E1, E2). Each sample was subjected to 12.5% SDS-PAGE (A). The elution fractions (E1 in Fig. 3A) on days 0 (left) and 14 (right) were subjected to SDS-PAGE and mass spectrometry analysis. The excised proteins from the bands are indicated by the numbers (B). The molecular mass marker is indicated.

Fig. 4 Incorporation of pepK5 into the purified recombinant proteins for possible substrate proteins.

Incorporation of biotin-labeled pepK5 into possible substrate proteins (A, KLK-10: kalikrein-10; B, Cry: α-crystallin B; C, Gal-7: galectin-7) as well as recombinant SPR1 were detected by 12.5％SDS-PAGE and western blotting followed by development with streptavidin-conjugated peroxidase. TG1 was contained at a final concentration of 2 ng/μl, and the following substrate concentrations were used in each reaction (A; 10 ng/μl, B; 40 ng/μl, C; 100 ng/μl). Reaction products at the indicated times and also the negative control using the mutant peptide (QN) were subjected to the analyses. The closed and open triangles indicate each substrate protein and SPR1, respectively.

Supplement figure

Procedure of identification of possible substrates. The biotin-pepK5QN peptide was incubated to absorb non-specific binding proteins. Then the solution was mixed to avidin-immobilized gel and the flow-through fractions were harvested (left). By incubating with biotin-pepK5, the substrate proteins incorporated the peptide by the endogenous TG1. The biotin-incorporating proteins were affinity-purified by avidin-gel chromatography (right).

Table 1

Actin (4, 5, 6, 7)	Annexin A1 (1, 2, 6)	Annexin A2 (1, 5)
Alfa-crystallin B (1, 10)	Desmoplakin (1, 2)	Epiplakin (1)
Fibronectin (1)	Junction plakoglobin (1)	
Heat shock protein β-1 (1, 2, 10, 12)		Hornein (5, 7, 10)
Kallikrein-10 (8)	**Galectin-7** (11)	Involucrin (1)
Plectin-1 (1)	Plasminogen activator inhibitor 2 (1)	S100A10 (1)
S100A11 (1)	SPR1 (cornifin-A) (1)	SPR2D (1)
40S ribosomal protein S13 (1)		
60S ribosomal proteins (L6, L13, L13, L15) (1)		

List of the possible lysine-donor substrates identified. The numbers in the parenthesis mean the excised band shown in Fig. 3B. The underlined proteins have been reported as components of CE.

Figure

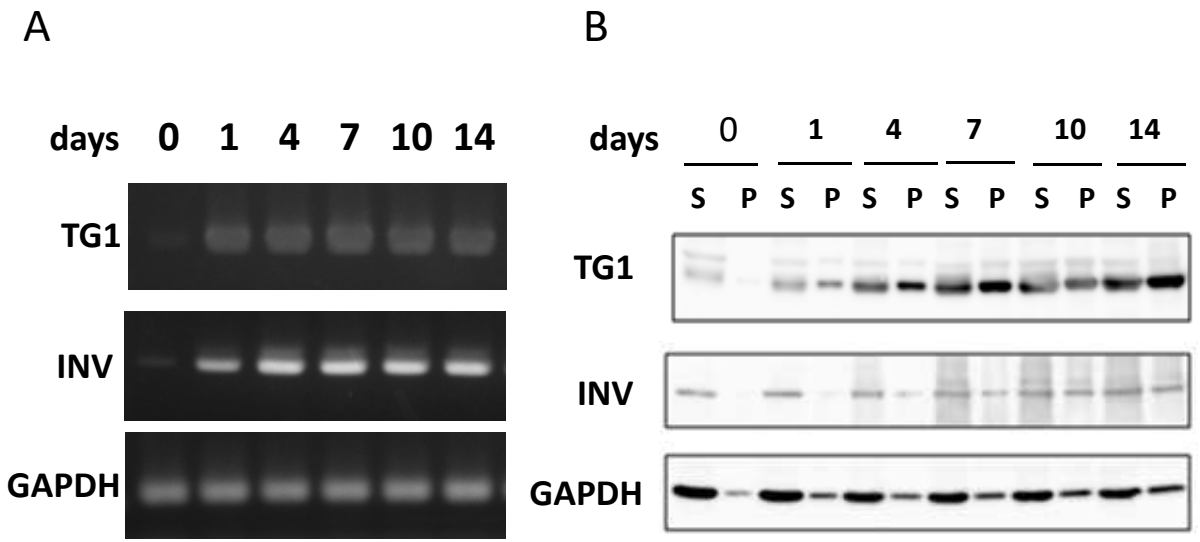
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Fig. 1

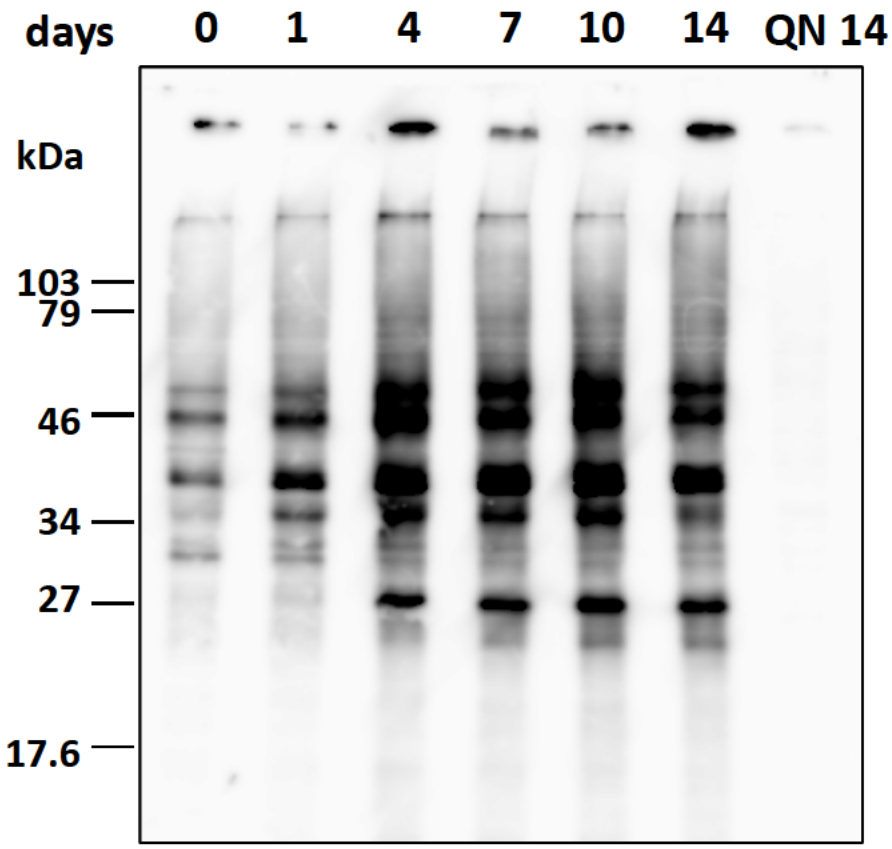

Fig. 2

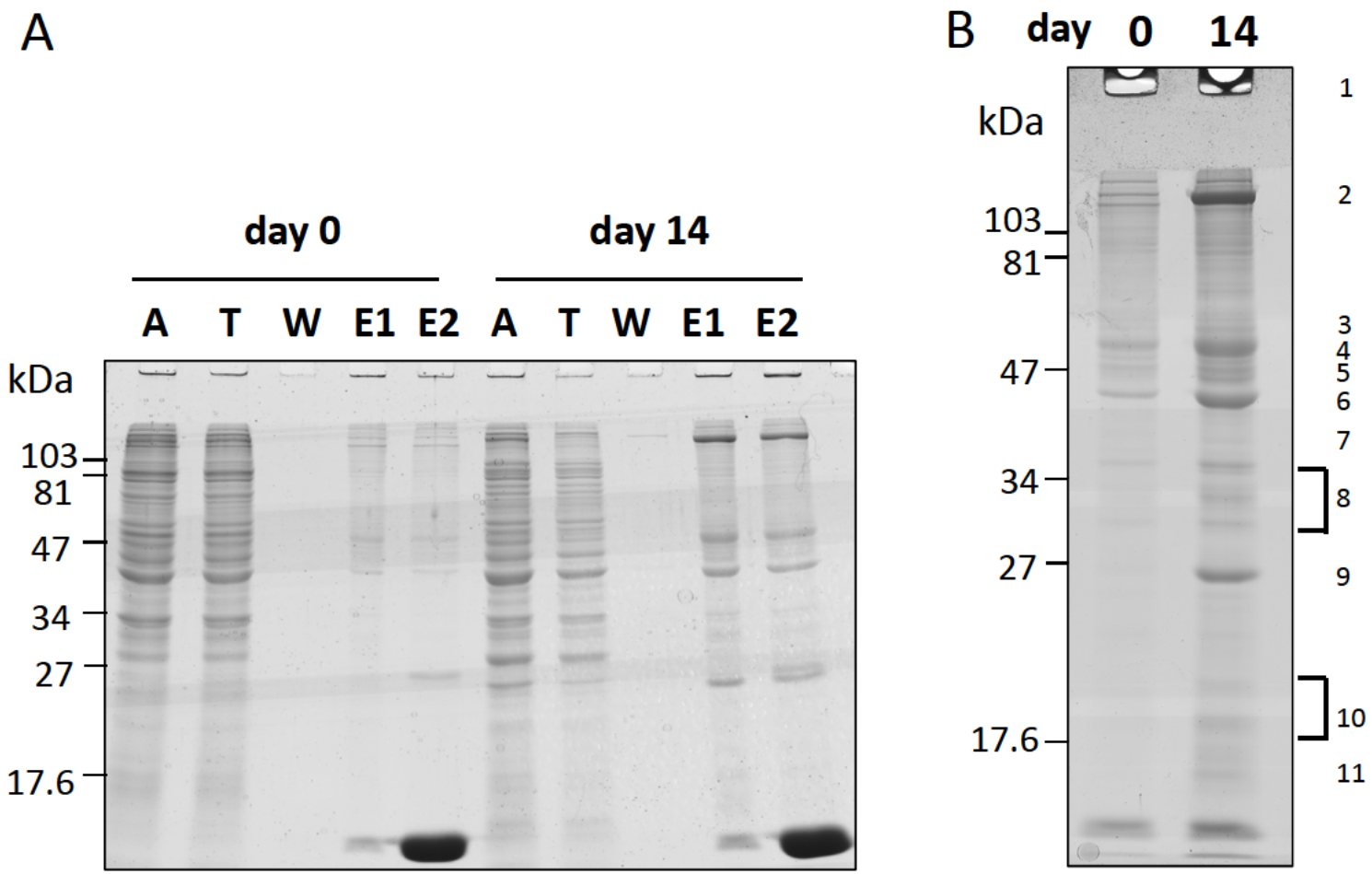
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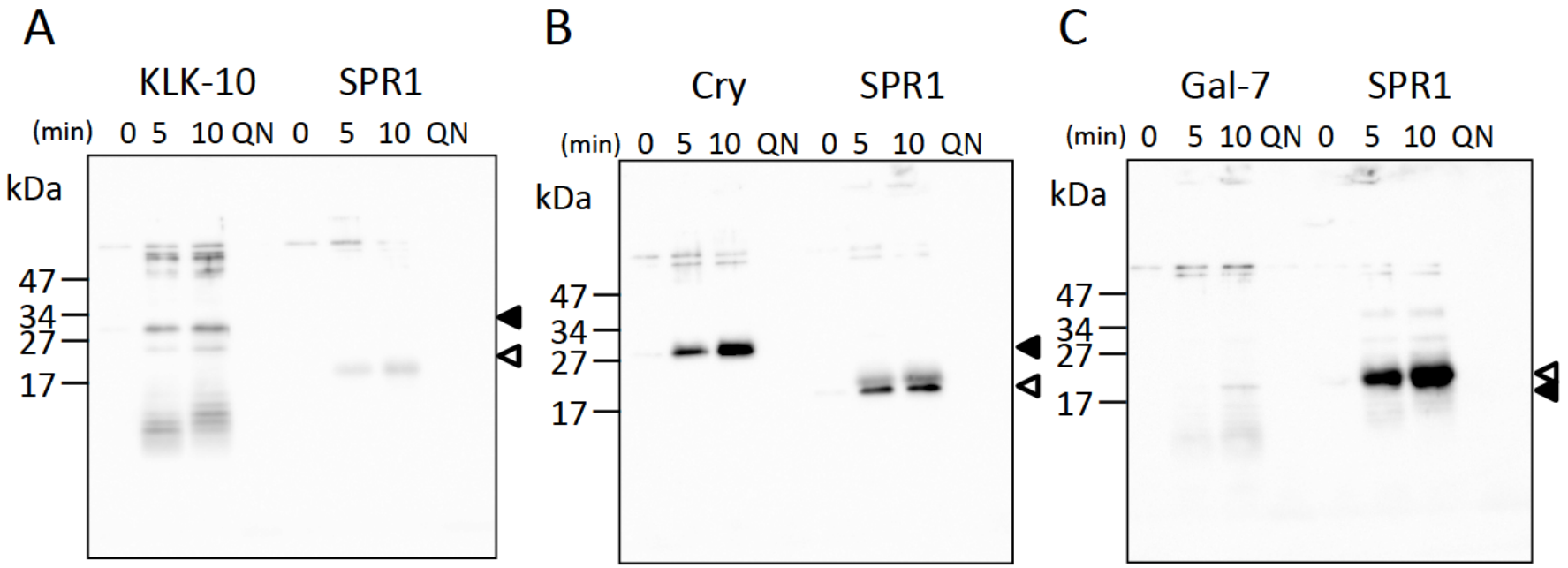
Fig. 3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Gal-7
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Fig. 4

Conflict of interest

The authors declare that they have no competing interests.

On behalf of the authors,

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