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Linkage between phosphorylated cystatin α and filaggrin by epidermal transglutaminase as a model of cornified envelope and inhibition of cathepsin L activity by cornified envelope and the conjugated cystatin α

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Abstract

Lysine-rich phosphorylated cystatin α (P-cystatin α) from newborn rat epidermis is a good substrate for epidermal transglutaminase (TGase) and also one of the component proteins of cornified envelope in the stratum corneum. Since the filaggrin linker segment peptide was efficiently conjugated with P-cystatin α and was mediated by epidermal TGase in the presence of Ca^{2+} ions, filaggrin is a candidate for the glutamine-rich linkage protein to conjugate with lysine-rich P-cystatin α. A conjugated protein was formed by epidermal TGase in the activated condition with Ca^{2+} ions and dithiothreitol. In contrast, the conjugated protein was not formed under chelated conditions with EDTA. The conjugated protein reacted positively with anti-P-cystatin α polyclonal antibody (PoAb). The conjugated protein and purified cornified envelope showed an inhibitory effect against papain and cathepsin L, but cathepsin B and H were not inhibited by these P-cystatin α conjugates. Although the component protein, P-cystatin α itself, inhibited cathepsin H strongly, these conjugated proteins inhibited specifically the cathepsin L family. The amino acid composition of cornified envelope protein and the conjugated protein of P-cystatin α and filaggrin linker segment peptide was not completely the same. The conjugated protein of P-cystatin α and filaggrin linker segment peptide showed the same inhibitory properties against cysteine proteinases as the cornified envelope. These findings suggest that the linkage protein between P-cystatin α and filaggrin linker segment peptide may be considered a model of cornified envelope, although skin cornified envelope may be conjugated with some additional proteins.

Key words: Cornified envelope; Transglutaminase; Cystatin α; Cysteine proteinase inhibitor; Filaggrin peptide

1. Introduction

We have previously reported that cystatin α phosphorylated by protein kinase C (P-cystatin α) is located in keratohyalin granules of the stratum granulosum and also in the cornified envelope of stratum corneum [1]. Cornified envelope is formed from specific substrates by epidermal transglutaminase (TGase) during the terminal differentiation of epidermal cells [2]. Since cystatin α contains many lysine residues [3,4], it is a good substrate of epidermal TGase in vitro [5]. A suitable glutamine-rich protein or peptide to cross-link with P-cystatin α by epidermal TGase is necessary to form the cornified envelope [6]. Filaggrin, a component of cornified envelope protein in the stratum corneum [7], is a glutamine-rich protein, particularly its linker segment peptide which has 6 glutamine residues among the 26 amino acid residues [8]. Although it has been clarified by our group that P-cystatin α is a component protein of cornified envelope [1], the glutamine-rich linkage protein has not been determined. Therefore, we propose the linkage reaction between P-cystatin α and filaggrin linker segment peptide by epidermal TGase as a model of cornified envelope formation. Also inhibition of various cysteine proteinases by cornified envelope and conjugated protein are examined.

2. Materials and methods

2.1. Materials

Newborn Sprague–Dawley rats were purchased from SLC, Japan, Superose 12, Mono-Q and Superdex 200 columns and ampholines were obtained from Pharmacia-LKB, Sweden. Gradient SDS-PAGE gel (15–25%) was ordered from Daiichi Pure Chemical Co. Ltd., Japan, and a protein assay kit was obtained from Bio-Rad, USA. PVDF membrane was purchased from Nippon Millipore Limited, Japan. Other chemicals, analytical grade, were purchased from commercial sources.

2.2. Purification of P-cystatin α and formation of polyclonal antibody (PoAb) against P-cystatin α

Newborn rat epidermis was separated with EDTA solution as described previously [9], and P-cystatin α was purified by isoelectric focussing. Superose 12 gel filtration and Mono Q anion exchange column chromatography. This was then used to raise a PoAb.

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Abbreviations: P-cystatin α, phosphorylated cystatin α; TGase, transglutaminase; PoAb, polyclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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2.3. Preparation of cornified envelope

Cornified envelope was prepared as follows. Newborn rat stratum corneum was sonicated in 50 mM Tris-HCl (pH 9.0) containing 8 M urea and 10 mM 2-mercaptoethanol, stirred for 30 min and centrifuged at 15,000 rpm. After this procedure was repeated 3 times, the residue was rinsed with 50 mM Tris-HCl (pH 7.4) 5 times. The residue was rinsed with distilled water and lyophilized.

2.4. Partial purification of epidermal TGase

Cow snout epidermis was lyophilized and the powder was extracted and partially purified by the modified method of Buxman and Wuepper [10] by anion-exchange chromatography and gel filtration.

2.5. Synthesis of filaggrin linker segment peptide

Filaggrin linker segment peptide, whose sequence was -Y-Y-Y-E-Q-E-H-S-E-E-E-S-D-S-Q-H-Q-H-G-H-Q-H-E-Q-Q-R- [8], was synthesized using a Shimadzu peptide synthesizer.

2.6. Conjugation of P-cystatin α and filaggrin linker segment peptide by epidermal TGase

We examined the conjugation of P-cystatin α alone and with filaggrin linker segment peptide by epidermal TGase as follows: P-cystatin α (0.05 nmol) was incubated, alone and with filaggrin linker segment peptide (0.5 nmol), was incubated with epidermal TGase in the activated condition containing 10 mM $CaCl_2$ and 10 mM dithiothreitol or in the chelated condition containing 10 mM EDTA. These samples were then treated with 1% SDS and 2-mercaptoethanol and subjected to SDS-PAGE.

2.7. Immunoblotting analysis using anti-P-cystatin α PoAb

The proteins in SDS-PAGE gel were transferred to PVDF membrane. The membrane was reacted with anti-P-cystatin α PoAb and then it was reacted with peroxidase-conjugated goat anti-rabbit IgG. The reactive proteins were stained with 3,3'-diaminobenzidine and H_2O_2.

2.8. Collection of the conjugated protein

The conjugated protein of P-cystatin α and filaggrin linker segment peptide formed by epidermal TGase was collected by Superdex 200 column gel filtration. The amount of protein was determined utilizing the Bio-Rad protein assay kit. Bovine serum albumin was used as a standard protein for calibration.

Fig. 1. SDS-gel electrophoresis profiles on the conjugated proteins by epidermal TGase between P-cystatin α and filaggrin linker segment peptide. (A) Immunoblotting analysis using anti-P-cystatin α PoAb; (B) Coomassie brilliant blue staining. (a,b) P-cystatin α with TGase; (c,d) P-cystatin α and filaggrin linker segment peptide with TGase; (a,c) Under activated conditions containing 10 mM $CaCl_2$ and 10 mM 2-mercaptoethanol; (b,d) Under chelated conditions containing 10 mM EDTA. Arrows and arrowheads indicate the conjugated protein formed and P-cystatin α, respectively.

2.9. Inhibitory properties of cornified envelope and the conjugated protein

The inhibitory properties of cornified envelope and the conjugated protein against cysteine proteinase activities were determined using MCA conjugated synthesized substrates. Because cornified envelope was not soluble, it was suspended in Tris-HCl buffer containing Brij-35. Papain, cathepsin B, H and L were used for inhibitory activity assay of cornified envelope and the conjugated protein. Fluorescence was measured at excitation 370 nm and emission 460 nm [11].

2.10. Amino acid composition of the conjugated protein

The conjugated protein was subjected to reverse-phase column chromatography and its amino acid composition was determined using Derivatizer, Applied Biochemicals, USA. Cornified envelope, which was prepared by the above method, was hydrolyzed in 6 N HCl for 24 h. Amino acid composition was also determined using Derivatizer.

3. Results

Conjugated proteins between P-cystatin α and filaggrin linker segment formed by epidermal TGase are shown in the SDS-gel electrophoresis pattern in Fig. 1. Two kinds of high molecular weights proteins conjugated with P- cystatin α were formed by partially purified epidermal TGase under activated conditions. However, the yield was not enough, because of a small amount of contamination of glutamine-rich protein(s) in the partially purified epidermal TGase preparation were used as the glutamine-donor protein. Therefore, filaggrin linker segment peptide, one of the glutamine-rich peptides found in the epidermis, was synthesized using the method of Resing et al. [8]. In contrast, sufficient

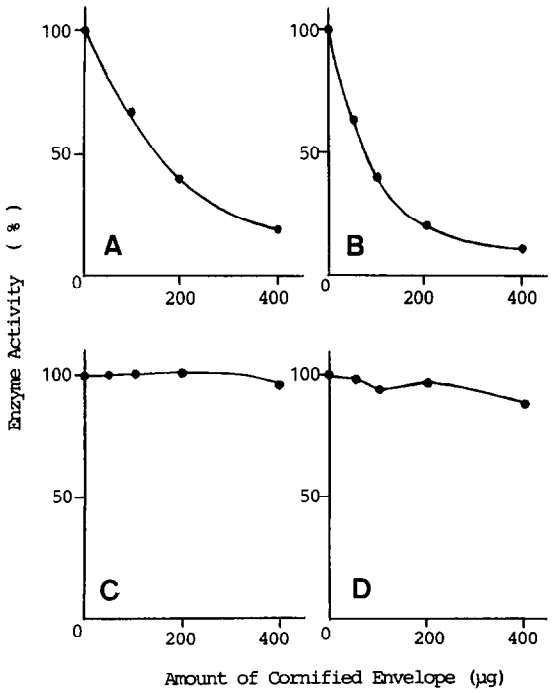

Fig. 2. Inhibition of papain, cathepsin B, H and L by cornified envelope. (A) Papain; (B) cathepsin L; (C) cathepsin B; (D) cathepsin H. Cornified envelope is insoluble, therefore this is used as the suspension The amounts indicated are dry weight of cornified envelope.

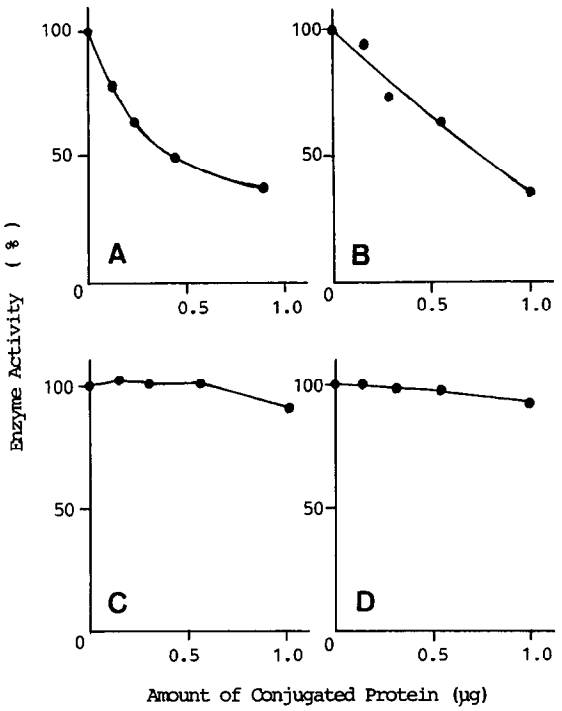

Fig. 3. Inhibition of papain, cathepsin B, H and L by the conjugated protein synthesized by epidermal TGase. (A) Papain; (B) cathepsin L; (C) cathepsin B; (D) cathepsin H. The amounts of proteins were assayed by Bio-Rad protein assay kit using bovine serum albumin as a standard protein.

amounts of the conjugated protein were synthesized by addition of the filaggrin linker segment peptide under the same conditions. These conjugated proteins reacted positively with anti-P-cystatin α PoAb by immunoblotting as shown in Fig. 1A and also with anti-filaggrin PoAb (data not shown). These products, however, were not formed under chelated conditions. The purified cornified envelope (dry weight of cornified envelope: 0 to 400 μg) showed strong inhibition against papain and cathepsin L, but not against cathepsin B and H as shown in Fig. 2, while P-cystatin α itself inhibited cathepsin H considerably. The inhibitory profile of the enzymatic conjugated proteins between P-cystatin α and filaggrin linker segment peptide are presented in Fig. 3. The conjugated protein strongly inhibited the activities of papain and cathepsin L, and the inhibition profile was the same as that of the cornified envelope. However, since the enzymatic synthesized proteins were water soluble, the inhibitory intensity was much stronger than that of cornified envelope. The amino acid composition of the conjugated protein is compared with those of P-cystatin α and cornified envelope in Table 1.

Table 1
Comparison of amino acid compositions of the conjugated protein of P-cystatin α and filaggrin linker segment peptide, P-cystatin α and cornified envelope

Amino acid	Conjugated protein	P-Cystatin α	Cornified envelope
Asp	3.6	12.0	2.3
Glu	8.7	14.3	7.7
Ser	8.1	3.6	20.2
Gly	21.6	10.0	37.3
His	4.5	1.2	0.7
Arg	6.5	3.2	2.4
Thr	5.2	7.3	2.3
Ala	7.5	4.5	2.6
Pro	4.4	3.3	4.6
Tyr	2.3	2.9	3.0
Val	6.3	8.0	2.9
Met	1.8	2.6	0.5
Cys	0.8	0	5.4
Ile	5.0	2.2	1.3
Leu	7.8	8.7	2.2
Phe	5.6	4.0	1.4
Lys	10.3	13.7	3.3

4. Discussion

We have previously reported that P-cystatin α is a substrate of epidermal TGase and a component protein of cornified envelope [1]. Cornified envelope seems to be formed by a cross-linking reaction of epidermal TGase between P-cystatin α and glutamine-rich protein(s). Filaggrin [7], involucrin [12], loricrin [13] and cystine-rich protein [14] in the epidermis have been reported as suitable substrates of TGase. When P-cystatin α was reacted with a partially purified epidermal TGase, a small amount of the conjugated proteins was formed. This high molecular weight protein may be formed from P-cystatin α and an unknown glutamin-rich protein contaminating in the partially purified epidermal TGase fraction. The synthesized filaggrin linker segment peptide was used as a substrate of glutamine-rich peptide to conjugate with P-cystatin α by epidermal TGase. The same high molecular weight conjugated proteins were formed as when filaggrin linker segment peptide was not added. Cornified envelope and the conjugated protein inhibit the activities of papain and cathepsin L, but not the activities of cathepsin B and H. Although the amino acid compositions between the conjugated protein formed by epidermal TGase in vitro and natural cornified envelope were not the same, they became similar when the amino acid composition of conjugated protein was plus that of loricrin [13] which was major protein of cornified envelope [15] as shown in Fig. 4. It is speculated that natural cornified envelope could be conjugated with protein(s) other than filaggrin, such as loricrin. Furthermore, the conjugated protein showed the same inhibitory properties as natural cornified envelope. P-cystatin α inhibited cathepsin H activity to the same degree as that cathepsin L activity, but natural cornified envelope and conjugated protein did not inhibit cathepsin H activity. Therefore, the conjugation of P-cystatin α and filaggrin linker segment peptide could be considered a model of

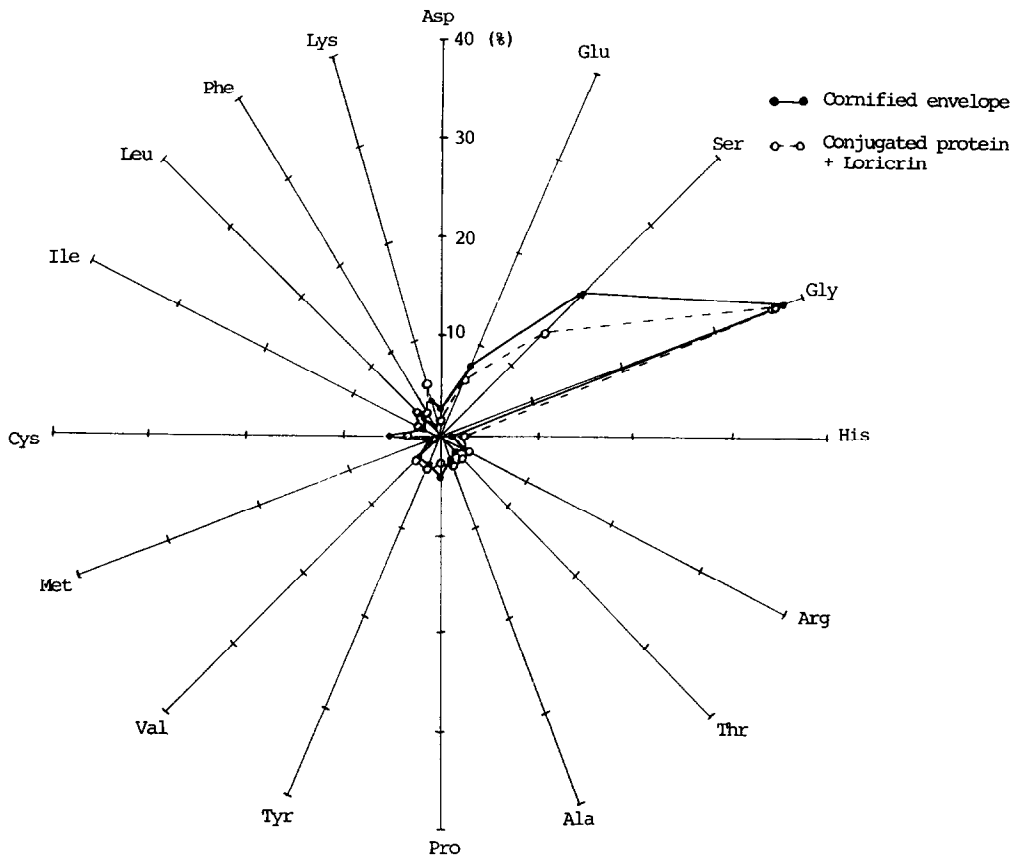

Fig. 4. Comparison of the amino acid profiles of cornified envelope and the sum of those of the conjugated protein and loricrin. Closed circles and open circles indicate the amino acid composition of cornified envelope and that of sum of the conjugated protein and loricrin, respectively.

cornified envelope formation. Korant et al. reported that egg white cystatin inhibited cysteine proteinase of picorna virus and processing of the virus precursor proteins [16,17]. Cornified envelope and conjugated protein in the stratum corneum may act as a barrier by inhibition of cysteine proteinases of bacteria and viruses.

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