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Phosphorylated cystatin α is a natural substrate of epidermal transglutaminase for formation of skin cornified envelope

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Both keratohyalin granules (KHG) and cornified envelopes were stained histochemically in an indirect immunofluorescent study by anti-phosphorylated cystatin α antibody, indicating that phosphorylated cystatin α is a component of the cornified envelope proteins. When phosphorylated cystatin α (P-cystatin α) was incubated with epidermal transglutaminase (TGase) and Ca^{2+} ions, polymerized protein was produced by formation of ε-(γ-glutamyl)lysine cross-linking peptide bonds between lysine residues of cystatin α and glutamine residues of suitable protein(s) in the enzyme preparation. However, phosphorylated and non-phosphorylated cystatins were polymerized to similar extents by the TGase. Immunofluorescent and immunoelectron microscopic observations revealed that P-cystatin α could be detected in vivo in the KHG and cornified envelopes. Treatment of sphingosine, a specific inhibitor of protein kinase C, markedly suppressed the incorporation of cystatin α into KHG. Thus phosphorylation of cystatin α by protein kinase C may play an important role in targeting cystatin α into KHG.

Cysteine proteinase inhibitor; Phosphorylated cystatin α; Cornified envelope; Epidermal transglutaminase

1. INTRODUCTION

Cysteine proteinase inhibitors of the rat skin were reported by Järvinen et al. in 1975 [1], and they have subsequently been characterized by other investigators [2–4]. Recently, we reported that cystatin α, a cysteine proteinase inhibitor, is located in keratohyalin granules (KHG) of the epidermis as a hematoxylin-stainable protein, and that cystatin α located in the granules is phosphorylated by protein kinase C [5,6]. We found, by an immunofluorescent technique, that KHG of the epidermis were stained histochemically with both anti-phosphorylated cystatin α (P-cystatin α) [7] and anti-cystatin α antibodies, using Lowicryl K4M-embedded sections, and that the cornified envelope of the skin in the cryostat sections also reacted strongly to both antibodies. These findings suggested that polymerized P-cystatin α is a main component of cornified envelope proteins. Transglutaminase (TGase) catalyzes the formation of an ε-(γ-glutamyl)lysine cross-linking peptide bond between a lysine residue of one protein and a glutamine residue of another [8]. P-cystatin α is quite rich in lysine residues (13.7%) [9] and, furthermore, keratinocytes contain a large amount of TGase.

TGase in keratinocytes is known to act on soluble protein substrates, such as involucrin [10], keratolinin [11], loricrin [12] and filaggrin [13]. The resulting insoluble cornified envelope is formed beneath the plasma membrane of the stratum corneum cells during terminal differentiation of the epidermis [14]. Therefore, this reaction is important for the formation of the stratum corneum and the barrier function of the skin.

In this study, we examined whether P-cystatin α was polymerized by the action of epidermal TGase forming the cornified envelopes, and we clarified the location of P-cystatin α on cornified envelopes. We also investigated the significance of phosphorylation of cystatin α by inhibiting its phosphorylation with a protein kinase C inhibitor, sphingosine [15]. This resulted in a marked suppression of the targeting of cystatin into KHG. The process of cornified envelope formation from cystatin α is discussed at the molecular level in this paper.

2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant cystatin α, which was prepared by the method of Katunuma et al. [16], an antibody against cystatin α, and recombinant human cystatin C, were provided by Dr. Ohshita and Dr. Nikawa, respectively, of the Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima. Egg white cystatin and sphingosine were from Sigma, USA. FITC-conjugated anti-rabbit IgG goat IgG was from Cappel, USA. Gold-conjugated anti-rabbit IgG goat IgG was from Amersham, UK. Superose-12, Mono-Q columns

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Abbreviations: KHG, keratohyalin granules; TGase, transglutaminase; P-cystatin α, phosphorylated cystatin α; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PoAb, polyclonal antibody.

and ampholines were ordered from Pharmacia-LKB, Sweden. Lowicryl K4M and Epon embedding kits were obtained from TAAB Laboratories, UK.

2.2. *Purification of P-cystatin α and formation of PoAb against P-cystatin α*

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

2.3. *Indirect immunofluorescent study*

Lowicryl K4M-embedded sections [17] of newborn rat skin previously fixed in 2% paraformaldehyde–0.025% glutaraldehyde solution at 4°C for 1 h, or cryostat sections of newborn rat skin fixed in cold ethanol (−20°C) for 4 min, were incubated with anti-P-cystatin α PoAb (1:200) at 37°C for 30 min. The sections were rinsed in phosphate-buffered saline (PBS) and incubated with FITC-conjugated anti-rabbit IgG goat IgG (1:200) at 37°C for 30 min. They were rinsed in PBS and visualized by fluorescent microscopy.

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 [18] by anion-exchange chromatography and gel filtration. The resulting preparation was purified about 10-fold over the crude extract as judged by its activity.

2.5. *Polymerization of P-cystatin α and other cystatins by epidermal TGase*

We examined the properties of P-cystatin α as a substrate of epidermal TGase as follows: a mixture of P-cystatin α and epidermal TGase was incubated in either Tris-HCl activation solution containing 10 mM $CaCl_2$ and 10 mM dithiothreitol, or Tris-HCl chelation solution containing 10 mM EDTA overnight at 37°C. Samples were then treated with 1% SDS and 2-mercaptoethanol and subjected to SDS-PAGE. Other cystatins, such as a recombinant cystatin α, a recombinant human cystatin C, and egg white cystatin, were also examined for polymerization.

2.6. *Immunofluorescent and immunoelectron microscopic observations of isolated cornified envelopes*

Cornified envelopes were prepared according to the modified method of Richards et al. [13]. Briefly, powdered newborn rat stratum corneum was heated, with stirring, in Tris-HCl buffer (pH 8.0) containing 2% SDS, 20 mM dithiothreitol and 5 mM EDTA for 10 min in boiling water. After centrifugation at 10,000 rpm for 15 min, the residue was suspended in the above buffer and sonicated. These treatments were repeated again. The sample was kept for 10 min and the floating envelopes were collected by centrifugation at 10,000 rpm for 15 min. The residue was re-suspended and washed with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1% Triton X-100 and 0.1% BSA. For the immunofluorescent study, thus prepared cornified envelopes were embedded in OCT compound, and cryostat sections of 6 μm thickness were prepared. The specimens were stained as reported in section 2.3. For immuno-electron microscopic analysis, suspended cornified envelopes were reacted with either anti-P-cystatin α PoAb or pre-immune rabbit serum (1:200). After a rinse, they were incubated in gold-conjugated anti-rabbit IgG goat IgG (1:10). They were fixed in 2% glutaraldehyde, rinsed and post-fixed in 2% osmium tetroxide. The specimens were serially dehydrated and embedded in Epon. Thin sections were stained with uranyl acetate and lead acetate.

2.7. *Effect of inhibition of protein kinase C by sphingosine on formation of KHG*

Newborn rat skin was pre-treated with chloroform:methanol (1:1) solution to remove the intercellular lipids of the stratum corneum cells. Filter paper soaked in sphingosine–DMSO solution (0.5 mg/ml DMSO) or DMSO alone was applied overnight by the occlusive dressing technique to skin that had been treated with chloroform:methanol solution. Lowicryl K4M-embedded sections of the previously sphingosine-treated skin portion, which were fixed in the mixture of 2% paraformaldehyde and 0.025% glutaraldehyde solution, were stained with anti-cystatin α PoAb (1:200) and examined by the indirect immunofluorescent technique as described above.

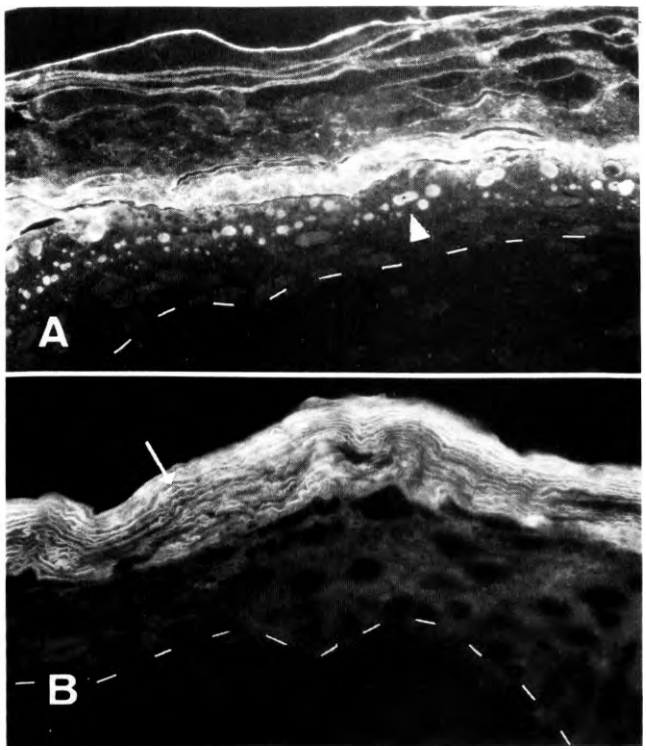

Fig. 1. Indirect immunofluorescent examination using a cryostat section of newborn rat skin. (A) Lowicryl K4M-embedded section: the arrowhead indicates the specific fluorescence of anti-P-cystatin α PoAb in KHG. (B) Cryostat section: the arrow indicates the specific fluorescence of anti-P-cystatin α PoAb in the cell membrane region of the stratum corneum. Dotted line indicates the dermal-epidermal junction. Magnification ×350.

3. RESULTS

We examined Lowicryl K4M-embedded sections (Fig. 1A) or cryostat sections (Fig. 1B) of newborn rat skin by the indirect immunofluorescent technique with anti-P-cystatin α antibody to determine the distribution of P-cystatin α. As shown in Fig. 1B, the layers of cornified envelopes were clearly stained with the antibody, suggesting that the cornified envelopes contain P-cystatin α protein.

As P-cystatin α contains thirteen lysine residues per mole (13.7%), P-cystatin α is a possible substrate for TGase. After incubation of P-cystatin α with partially purified epidermal TGase, the reaction products were analyzed by SDS-PAGE. After incubation in the activated condition, P-cystatin α was polymerized to the high molecular weight protein, whereas in chelated conditions, or with P-cystatin α or TGase alone, no polymer formation occurred. The partner substrate protein

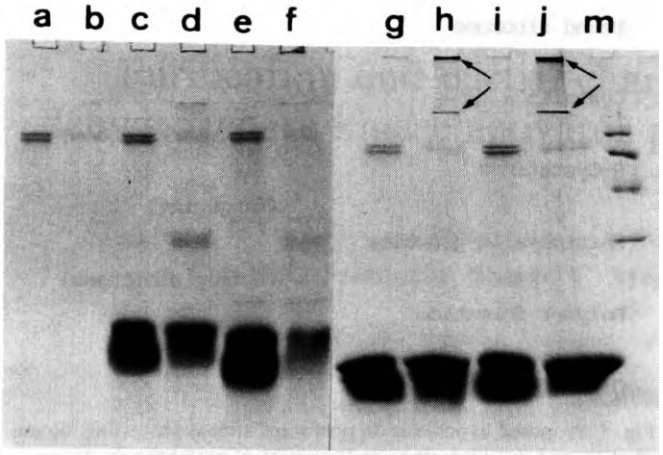

Fig. 2. Polymerizations of P-cystatin α and other cystatins by epidermal TGase. (Lanes a,b) Epidermal TGase; (lanes c,d) egg white cystatin; (lanes e,f) human cystatin C; (lanes g,h) cystatin α; (lanes i,j) P-cystatin α. (Lanes a,c,e,g,i) With 10 mM EDTA; (lanes b,d,f,h,j) with 10 mM Ca^{2+} ions and 10 mM dithiothreitol. (Lane m) Molecular weight markers; (from top to bottom in kDa) 94, phosphorylase b; 67, bovine serum albumin; 43, ovalbumin; 30, carbonic anhydrase; 20.1, soybean trypsin inhibitor; 14.4, lactalbumin. Arrows indicate polymerized high molecular weight protein. Coomassie brilliant blue staining.

that supplied glutamine residues to bind with the lysine residues of P-cystatin α in the TGase reaction is not known. Under the same conditions, cystatin α also formed high molecular weight proteins, but egg white cystatin and human cystatin C formed only trace amounts of high molecular weight proteins, as shown in Fig. 2.

Under in vitro conditions both types of cystatin derivatives, phosphorylated and non-phosphorylated, were equally susceptible to the TGase in their two forms. The immunofluorescent and immunoelectron microscopic examinations revealed that P-cystatin α was located on the cornified envelope, as shown in Fig. 3. Therefore, the phosphorylation does not participate in the polymerization step.

What is the biological significance of the phosphorylation of cystatin α in epidermal differentiation? To answer this, we examined the effect of inhibition of phosphorylation by protein kinase C on the targeting of cystatin α into KHG. We found that sphingosine, a specific inhibitor of protein kinase C, markedly inhibited the incorporation of cystatin α into KHG based on the observation by the immunofluorescent study shown in Fig. 4. These results indicate that phosphorylation is important in targeting of cystatin α into KHG.

4. DISCUSSION

Cystatin α is located in the epidermis of the skin, and in smaller amounts in the upper parts of the mucosal epithelium of the esophagus and digestive tract. This distribution is unique, so it seemed interesting to investigate why cystatin α is mainly located only in the epithelium, especially the epidermis. Cystatin α is located in KHG and the cornified envelopes of the skin, as shown by immunofluorescent and immunoelectron studies. Although the reason why the specific fluorescence on KHG is not observed in cryostat sections is not understood, the fluorescence of KHG in Lowicryl

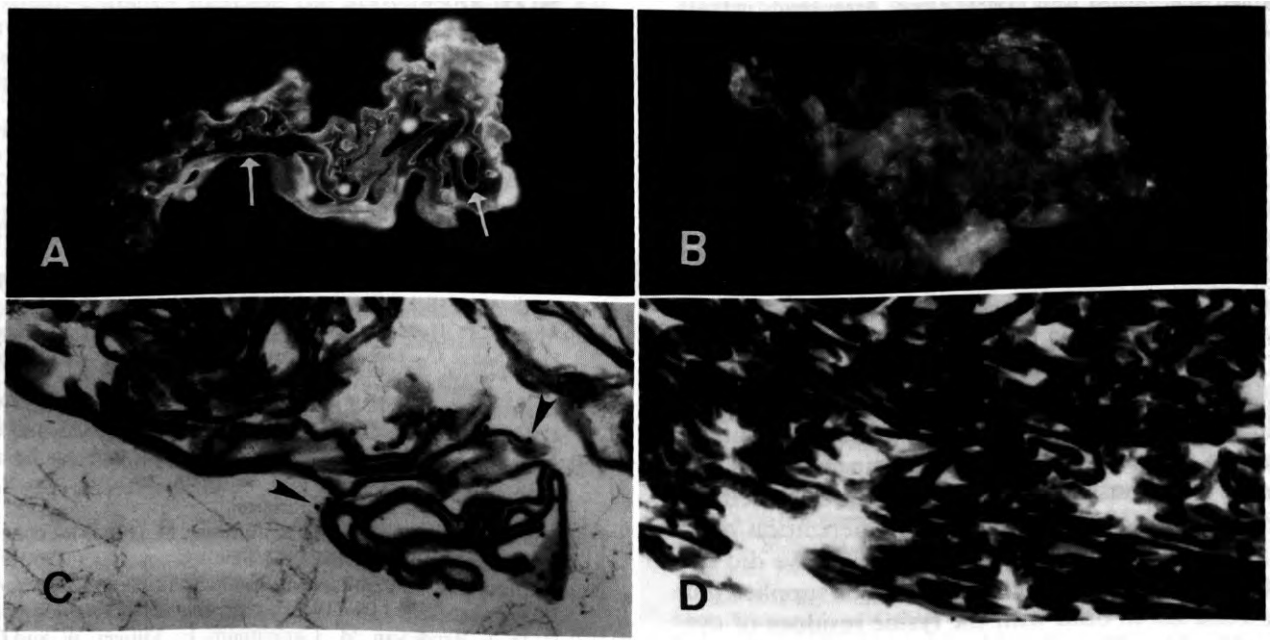

Fig. 3. Immunofluorescent and immunoelectron microscopic examinations of cornified envelopes using anti-cystatin α PoAb. (A,B) Immunofluorescent examination. (C,D) Immunoelectron microscopic examination. (A,C) Stained with anti-cystatin α PoAb. (B,D) Stained with preimmune rabbit serum. Arrows indicate specific fluorescence of cornified envelopes. Arrowheads indicate gold particles bound to cornified envelopes. Magnification A,B, ×350; C,D, ×45,000.

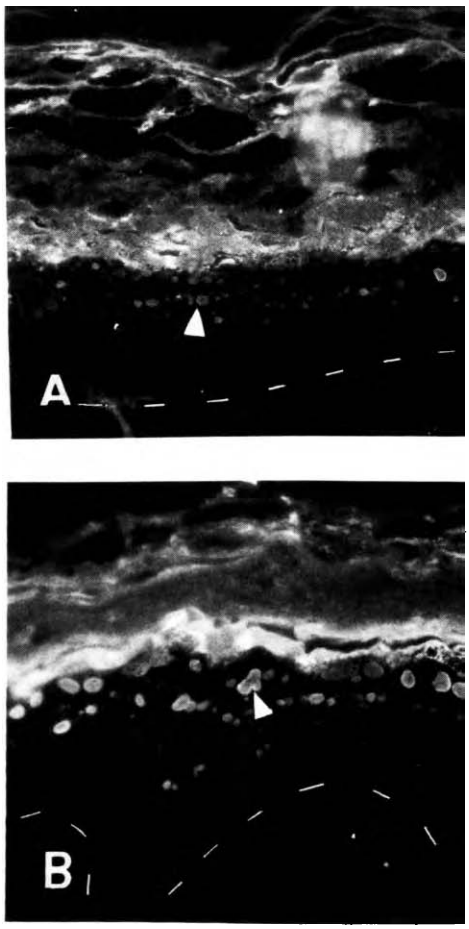

Fig. 4. Inhibition of cystatin α incorporation into KHG by sphingosine treatment; indirect immunofluorescent study of newborn rat skin using anti-cystatin α PoAb. (A) Skin treated with sphingosine-DMSO solution. (B) Skin treated with DMSO alone. Arrowheads indicate KHG and dotted line indicates the dermal-epidermal junction. Magnification ×350.

K4M-embedded sections is specific, because it could not be observed when either the antiserum previously absorbed by the purified P-cystatin α or the pre-immune serum were used. This may be caused by masking of the antigenic site. Also data from an ELISA indicate that P-cystatin α is contained in the prepared cornified envelopes (data not shown).

Cystatin α in KHG is phosphorylated by protein kinase C, and this phosphorylation is important in targeting cystatin α into the granules. There is probably a specific receptor for P-cystatin α on the KHG, but the mechanism of recognition of P-cystatin α is still unknown. We demonstrated that one of the cornified envelope proteins was produced by polymerization of P-cystatin α with epidermal TGase. However, we did not identify the partner substrate protein that supplies glutamine residues to bind with the lysine residues of cystatin α. The reversible reaction of TGase may be useful for separating these proteins from the conjugate polymer [8] for its identification.

From the above results, we speculate that post-translational modification of cystatin α and its translocation during epidermal differentiation may occur as illustrated in Fig. 5.

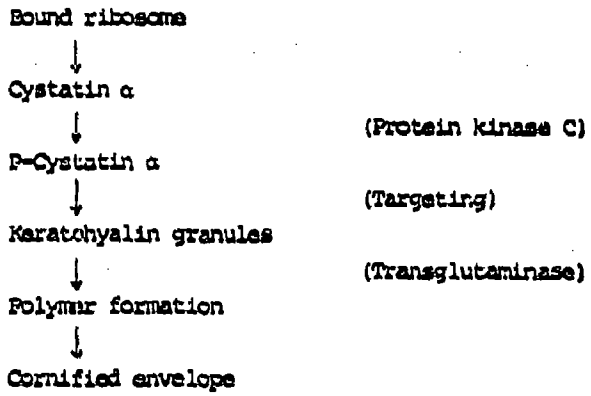

Fig. 5. Proposed hypothesis on post-translational processing, targeting and cornified envelope formation of cystatin α in skin.

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