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Identification of hematoxylin-stainable protein in epidermal keratohyalin granules as phosphorylated cystatin α by protein kinase C

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The hematoxylin-stainable protein (HSP) in keratohyalin granules of the newborn rat epidermis was found to have the same amino acid composition and the same inhibitory and immunological properties as cystatin α. However, only its pI value (4.7) differed from that of cystatin α (5.3). Alkaline phosphatase treatment of HSP changed its pI value from 4.7 to 5.3. This pI change was inhibited by EDTA, an inhibitor of alkaline phosphatase. Furthermore, ^{32}P from [γ-^{32}P]ATP was incorporated into recombinant cystatin α by a protein kinase C (PKC) preparation in the presence of phosphatidyl serine and Ca^{2+} ions as co-factors. The incorporation increased dose-dependently with the added cystatin α and was inhibited significantly by H-7, a specific inhibitor of PKC. SDS-PAGE autoradiography of the ^{32}P-labeled proteins showed that ^{32}P was incorporated into the cystatin α. This incorporation was not observed by the action of cAMP-dependent protein kinase. Therefore, it is highly possible that the HSP is a phosphorylated cystatin α and that the phosphorylation is catalyzed specifically by PKC.

Phosphorylated cysteine proteinase inhibitor; Hematoxylin stainable protein; Cystatin α

1. INTRODUCTION

In a previous study we demonstrated the presence of cystatin α in keratohyalin granules in the rat epidermis in vivo and proposed that the hematoxylin-stainable protein (HSP) in these granules is a cystatin α derivative [1]. Keratohyalin granules are known to contain a basic, histidine-rich protein, filaggrin [2], which is thought to be functionally related with the aggregation of keratin filaments [3] or the water-holding capacity of the stratum corneum [4]. In contrast, HSP, which is an acidic protein component of keratohyalin granules [5], is similar to cystatin α; both are cysteine proteinase inhibitors, and the two proteins have the same amino acid compositions, molecular weights, and inhibitory and immunological properties. However, the pI value of cystatin α is 5.3 whereas that of HSP is 4.7, as shown by analytical isoelectric focusing [1]. This finding suggests that HSP is a phosphorylated form of cystatin α, like the egg white cystatin reported by Laber et al. [6].

In this study, we investigated the dephosphorylation of HSP using an alkaline phosphatase and the phosphorylation of recombinant cystatin α using a partially purified protein kinase C (PKC).

Abbreviations: HSP, hematoxylin-stainable protein; PKC, protein kinase C

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2. MATERIALS AND METHODS

2.1. *Materials*

Alkaline phosphatase, histone III-S and cyclic AMP-dependent protein kinase were purchased from Sigma (USA). Superose 12 and Mono Q anion-exchange columns and ampholines were from Pharmacia-LKB (Sweden). [γ-^{32}P]ATP was obtained from NEN (USA). Recombinant cystatin α was prepared by the method of Katunuma et al. [7].

2.2. *Purification of HSP*

Rat HSP was purified to homogeneity in 50 mM Tris-HCl (pH 7.4) from 3-day-old rat (Sprague–Dawley strain) epidermis by preparative isoelectric focusing, Superose 12 gel filtration and Mono Q anion-exchange column chromatography were carried out as described previously [8].

2.3. *Alkaline phosphatase treatment of HSP*

HSP was dissolved in 50 mM Tris-HCl (pH 9.0) containing 1 mM $MgSO_4$ and 0.1 mM $ZnSO_4$, incubated at 37°C for 6 h with 7.5 μg of alkaline phosphatase and subsequently analyzed by isoelectric focusing. The inhibitory effect of 10 mM EDTA on the reaction was also tested.

2.4. *Partial purification of PKC*

PKC was partially purified from rat brain by Ono's method [9]. Briefly, rat brain was homogenized in 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100000 × g for 1 h, and the supernatant was applied to a Mono Q anion-exchange column, previously equilibrated with 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA and 10 mM 2-mercapthoethanol. PKC was eluted from the column with a linear gradient of NaCl. The fraction of the eluate with PKC activity was applied to a hydroxyapatite column (Koken, Tokyo, Japan) equilibrated with 20 mM potassium phosphate buffer (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol and 10 mM 2-mercaptoethanol, and PKC activity was eluted with a linear gradient of potassium phosphate.

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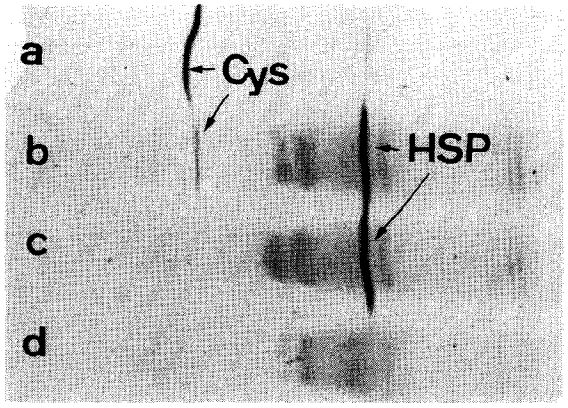

Fig. 1. Analytical isoelectric focusing of HSP after treatment of alkaline phosphatase. (a) Recombinant cystatin α; (b) HSP treated with alkaline phophatase, $ZnSO_4$ and $MgSO_4$; (c) HSP treated with alkaline phosphatase, EDTA; (d) alkaline phosphatase. The arrow indicates newly produced cystatin α by treatment with alkaline phosphatase, which showed the same pI value as cystatin α

2.5. *Cystatin α-dependent incorporation of ^{32}P into protein by PKC*

Recombinant cystatin α or histone III-S was incubated with 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, 20 μl of 0.5 mg/ml phosphatidyl serine, 10 mM 2-mercaptoethanol and partially purified PKC in 200 μl of 25 mM Tris-HCl buffer (pH 7.5) for 10 min at 37°C. The reaction was terminated by adding 20% trichloroacetic acid (TCA) and then 5% TCA and 0.1% BSA were added to precipitate the proteins. The precipitated protein was washed with 5% TCA and solubilized in 1 M NaOH [10]. The inhibition analysis was also performed in the presence of 2 mM of H-7. Cystatin α-dependent incorporation of ^{32}P was assayed by the Cerenkov effect [11].

2.6. *Analytical isoelectic focusing and SDS-PAGE autoradiography of cystatin α with incorporated ^{32}P*

The materials obtained in the alkaline phosphatase reaction were subjected to analytical isoelectric focusing in the pH range of 4.0 to 6.5 [12], and then the gel was stained with Coomassie brilliant blue.

For the observation of the incorporation of ^{32}P into cystatin α by the action of PKC, cystatin α (100 μg) was incubated with a partially purified PKC, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 10 μM [γ-^{32}P] ATP, 20 μl of 0.5 mg/ml phosphatidyl serine and 10 mM 2-mercaptoethanol in 200 μl of 25 mM Tris-HCl buffer (pH 7.5), for 10 min at 37°C [10]. The inhibitory effect of 20 mM H-7 was also examined. For the observation of the incorporation of ^{32}P into cystatin α by the action of cAMP-dependent protein kinase, cystatin α (100 μg) was incubated with 10 mM $Mg(CH_3COO)_2$, 20 μM [γ-^{32}P]ATP, 1 μM cAMP and cAMP-dependent protein kinase in 200 μl of 25 mM Tris-HCl buffer (pH 7.5) for 10 min at 37°C [13]. This inhibitory effect of 1 mM H-8 was also examined. The reactions were terminated by the addition of TCA and then 5% TCA and 0.1% BSA were added to precipitate proteins. The precipitates were washed with cold 90% acetone to remove TCA and then solubilized in 1% SDS solution and subjected to SDS-PAGE in 15% acrylamide gels [14].

3. RESULTS

After treatment with alkaline phosphatase, HSP changed from pI 4.7 to a protein band of pI 5.3 on isoelectric focusing (Fig. 1b). This newly formed protein band corresponded with that of recombinant cystatin α (Fig. 1a). This pI 5.3 protein band was not formed in the co-existence with an inhibitor of alkaline phosphatase (Fig. 1c).

Results on cystatin α-dependent and histone III-S-dependent incorporations of ^{32}P into protein fractions by partially purified PKC are shown in Table I. ^{32}P was incorporated into both cystatin α and histone III-S by activated PKC. The incorporation increased dose-dependently with added cystatin α and was inhibited significantly by the addition of H-7, a specific inhibitor of PKC. To reconfirm that ^{32}P was incorporated into the cystatin α molecule, cystatin α was treated with activated PKC and [^{32}P]ATP under the same conditions as described above and then the ^{32}P-labeled proteins were examined by SDS-PAGE and autoradiography. As shown in Fig. 2, a clear band of ^{32}P-labeled protein was observed in the position of cystatin α (arrow). However, the incorporation of ^{32}P into the cystatin band is inhibited completely in the presence of H-7 inhibitor. In addition, this ^{32}P incorporation into cystatin α was not observed by the action of cAMP-dependent protein kinase, although added histone was phosphorylated by the cAMP-dependent protein kinase. These findings suggested that HSP in keratohyalin granules of the epidermis could be a phosphorylated cystatin α and that the cystatin α could be phosphorylated by the action of PKC during targetting of cystatin α into the granules.

Table I

Incorporation of ^{32}P into cystatin α by protein kinase C

Substrate	Inhibitor	^{32}P incorporated into protein fraction (cpm)	^{32}P incorporated into cystatin α or histone (cpm)
-	-	523.9	
-	+ H-7	448.0	
Cystatin α (86 μg)	-	1146.4	622.3
Cystatin α (86 μg)	+ H-7	711.4	263.3
Cystatin α (172 μg)	-	1363.1	839.3
Cystatin α (172 μg)	+ H-7	789.1	341.1
Histone (86 μg)	-	6820.4	6296.9
Histone (86 μg)	+ H-7	792.3	344.3

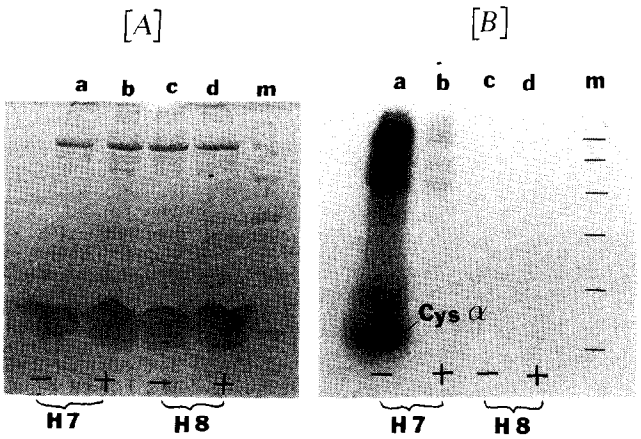

Fig. 2. SDS-PAGE autoradiography of ^{32}P incorporation into cystatin α. The gel was stained with Coomassie brilliant blue solution (A) and also examined by autoradiography (B). For the latter, the dried gel was exposed to Fuji X-ray film for 5 days. The arrows indicate the band of cystatin α. (a) cystatin α treated with PKC; (b) cystatin α treated with PKC in the presence of H-7; (c) cystatin α treated with cAMP-dependent protein kinase; (d) cystatin α treated with cAMP-dependent protein kinase in the presence of H-8; (m) molecular weight markers (top to bottom): phophorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), lactalbumin (14.4 kDa).

4. DISCUSSION

The inhibitory activity of HSP was quite similar to cystatin α as previously reported [1]. Since both these proteins immunologically cross-reacted [1], they should have many common epitopes in their molecular structure. The only known difference between these two proteins is that the isoelectric point of HSP is 4.7, whereas that of cystatin α is 5.3. Takeda et al. reported cystatin α derivatives with higher isoelectric points than that of cystatin α, due to the elimination of acidic amino acids from the N-terminal region [15]. In preliminary studies, we found that the N-terminus of HSP is masked, but that several amino acids in its C-terminal region are identical with those in cystatin α. Moreover, no protein spot with a higher isoelectric point was observed when the epidermal extract was examined with antibodies to cystatin α or by protein staining for cystatin α. These findings suggested that HSP might have a different isoelectric point from that of cystatin α because it is phosphorylated, like egg white cystatin [6]. In the present investigation we found that on treatment of HSP with alkaline phosphatase, its pI value shifted to that of cystatin α, although the amount of protein migration was low. The negative charges of the phosphate moiety could be responsible for lowering the isoelectric point from 5.3 (cystatin α) to 4.7 (HSP). It is unknown why so little reaction product was obtained on treatment with alkaline phosphatase (Fig. 1b).

The preparation of PKC used in this work was not pure enough, and still contained some phophorylatable proteins by CPK. Also, phosphorylatable site(s) in cystatin α molecule must be small in number(s) compared to histone. Although only a small amount of ^{32}P was incorporated into cystatin α, the incorporation increased with higher concentrations of cystatin α added. X-ray crystallographic studies have shown that the binding site with the cathepsins is located in N-terminal region [16]. Moreover, previous observations showed that the inhibitory profiles of cystatin α and HSP are similar. Thus the site is probably phosphorylated in the C-terminal region which is not important for the inhibitory activity. The amino acid in the HSP molecule that is phosphorylated is probably a serine or threonine residue because HSP did not react with anti-phosphotyrosine monoclonal antibody (data not shown). In addition, this ^{32}P incorporation could be a specific action of PKC, because ^{32}P was not incorporated into cystatin α by the action of cAMP-dependent protein kinase.

In general, serine or threonine located between basic amino acids are easily phosphorylated by PKC. From these aspects, it is possible to speculate that the possible phosphorylating site of cystatin α is the threonine in the -N-K-T-K-N- sequence located near the C-terminus. It will be confirmed in the near future.

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