Hydrolysis of histones by proteinases

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Hydrolysis of histones by proteinases from rat liver, skin and other sources was studied by using a rat thymus histone preparation as the substrate and polyacrylamide-gel electrophoresis and densitometric analysis as the methods to detect histone subtypes and their hydrolysis. The rat mast-cell proteinase I effectively hydrolysed histones except type H4. Thrombin hydrolysed effectively histones H1 and H2A, whereas plasmin hydrolysed all types of histones. Cathepsin D hydrolysed especially histone H2A. Cathepsins B and L hydrolysed all histones more slowly, and cathepsin H hydrolysed them extremely slowly. Epidermal aminoendopeptidase did not hydrolyse histones. Trypsin and chymotrypsin were used as reference enzymes, which hydrolysed all types of histones in very low concentrations. This study suggests that a variety of proteinases could play a role in histone hydrolysis. Hydrolysis of a specific subtype of histones, such as histone H2A at pH 6 by cathepsin D, may be directly involved in regulation of epidermal-cell differentiation.

INTRODUCTION

One of the unique features of epidermal keratinocyte differentiation is the loss of the nucleus. During terminal differentiation nucleic acids and proteins disappear from the nucleus, although an immunohistochemical study has demonstrated that the nuclear envelope is retained in keratinized cells [1]. Apparently proteinases and DNAases are involved in the processes, and hydrolysis of histones must be one of the major catabolic events. Histone-hydrolysing activity has been demonstrated in psoriasis, the skin disease associated with increased cell turnover [2]. Plasmin has been suggested to be involved in the final epidermal-cell maturation [3], and the amounts of plasminogen activator were found to be increased in psoriatic epidermis [2,4].

Tissues rich in cells with rapid turnover rates, such as thymus, have been shown to contain histone-hydrolysing proteinases [5–8]. Histone-hydrolysing proteinase activity has also been described in rat liver chromatin [9–11], chicken erythrocytes [12], and various other rat tissues [13]. Principally, two different kinds of enzyme activities have been demonstrated to hydrolyse histones; one is a neutral serine enzyme present in high-salt extracts [2,5,9] and the other has an acidic pH optimum [5]. In addition, enzymes which hydrolyse a specific type of histone, such as H1 [6–8,11], H2A [7,8,13,14], H2B [6,13], H3 [6–8] and H4 [13], have been recognized, although biochemical characterization and identification of most of these proteinases have not been completed. Furthermore, the cellular distribution of the histone hydrolases is still unclear, and contamination(s) from other cells and plasma is possible [15,16]. Degradation of histones, by various proteolytic enzymes has been described by many authors, but has not been studied systematically.

MATERIALS AND METHODS

Chemicals

[1,3-³H]Di-isopropyl fluorophosphate (5.5 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Human urokinase was from Green Cross (Osaka, Japan); CM-cellulose CM-52 was from Whatman (Maidstone, Kent, England); Sephadex G-75 and G-100, lysine-Sepharose CL 4B, Sepharose 4B and protein standards for electrophoresis were from Pharmacia (Piscataway, NJ, U.S.A.); dithiothreitol (DTT) was from Calbiochem-Behring (La Jolla, CA, U.S.A.); 7-amino-4-trifluoromethylcoumarin (AFC), Arg-AFC, Z-Arg-AFC, Z-Ala-Arg-Arg-AFC, Bz-Val-Lys-Lys-Arg-AFC and Z-Phe-Arg-AFC were from Enzyme Systems Products (Livermore, CA, U.S.A.); Spectrapor dialysis tubing with a *M_r* cut-off limit of 5000–8000 was from Spectrum Medical Industries (Los Angeles, CA, U.S.A.); all other chemicals were of analytical grade, from Mallinkrodt (Paris, KY, U.S.A.), Eastman Kodak (Rochester, NY, U.S.A.) or Sigma (St. Louis, MO, U.S.A.); YM-5 and YM-10 filter membranes were from Amicon (Lexington, MA, U.S.A.); Transpore surgical tape was from 3M Medical Products Division (St. Paul, MN, U.S.A.). Double-distilled water was used.

Purification of histones and detection of histone hydrolysis

Histones were purified from thymuses (total wt. about 6 g) from 12 Sprague–Dawley rats (about 2 months old) in accordance with the principles of Martinson *et al.* [17], with slight modifications. Thymuses were homogenized in 5 vol. of ice-cold 75 mM-NaCl/24 mM-EDTA, containing 0.2% (v/v) octanol, 0.2 mM-*p*-hydroxymercuribenzoate, 2 mM-phenylmethanesulphonyl fluoride and

Abbreviations used: AFC, 7-amino-4-trifluoromethylcoumarin; NA, naphthylamide; Bz-, benzoyl-; DTT, dithiothreitol; RMCP-I, rat mast-cell proteinase I (= chymase); Z-, benzyloxycarbonyl-.

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1 mM-*p*-chloromercuribenzenesulphonic acid (pH 4.5–5.0). After extraction of nuclei with 200 mM- H_2SO_4 , the histones were precipitated in 10 vol. of ice-cold acetone at -20°C overnight. The histone preparation was washed three times with cold acetone, centrifuged (8000 *g* for 30 min), freeze-dried, and stored at -20°C . The yield of histone protein was approx. 50–80 mg. The purity of histone preparations was confirmed by SDS/polyacrylamide-gel electrophoresis [18].

The incubation mixture for analysis of histone hydrolysis consisted of 25 μl of histones (4 mg/ml in 1 M-NaCl), 25 μl of sample, 25 μl of 200 mM buffer solution (at appropriate pH) and 25 μl of water or modifier solution. The incubation was carried out in silicone-treated glass tubes at 37°C for 60 min. The reaction was terminated by adding 300 μl of solution containing 100 mM-glycine, 0.2% (w/v) SDS and 4 M-urea (pH 10.0) and heating the mixture at 90°C for 10 min. A 60 μl sample of the final mixture was taken for SDS/polyacrylamide-gel electrophoresis to analyse hydrolysis of histones as described below. The amount of histones was kept constant in the incubation mixture, whereas different enzyme (sample) dilutions were used.

Assay procedures

Cathepsin D assay was performed with haemoglobin as substrate, by the method of Barrett [19], with slight modifications, in citrate buffer, pH 3.5. L-Tyrosine was used for standardization; its absorption coefficient, $A_{280}^{1\text{ cm}}$, was 1080 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$.

Cathepsin L assay was performed with azocasein as substrate, as described by Kirschke *et al.* [20], with slight modifications, in acetate buffer, pH 5.0. The hydrolysis rate was expressed as the change in A_{366} in 1 min.

Cathepsins B, H and L were assayed with synthetic peptide substrates with the AFC detection group at the C-terminal end, in accordance with the principles of Pearson *et al.* [21] and Mason *et al.* [22], with slight modifications. Z-Arg-Arg-AFC, Z-Ala-Arg-Arg-AFC and Bz-Val-Lys-Lys-Arg-AFC were used for cathepsin B assay. Arg-AFC was used for cathepsin H and Z-Phe-Arg-AFC for cathepsin L assay. The reaction mixture, in a 1 ml cuvette, contained 25 μl of 10 mM substrate (in dimethylformamide), 175 μl of water and 250 μl of 200 mM-sodium phosphate buffer, pH 6.2, containing 2 mM-EDTA and 2 mM-DTT, and 50 μl of sample. The fluorescence was measured at 37°C with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. For cathepsin L the buffer was 200 mM-acetate buffer, pH 5.5, containing 2 mM-EDTA and 2 mM-DTT. The calibration was done as described by Smith *et al.* [23]. Standardization was done with AFC (in dimethylformamide) solubilized in the reaction mixture, except that the sample was replaced with water.

Cathepsin H was assayed with Arg-NA and Bz-Arg-NA substrates as described by Barrett & Kirschke [24], with the modification of Ito *et al.* [25], in phosphate buffer, pH 6.0. β -Naphthylamine was used as standard, at 545 nm.

Protein was assayed as described by Lowry *et al.* [26], with bovine serum albumin as standard.

Electrophoretic procedures

SDS/polyacrylamide-gel electrophoresis for analysis of histone hydrolysis was performed as described by Panyim & Chalkley [18], with slight modifications, in

15%-acrylamide gels. The proteins in the gels were stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany). Separated histone proteins were quantified by densitometric scanning of the gels (6 mm $\times$ 110 mm) at 610 nm.

Extraction and fractionation of epidermal proteinases

Separation of epidermis and extraction of epidermal proteins were done as described previously [27].

The low-salt extract (in 20 mM-Pipes/150 mM-NaCl/5 mM-EDTA/5 mM-DTT, pH 6.5) was dialysed for 16 h against 40 vol. of 20 mM-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA and 1 mM-DTT, and centrifuged at 8000 *g* for 30 min. The proteins in the supernatant were further fractionated on a CM-cellulose CM-52 column [27]. U.v. absorbance at 280 nm and hydrolysis rates of Arg-NA at pH 6.0, Z-Arg-Arg-AFC at pH 6.2, haemoglobin at pH 3.5 and azocasein at pH 5.0 by the fractions (3.2 ml) were measured. The fractions were pooled into five groups, and each was concentrated by use of an Amicon YM-5 membrane and tested for histone-hydrolysing activity at pH 6.0. The fractions hydrolysing histone H2A were pooled, adjusted to pH 3.5 with HCl, and the enzyme preparation was further purified on a pepstatin-aminohexyl-Sepharose 4B column. The affinity material was prepared as described by Huang *et al.* [28]. The enzyme preparation was applied at 50 ml/h to a pepstatin-aminohexyl-Sepharose 4B column (2.0 cm $\times$ 3.0 cm) equilibrated with 20 mM-sodium citrate buffer, pH 3.5, containing 200 mM-NaCl. The column was washed with 140 ml of equilibration buffer and the proteins were eluted with 70 ml of 50 mM-Tris buffer (pH 8.5)/200 mM-NaCl. The protein peak was dialysed against distilled water for 2 days, freeze-dried and stored at -70°C .

A peptidase from the newborn-rat skin hydrolysing both Leu-NA and Bz-Arg-NA, which has been termed 'aminoendopeptidase', was purified as described by Ito *et al.* [25].

Purification and activation of plasminogen

Plasminogen was purified from plasma of adult Sprague-Dawley rats by using the modification of Deutsch & Mertz [29], on a lysine-Sepharose CL-4B affinity column. Plasminogen was separated from ϵ -aminohexanoic acid by using a Sephadex G-25 column equilibrated with 10 mM- NH_4HCO_3 . Plasminogen was freeze-dried and stored at -70°C .

Rat plasminogen was activated by adding 25 μl of human urokinase (solubilized at a concentration of 200 i.u./ml in 20 mM-Tris/HCl, pH 7.5, containing 0.14 M-NaCl) to 225 μl of plasminogen (0.44 mg/ml, solubilized in 20 mM-Tris/HCl, pH 7.5, containing 0.14 M-NaCl) and incubating for 10 min at 37°C . Active plasmin concentration was calculated by active-site titration with [^3H]di-isopropyl fluorophosphate as described by Schechter *et al.* [30].

Purification of rat liver cathepsins B, D, H and L

The proteinases were purified from adult-rat liver in accordance with the principles of Barrett and co-workers [22,24], with some modifications. Rat livers were gently homogenized in 10 mM-sodium phosphate buffer (pH 6.5)/150 mM-NaCl/1 mM-EDTA/1 mM-DTT, followed by stepwise centrifugation to obtain the lysosomal fraction [20], which was homogenized in 10 mM-

sodium acetate buffer (pH 5.0)/150 mM-NaCl/1 mM-EDTA/1 mM-DTT. The soluble proteins in the concentrated supernatant were further fractionated (flow rate 25 ml/h) on a Sephadex G-100 column (1.9 cm $\times$ 114 cm) equilibrated with the homogenization buffer. Fractions (each 5.3 ml) possessing cathepsin B, D, H and L activities were pooled, and dialysed against 80 vol. of 20 mM-sodium acetate buffer (pH 5.0)/1 mM-EDTA/1 mM-DTT, for 16 h. The proteins were further fractionated on a CM-Sephadex column (1.5 cm $\times$ 15 cm; flow rate 43 ml/h) with a stepwise NaCl gradient [80 mM (cathepsin H), 150 mM (cathepsins B and D) and 400 mM (cathepsin L)] in the equilibration buffer. The pH of the pool containing cathepsins B and D was adjusted to pH 3.5 with 6 M-HCl, and the pool was applied to a pepstatin-aminohexyl-Sepharose affinity column. The fractions eluted through the column were collected and pooled, and the pH was adjusted to 5.0 with 1 M-NaOH. All the pooled fractions were thereafter concentrated with an Amicon YM-5 membrane and stored at -70°C . Cathepsin D bound to the pepstatin-aminohexyl-Sepharose was further purified as described above for epidermal cathepsin D.

Purification of rat mast-cell proteinase I

Rat mast-cell proteinase I (RMCP-I, chymase) was purified from adult-rat skin in accordance with the principles of Seppä & Järvinen [31]. Skin proteins were extracted first in low-salt buffer [27]. The high-salt extract was prepared in 20 mM-sodium phosphate buffer, pH 7.5, containing 2 M-KCl. The soluble fraction was dialysed for 24 h against 100 vol. of 20 mM-sodium phosphate buffer, pH 6.0. The white precipitate formed was collected by centrifugation at 15000 *g* for 30 min. The precipitate was dissolved in 10 mM-sodium phosphate buffer (pH 8.0)/2 M-KCl. After centrifuging at 100000 *g* for 30 min, 3.5 ml of the supernatant fraction was applied to a Sephadex G-100 column (1.9 cm $\times$ 113 cm) equilibrated with 10 mM-sodium phosphate/2 M-KCl (pH 7.5), at 20 ml/h. Fractions hydrolysing azocasein at pH 8.0 were pooled (55 ml), and applied to a D-tryptophan-agarose column (1.6 cm $\times$ 3.2 cm) equilibrated previously with 10 mM-sodium phosphate/2 M-KCl, pH 7.5. The column was washed with equilibration buffer, and the enzyme was eluted with 15 mM-acetyltyrosine ethyl ester (in equilibration buffer), which was removed by dialysis against 100 vol. of 10 mM-sodium phosphate buffer (pH 7.5)/2 M-KCl, for 36 h.

RESULTS

Hydrolysis of histones by crude epidermal extracts

The low-salt extract of newborn-rat epidermis hydrolysed histone H2A selectively at pH 6 (Fig. 1) during a 24 h incubation period, and the optimal pH for H2A hydrolysis was found to be about pH 5 in a 60 min incubation. At lower pH (2–4), hydrolysis of histones H2B and/or H3 and H1, as well as H2A, was detected (results not shown). No hydrolysis of histones by the same extract was detectable at pH 7.5–9 during 24 h of incubation. The high-salt extract did not hydrolyse histones at pH 6–9 in 24 h, nor at pH 3–6 in 60 min incubation (results not shown). The histone H2A hydrolysis by low-salt extract during 60 min incubation was inhibited by 10 μM -pepstatin, but not by phenyl-

Fig. 1. Hydrolysis of rat thymus histones by crude rat epidermal low-salt extract incubated at various pH values for 24 h

Tracks: 1, histones alone; 2, histones + extract; 3, extract alone.

methanesulphonyl fluoride, *p*-hydroxymercuribenzoate and 1,10-phenanthroline in 1 mM concentrations (Fig. 2). Di-isopropyl fluorophosphate, iodoacetamide and *N*-ethylmaleimide in 1 mM concentrations and leupeptin in 0.1 mM concentration did not inhibit histone H2A hydrolysis by low-salt extract.

Hydrolysis of histones by partially purified epidermal proteinases

The low-salt extract of newborn-rat epidermis was fractionated by CM-cellulose chromatography. Fractions were tested for hydrolysis of substrates for cathepsins B, H, L and D and for epidermal aminoendopeptidase (Fig. 3). The appropriate pooled and concentrated enzyme fractions shown in Fig. 3 were tested for histone hydrolysis. Only the enzyme pool 3 that hydrolysed haemoglobin at pH 3.5 readily hydrolysed histone H2A at pH 6.0 (results not shown).

Hydrolysis of histones by purified rat liver cathepsins B, D, H and L and rat skin aminoendopeptidase

Cathepsins B, D, H and L and aminoendopeptidase were tested for hydrolysis of histones in a 60 min incubation. Cathepsins B, H and L hydrolysed all types of histones, whereas cathepsin D hydrolysed especially histone H2A and to some extent also H2B and/or H3, and very slowly H1. Epidermal aminoendopeptidase did not show detectable histone hydrolysis at a concentration of 32 $\mu\text{g}/\text{ml}$, even after 16 h of incubation. The enzyme concentrations giving 50% hydrolysis of histones are shown in Table 1.

Hydrolysis of histones by plasmin, thrombin, RMCP-I, trypsin and chymotrypsin

Plasmin at a concentration of about 20–30 $\mu\text{g}/\text{ml}$ hydrolysed all types of histones in a 60 min incubation,

Fig. 2. Inhibition profile of histone H2A hydrolysis by the crude rat epidermal extract at pH 6.0 in a 60 min incubation with inhibitors of different proteinase classes

Tracks: 1, histones alone; 2, histones+extract; 3, histones+extract+1 mM-*p*-hydroxymercuribenzoate; 4, histones+extract+1 mM-phenylmethanesulphonyl fluoride; 5, histones+extract+1 mM-1, 10-phenanthroline; 6, histones+extract+10 μ M-pepstatin; 7, extract alone.

as shown in Table 1. Trypsin also hydrolysed all types of histones, at a markedly lower concentration than did plasmin. The enzyme concentrations of various serine proteinases giving 50% hydrolysis of histones are presented in Table 1. All but histone H4 were hydrolysed by RMCP-I. Histone H1A (H1 with higher M_r) and to some extent also H2A were readily hydrolysed by thrombin. Urokinase did not hydrolyse histones even in a 10-fold higher concentration than that used for activation of plasminogen.

DISCUSSION

The low-salt extract of newborn-rat epidermis hydrolysed histone H2A selectively. The enzyme responsible for this hydrolysis was found to be the aspartic proteinase cathepsin D, because of its pH optimum, substrate specificity and inhibitor profile [27,32]. Cathepsin D purified from rat liver lysosomes also hydrolysed, more slowly, histones H2B and/or H3 and H1, whereas H4 was relatively resistant. Other lysosomal cathepsins from rat liver, such as the cysteine proteinases, cathepsins B, H and L, also present in epidermis [27], hydrolysed non-selectively all types of histones. With the crude epidermal extract the hydrolytic effects of cysteine proteinases were not seen, most probably because of the presence of the cysteine proteinase inhibitor [33,34] and/or the requirement for a high concentration of cathepsins B, H and L. These enzymes may, however, be involved in histone hydrolysis in an intact tissue, where enzymes and their

Fig. 3. CM-cellulose chromatography of newborn-rat epidermal extract

Bars (1-5) indicate pools which were used for analysis of histone hydrolysis.

inhibitor(s) could be located in different cell compartments.

Epidermal cells produce plasminogen activator [35], and plasminogen could be activated in epidermis to plasmin, which has been considered to be involved in the final maturation of epidermal cells [3]. We found that plasmin effectively hydrolysed all types of histones. An immunohistochemical study [36] has suggested that plasminogen activator is in the epidermal-cell nuclear region; this interesting finding supports the hypothesis that plasmin may be involved in hydrolysis of histones in epidermis *in vivo*.

There are several serine proteinases which have been detected in the crude epidermal extract [37], although chemical characterization of the enzymes is not complete. Trypsin and thrombin, which hydrolyse peptides with arginine and lysine (the major amino acids of histone peptide chains) at the P1 subsite, were also found to hydrolyse histones effectively. However, chymotryptic proteinases, such as chymotrypsin and RMCP-I, which

Table 1. Hydrolysis of histones by various proteolytic enzymes

Hydrolysis is expressed as the concentration of proteinase (μg of protein/ml) causing 50% hydrolysis of histones (1 mg/ml) during 60 min incubation at 37 °C in the final incubation volume of 100 μl . Enzyme activities in assay conditions are (munits/mg of protein if not otherwise stated): cathepsin B, 73.4 with Z-Arg-Arg-AFC; cathepsin H, 2.97 with Arg-AFC; cathepsin L, 0.59 with Z-Phe-Arg-AFC; aminoendopeptidase 1200 with Leu-NA; cathepsin D, 480 with haemoglobin (tyrosine equivalent; see the Materials and methods section); trypsin, 13900000 with Bz-Arg-OEt (Sigma T-2395); chymotrypsin, 62000 with Bz-Tyr-OEt (Sigma C-7762); RMCP-I, 0.15 A_{366} unit/min per mg with azocasein; thrombin, 4200 NIH units/mg (Sigma T-6759); plasmin with [^3H]di-isopropyl fluorophosphate (see the Materials and methods section). Abbreviation: ND, no hydrolysis was detected under the conditions described.

Enzyme	pH	Concn. giving 50% hydrolysis of histones				
		H1A	H1B	H2A	H2B/H3	H4
Cathepsin B	5.0	110	110	290	190	400
Cathepsin H	6.0	1230	650	760	1500	1430
Cathepsin L	5.0	81	84	310	170	180
Cathepsin D	6.0	5.6	9.0	1.8	6.9	ND
Aminoendopeptidase	7.5	ND	ND	ND	ND	ND
Trypsin	7.5	0.062	0.085	0.14	0.048	0.10
Chymotrypsin	7.5	0.96	1.30	2.10	4.60	1.50
RMCP-I	7.5	55	25	23	25	ND
Thrombin	7.5	23	45	49	ND	ND
Plasmin	7.5	15	14	34	35	41

hydrolyse mainly peptides with tyrosine and phenylalanine at the P1 subsite, also hydrolysed histones. Trypsin- and chymotrypsin-like enzymes are present in skin, especially in mast cells, and they might be involved in the keratinization processes.

This study was done mainly with rat thymus histones and rat proteinases. There could be differences in enzymes that hydrolyse histones in different species. Recently it has been reported [38] that rabbit lung cathepsin D is not able to hydrolyse calf thymus histone H1, whereas in our study rat thymus histone H4 was resistant to rat liver cathepsin D.

Whether or not our results *in vitro* directly apply to the situation *in vivo* is unknown. It could be speculated that histones separated from DNA and used as substrates *in vitro* would not serve as natural substrates *in vivo*. This argument is supported by the report [39] that histones except H1 are resistant to proteinase B in the presence of DNA. However, there are reports that trypsin [12], chymotrypsin [40] and clostripain [41] are able to digest nucleosome core histones, which are bound to DNA. In addition, a nuclear thiol proteinase is involved in degradation of newly synthesized histones which are not bound to DNA [10].

There could be artefactual findings [15,16] in earlier studies reporting proteinases in nuclear fraction of cells, because chromatin effectively binds other proteins with an ionic charge. On the other hand, accumulation of proteins, including proteinases, in the nucleus may result in degradation of nuclear proteins [42]. It could be speculated that a proteinase present within the chromatin structure may be able to destabilize the nucleosome [14], and the chromatin modifications may allow hydrolysis by an enzyme.

A chromatin-associated proteinase has been described [8] which hydrolysed selectively histones H2A and H3 at pH 5–7. In the present study we found that cathepsin D also hydrolysed the same histones in the same pH range. However, cathepsin D distribution in the epidermis is

rather diffuse [43]. There is no direct evidence to support the idea that hydrolysis of histone H2A by cathepsin D results in the disappearance of the nucleus during terminal differentiation.

The present paper demonstrates that several proteinases hydrolyse histones and that some of the proteinases may be involved in hydrolysis of certain specific histone subtypes. Evidently histone-hydrolysing proteinases present in tissues and cells where rapid degradation of the nucleus takes place, such as epidermis, could play an important role in regulation of cell maturation. The hydrolysis of specific histone types by certain proteinases, such as that of H2A by cathepsin D, against which no tissue inhibitors have been found, could play a role in degradation of nucleosomes in epidermis.

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