Partial Purification and Characterization of Two Distinct Types of Caspases from Human Epidermis

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Recent observations demonstrated that interleukin-1β converting enzyme family proteases, now referred to as caspase family, play central roles in apoptosis, or programmed cell death. In this study, we tried to isolate and characterize epidermal caspases. By DEAE-Sephacel anion-exchange chromatography, human cornified cell extract showed two caspase-like fractions (F-I and F-II) with different substrate specificities. These were further purified by Sephacryl S-200, Mono Q ion exchange and Superose 6 gel chromatography. F-I showed a molecular weight of 30 kDa and specifically hydrolyzed acetyl-Asp-Glu-Val-Asp-methylcoumarinamide, a fluorogenic substrate for caspase-3 (CPP32) with a $K_m$ value of 13.8 $\mu$M. F-I generated a characteristic 85 kDa fragment from poly(ADP-ribose) polymerase. Inhibitor susceptibility of F-I was very similar to that of caspase-3, further confirming the caspase-3-like properties of F-I. In contrast, the molecular weight of F-II was estimated to be 110 kDa, which was much higher than the other caspases. F-II equally hydrolyzed acetyl-Asp-Glu-Val-Asp-methylcoumarinamide, and acetyl-Tyr-Val-Ala-Asp-methylcoumarinamide, caspase-1 (interleukin-1β converting enzyme)-specific substrate, and was inhibited by acetyl-Tyr-Val-Ala-Asp-aldehyde and acetyl-Tyr-Val-Ala-Asp-aldehyde. Affinity labeling using biotinylated YVAD-cmk demonstrated several positive bands ranging from 25 to 35 kDa, supporting the hypothesis that F-II is a complex of multiple caspases. Reverse transcriptase-polymerase chain reaction analysis demonstrated that among known caspases tested, caspase-1, -2, -3, -4, and -7 were expressed in cultured human keratinocytes. These results suggest that multiple caspases are synthesized in human keratinocytes and are involved in terminal differentiation.


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poptosis, or programmed cell death, plays important roles in various physiologic processes, such as development of organisms, tissue homeostasis, and recovery from pathologic disorders. Epidermis is one of the tissues in which apoptosis-like phenomena are often found. For example, “sunburn cells” were frequently observed in UVB-irradiated epidermis, in which nuclear condensation was a characteristic feature of these cells (Young, 1987; Schwarz et al., 1995). Regression phase of hair follicles (catagen) was thought to be an apoptotic process (Seiberg et al., 1991; Haake and Polakowska, 1993; Polakowska et al., 1994). The molecular mechanism of epidermal apoptosis has not been well understood, however.

Detailed studies on genetic pathways of programmed cell death in nematode C. elegans demonstrated that ced-3 was essential for the execution of the cell death program (Yuan and Holvitz, 1990). Cloning of ced-3 gene showed significant sequence homology to the mammalian cysteine protease, interleukin-1β converting enzyme (ICE) (Yuan et al., 1993). In addition, over-expression of ICE was found to induce apoptotic cell death in rat fibroblasts (Miura et al., 1993). The fact that inhibition of ICE activity by a potent ICE inhibitor, cytokine response modifier A (CrmA) from cowpox virus (Ray et al., 1992), prevented apoptosis in various cells (Miura et al., 1993; Gagliardini et al., 1994; Tewari and Dixit, 1995a) further supported critical roles of ICE in higher organisms. A number of attempts to hunt for enzymes similar to ICE have been reported elsewhere, and 10 human ICE-related genes have been cloned until recently. Such ICE-like proteases were classified as ICE/ced-3 family, which was recently named caspase family (Alnemri et al., 1996).

On the other hand, very limited reports were available for the purification of active caspases from cells and tissues. Active caspase-1 (ICE) has been purified from human monocyte THP.1 cells (Thornberry et al., 1992; Miller et al., 1993). Caspase-3 (CPP32/YAMA/apopain), which was identified as one of the ICE homologs (Fernandes-Alnemri et al., 1994; Tewari et al., 1995b), has been purified from THP.1 cells as a protein responsible for cleavage of poly(ADP-ribose) polymerase (PARP) during apoptosis (Nicholson et al., 1995). And also, Pai et al. have partially purified two distinct types of proteases that cleave sterol-regulatory element binding protein (SREBP) from hamster liver. Peptide sequence analysis revealed that they were identical to caspase-3 and caspase-7 (Mch3/ICE-LAP3/CMH-1), respectively (Pai et al., 1996).

In this study, we tried to investigate participation of caspase-like proteases in keratinocyte differentiation. The involvement of caspases

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in proliferation and differentiation of keratinocytes is still being debated. Here we describe the partial purification and biochemical characterization of epidermal caspases. We successfully obtained two distinct types of caspase-like proteases from normal human cornified cell extract. These two fractions, which are referred to as F-I and F-II, were quite different from each other in terms of molecular weight, substrate specificity, and inhibitor susceptibility, yet both hydrolyzed PARP with the cleavage product of 85 kDa fragment. Our results suggest that multiple types of caspases participate in the epidermal apoptosis and would be related to the terminal differentiation of keratinocytes.

MATERIAL AND METHODS

Materials Synthetic fluorogenic tetrapeptide substrates [acetyl-Tyr-Val-Ala-Asp-methylcoumarinamide (Ac-YVAD-MCA), acetyl-Asp-Glu-Val-Asp-methylcoumarinamide (Ac-DEVD-MCA)], tetrapeptide inhibitors [acetyl-Tyr-Val-Ala-Asp-aledehyde (Ac-YVAD-CHO), acetyl-Asp-Glu-Val-Asp-aledehyde (Ac-DEVD-CHO)], and protease inhibitors (E-64 and pepstatin A) were purchased from Peptide Institute (Osaka, Japan). Biotinyl-Tyr-Val-Ala-Asp-chromolymethylethone (biotin-YVAD-cmk) was a product of Calbiochem (La Jolla, CA). Protease inhibitors phenylmethylsulfonyl fluoride and N-ethylmaleimide were obtained from Wako Pure Chemicals (Tokyo, Japan). Trypsyl-lysin chroomolymethylethone, aprotinin, leupeptin, and antipain were from Boehringer (Mannheim, Germany), and iodoacetic acid, soybean trypsin inhibitor were from Sigma (St. Louis, MO), DEAE-Sepacel, Sepacel H-250, FPLC Mono Q, and Precision Column Superose 6 for SMART system were purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

Assay of caspases Caspase activity was measured using fluorogenic substrates Ac-YVAD-MCA or Ac-DEVD-MCA (Miller et al, 1993). Briefly, 50 µl of samples were incubated at 37°C for 30 min with 50 µM of substrate in 150 µl of 25 mM HEPES containing 10% sucrose, 0.1% 3-[3-cholamidopropyl]dimethylammonio]propane-sulfonic acid (CHAPS) and 1 mM dithiothreitol (HSCD buffer). The reaction was stopped by adding 2 ml of 0.1 M chloroacetic acid. Liberation of aminomethylcoumarin was measured by the fluorescence intensity at 460 nm excited by 380 nm recorded on a FP-777 fluorophotometer (Jasco, Tokyo, Japan). Inhibitor assays were performed similarly by adding 50 µl of inhibitor samples. Inhibitor susceptibility was estimated by the residual caspase activity after incubation at 37°C for 30 min using phosphate-buffered saline as a control.

Protein assay Protein concentration was determined by bicinchoninic acid (BCA) method with BCA Protein Assay Reagent (Pierce, Rockford, IO) using bovine serum albumin as a standard.

Purification of caspase-like enzymes from cornified cells All purification steps were carried out at 4°C. Human cornified cells (30 g) scraped from heels of healthy volunteers (age 27–42) were homogenized in 0.1 M Tris-HCl, pH 8.0 containing 0.14 M NaCl, 0.1% CHAPS, and 1 mM dithiothreitol with a glass homogenizer (Wheaton, Millville, NJ). The resulting suspension was centrifuged for 2 h at 25,000 g and the supernatant was filtered with a bottle-top filter unit (0.22 µm) equipped with a glass fiber prefiter. The filtrate was concentrated by ultrafiltration using YM-10 membrane (Amicon, Beverly, MA) and dialyzed overnight against 20 mM Tris-HCl, pH 7.8 containing 10% sucrose, 0.1% CHAPS, and 1 mM dithiothreitol (buffer A). The crude extract was applied to a DEAE-Sepacel ion-exchange column (2.5 cm x 20 cm), equilibrated with buffer A, and was eluted with a two-step linear gradient of NaCl from 0 to 0.3 M and from 0.3 M to 1 M. Enzymatically active fractions were pooled, concentrated, and applied onto a Sepacel S-200 gel filtration column (1.6 x 90 cm) equilibrated with phosphate-buffered saline containing 10% sucrose, 0.1% CHAPS, and 1 mM dithiothreitol (buffer B). The active fractions were concentrated to 2.5 ml and passed through a PD-10 column equilibrated with buffer A. The enzyme fractions were collected and concentrated for further analysis. Finally, caspase-like enzymes were further purified by Superose 6 gel filtration using SMART system to determine their molecular weights using LMW Gel Filtration Calibration Kit (Pharmacia) consisting of blue dextran (2 x 106), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Kinetic studies Enzyme samples in HSCD buffer were incubated with various concentrations of fluorogenic substrates. Initial rate v was calculated from the slope of a double-reciprocal plot (Lineweaver–Burk plots). Kinetic parameters such as Km were determined by Dixon plots.

Western blot analysis of PARP cleavage PARP was purified from bovine thymus according to the method of Yoshuura et al (1978). Purified bovine PARP was incubated with enzyme fractions in HSCD buffer for 30 min at 37°C. Cleavage products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent western blotting. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) at 2 mA per cm² for 2 h. The membranes were immersed in 3% skim milk (Snow Brand, Sapporo, Japan), and incubated with anti-PARP antibody (Enzyme System Products, Dublin, CA), followed by horseradish peroxidase conjugated anti-rabbit IgG. Enhanced Chemiluminescence (ECL) Western Blotting Detection System (Amersham, Bucks, U.K.) was used for detection. Molecular weight of the cleavage product was determined by Prestained SDS-PAGE Standards (BioRad, Hercules, CA).

Affinity labeling and blotting Biotin-labeled YVAD-cmk was used as an affinity ligand to detect caspase-like enzymes. The purified protease samples were incubated with the biotin-YVAD-cmk for 30 min at room temperature, and applied onto 12.5% SDS or native-PAGE gels under nonreducing condition. After transfer to a polyvinylidene fluoride membrane, the enzyme-inhibitor complexes were stained with horseradish peroxidase-conjugated streptavidin (BioRad) and visualized using ECL detection system. Molecular weight was estimated using Biotinylated SDS-PAGE Standards (BioRad).

Cell culture and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis Human keratinocytes (Kurabo, Osaka, Japan) were cultured in a serum-free K-GM medium. Poly (A)⁺ RNA were isolated from the cultured keratinocytes at 50%, 80%, 100% confluency as well as 2 d after confluence (namely 120%) using FastTrack mRNA Isolation Kit (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. For the synthesis of oligo-dT-primed cDNA, 250 ng of poly (A)⁺ RNA was reverse transcribed using 200 Units of SuperScript II (Gibco/BRL, Gaithersburg, MD) primed by 500 ng of oligo-(dT)₁₂₋₁₅ primer (Promega, Madison, WI), in a total volume of 20 µl. After 1 h incubation at 37°C, the reaction was stopped by heating at 95°C for 5 min, and 1 µl of reverse transcriptase products were used as templates for each PCR reaction. The PCR primers of seven caspase family members (caspase-1–7) were designed based on the published mRNA sequences: caspase-1, 5’-CTGCCCAAGTTTGAAGA-3’ and 5’-TTTATGTCCTGGGAAAG-3’ (expected length 310 bp); caspase-2 (Ich-1), 5’-ATGCCGCGCTGA-
RESULTS

Two distinct types of caspases are obtained from cornified cell extract

Figure 1 shows an elution profile of DEAE-Sephasel chromatography. Two active fractions of caspase-like enzymes were eluted at ≈0.25 and 0.30 M NaCl concentrations. These are designated as F-I and F-II, respectively. F-I showed a hydrolytic activity on Ac-DEVD-MCA, a substrate designed for caspase-3, but had little effect on Ac-YVAD-MCA, a substrate for caspase-1. F-I was further purified by Sephacryl S-200 gel column and FPLC Mono Q, respectively. By Sephacryl S-200, F-I showed a DEVD-specific activity at a molecular weight of 30 kDa, and by Mono Q chromatography at pH 7.8, F-I was eluted at ≈0.25 M NaCl (data not shown).

On the other hand, F-II hydrolyzed both Ac-DEVD-MCA and Ac-YVAD-MCA to a similar extent. F-II from DEAE-Sephasel chromatography was further purified by the same steps. When we applied F-II to Sephacryl S-200 gel chromatography, caspase-like activity eluted at much higher molecular weight than 30 kDa (data not shown).

To determine the molecular weight of these enzyme fractions, we used a SMART system equipped with a Superose 6 gel filtration column. Figure 2 shows the elution profiles of F-I and F-II by Superose 6 chromatography, respectively. F-I showed a sharp and single peak at a molecular weight of 30 kDa (Fig 2A). Molecular weight of F-II was estimated to be ≈110 kDa (Fig 2B), which was much higher than those of caspase family members. F-II demonstrated hydrolytic activities on both Ac-YVAD-MCA and Ac-DEVD-MCA substrates.

Kinetic properties We used partially purified F-I and F-II from the Mono Q step as samples for biochemical and enzymologic characterization. Kinetic constants were determined for F-I and F-II using tetrapeptide substrates with a continuous fluorometric assay as shown in Table I. Cleavage activity of Ac-DEVD-MCA by F-I showed Michaelis–Menten kinetics with a \( K_m \) value of 13.8 \( \mu \)M. F-I did not show any detectable activity on Ac-YVAD-MCA in this assay condition. The \( K_m \) value against Ac-DEVD-CHO is 0.0389 nM, which means that Ac-DEVD-CHO is a very specific inhibitor for F-I. F-I demonstrated different kinetic properties from the known caspases.

Inhibitor susceptibility Table II summarizes the result of inhibitor susceptibility for F-I and F-II. F-I was inhibited by thiol-alkylating reagents, N-ethylmaleimide and iodoacetic acid. On the other hand, E-64, which was also a universal cysteine protease inhibitor, did not show any effect on F-I. Other serine, aspartic, and metallo-protease inhibitors had no effect on F-I (Table I). F-II showed unusual inhibitor susceptibility compared with F-I, though it retained basic nature as a caspase. F-II was almost insensitive at 1 mM concentration of N-ethylmaleimide and iodoacetic acid, whereas more than 90% of F-I activity was inhibited. Even at 0.1 M N-ethylmaleimide and iodoacetic acid, ≈30% of F-II activity was observed. When tetrapeptide inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO were used, F-I and F-II showed different inhibition profiles as shown in Fig 3. The activity of F-I was not inhibited by Ac-YVAD-CHO up to 10 \( \mu \)M, but was inhibited by Ac-DEVD-CHO in a concentration-dependent manner. F-II was inhibited by both inhibitors to the similar extent; however, inhibition was rather weak compared with F-I.

Purified caspases have an activity to cleave PARP Because caspase-3 was identified as an enzyme responsible for PARP inactivation during apoptosis, we examined PARP cleavage activity using purified bovine PARP as a substrate. Western blot analysis (Fig 4) showed cleavage product at 85 kDa after incubation with F-I, supporting the hypothesis that F-I is a caspase-3-like protease. When F-II was incubated with the purified PARP, it was also able to generate 85 kDa fragment, showing that F-II shared a caspase-3-like property.

Table I. Kinetic parameters for caspase-like enzymes from epidermis

<table>
<thead>
<tr>
<th>Caspase-like</th>
<th>( K_m ) (( \mu )M)</th>
<th>( K_i ) (nM)</th>
</tr>
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<tbody>
<tr>
<td>Ac-DEVD-MCA</td>
<td>Ac-YVAD-MCA</td>
<td>Ac-DEVD-CHO</td>
</tr>
<tr>
<td>F-I</td>
<td>13.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>F-II</td>
<td>402</td>
<td>1029</td>
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<table>
<thead>
<tr>
<th>Caspase-like</th>
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<tr>
<td>Caspase-3a</td>
<td>9.7a</td>
</tr>
<tr>
<td>Caspase-3b</td>
<td>&lt;1b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Caspase-like</th>
<th>( K_i ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3a</td>
<td>14.3b</td>
</tr>
<tr>
<td>Caspase-3b</td>
<td>0.76b</td>
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</tbody>
</table>

\( \text{n.d.} \) = not determined.
Figure 3. Inhibition profiles of F-I and F-II by synthetic tetrapeptide inhibitors demonstrate that F-I is specific to Ac-DEVD-CHO. F-I and F-II were incubated with tetrapeptide aldehyde inhibitors and the residual activities were measured. F-I was specifically inhibited by Ac-DEVD-CHO but was not inhibited by Ac-YVAD-CHO up to 10 mM. On the other hand, F-II was inhibited by both inhibitors to a similar extent.

Figure 4. F-I and F-II have a cleavage activity on purified bovine PARP. Purified bovine PARP was incubated with F-I and F-II for 30 min at 37°C. After SDS-PAGE, reaction products were analyzed by western blotting using anti-PARP antibody.

Table II. Effect of various protease inhibitors on F-I and F-II

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Residual activity (%)</th>
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</thead>
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<tr>
<td>PMSF</td>
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<td>100</td>
</tr>
<tr>
<td>SBTI</td>
<td>10 µg per ml</td>
<td>100</td>
</tr>
<tr>
<td>TLCK</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>aprotinin</td>
<td>10 µg per ml</td>
<td>100</td>
</tr>
<tr>
<td>antipain</td>
<td>10 µg per ml</td>
<td>100</td>
</tr>
<tr>
<td>pepstatin</td>
<td>50 µg per ml</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>100</td>
</tr>
<tr>
<td>leupeptin</td>
<td>10 µg per ml</td>
<td>100</td>
</tr>
<tr>
<td>E-64</td>
<td>50 µg per ml</td>
<td>100</td>
</tr>
<tr>
<td>iodoacetic acid</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
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<tr>
<td>N-ethylmaleimide</td>
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<tr>
<td></td>
<td>10 mM</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>2.56</td>
</tr>
<tr>
<td>Ac-YVAD-CHO</td>
<td>10 µM</td>
<td>100</td>
</tr>
<tr>
<td>Ac-DEVD-CHO</td>
<td>10 µM</td>
<td>12.6</td>
</tr>
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</table>

Figure 5. Optimum pH of F-I and F-II. The pH dependence of F-I and F-II was measured at various pH values using Ac-DEVD-MCA as a substrate. Buffers used were 25 mM sodium phosphate (pH 5.5–6.7) and 25 mM Tris-HCl (pH 7.0–8.5). Both caspases were most active at pH 7.5.

Figure 6. Affinity labeling reveals that F-II is an oligomer of multiple caspases. F-II from Superose 6 was incubated with biotin-YVAD-cmk for 30 min at room temperature and applied on 12.5% native-PAGE (lane 1) or SDS-PAGE (lane 2) gel under nonreducing conditions. After transfer to a polyvinylidene fluoride membrane, the enzyme-inhibitor complexes were stained with horseradish peroxidase-conjugated streptavidin and visualized using ECL detection system.

Optimum pH values. The pH dependence of F-I and F-II was measured at various pH values using Ac-DEVD-MCA as a substrate. As shown in Fig 5, both fractions were most active at pH 7.5. F-II, however, showed broader pH optimum. F-II showed more than 50% activity even at pH 5.5 compared with that of F-I at pH 7.5, whereas F-I showed sharp pH dependence.

F-II is an oligomer with multiple species. These data suggest that F-II is the most unusual type of caspase ever identified. For further evaluation of F-II, affinity-labeled complex was analyzed by horseradish peroxidase-conjugated streptavidin after incubation with large excess of biotin-YVAD-cmk for 30 min. In native-PAGE, F-II-YVAD complex was detected in a single band at high molecular weight region (Fig 6, lane 1). On the other hand, SDS-PAGE did not demonstrate
any band at high molecular weight, but several bands were observed in the 25–35 kDa range (Fig 6, lane 2). These results suggest that F-II is a complex consisting of different species of caspase family members, and the complex is dissociable in the presence of SDS.

Multiple caspase genes are expressed in human keratinocytes

We designed specific primer pairs for seven caspase family members, caspase-1 to caspase-7, based on the published cDNA sequences. As shown in Fig 7, mRNA of caspase-1 (310 bp), -3 (410 bp), -4 (285 bp), and -7 (917 bp) were detected in cultured human keratinocytes at different stages. Interestingly, expression levels of each caspase showed significant difference. After the cells reached confluency, expression of caspase-7 was no longer detectable. Quantities of amplified cDNA of caspase-1, caspase-2, and caspase-3 were gradually decreased, whereas caspase-4 did not show significant changes and maintained its expression level after the confluent condition.

DISCUSSION

Although apoptosis-like phenomena are often found in human epidermis, participation of caspases has not been reported to date. Thus, it is important to purify and identify the epidermal caspases. This study isolates caspase-like proteases from normal epidermis. Two distinct types of caspase-like proteases (F-I and F-II) were obtained from human cornified cell extract, which were iodoacetic acid and N-ethylmaleimide-sensitive but E-64-insensitive cysteine proteases. This kind of selective inhibitor susceptibility to general cysteine protease inhibitors is a characteristic feature for caspases (Thornberry et al, 1992; Nicholson et al, 1995); however, F-I and F-II showed considerably different properties in terms of activity, molecular weight, substrate specificity, and inhibitor susceptibility. Among human caspases, caspase-3, caspase-6, and caspase-7 possess similar specificity to tetrapeptide DEVD substrate and designated as caspase-3 subfamily (Duan et al, 1996). The low molecular weight fraction, F-I, showed almost identical properties to those of the caspase-3 subfamily. F-I specifically hydrolyzed Ac-DEVD-MCA and was inhibited by Ac-DEVD-CHO. F-I did not show any effect on Ac-YVAD-MCA, and Ac-YVAD-CHO had no effect on F-I activity. Molecular weight estimated by gel filtration is identical to the heterodimeric complex of p20 and p10 (Thornberry et al, 1992). The K_m value of F-I (13.8 µM) is comparable with those of 9.7 µM for caspase-3 from THP1 cells (Nicholson et al, 1995), 13 µM of recombinant caspase-3, and 51 µM of recombinant caspase-7 (Fernandes-Ahrenmi et al, 1997). The K_v (0.0389 nM) is also similar to that of caspase-3, which was lower than 1 nM (Nicholson et al, 1995). These kinetic parameters strongly suggest that F-I is a caspase-3-like protease. Proteolytic cleavage of PARP is a distinctive phenomenon during apoptosis and it is induced by a protease resembling ICE, but not by ICE itself (Lazebnik et al, 1994). Recent studies have shown that protease resembling ICE is a member of the caspase-3 subfamily. Our results also revealed that F-I possessed a limited proteolytic activity on PARP and generated a specific 85 kDa cleavage product. Taken together, F-I is a caspase-3-like cysteine protease.

F-II, on the other hand, has distinct properties compared with other caspases. F-II was not obtained when cornified cells were extracted in the absence of CHAPS (data not shown), thus, the addition of CHAPS is essential for the extraction of F-II. This result suggests that F-II is associated with cell membrane or contains membrane-bound proteins. Although F-II is also an E-64-insensitive cysteine protease, its properties are quite different from other caspases. It hydrolyzed both YVAD and DEVD tetrapeptide substrates with considerably lower affinity, and showed unusually higher molecular weight. The K_m values for Ac-DEVD-MCA and Ac-YVAD-MCA were considerably higher than 9.7 µM for caspase-3 (Nicholson et al, 1995) and 14.3 µM for caspase-1 (Thornberry et al, 1992). Taken together, these results suggest that F-II is different in protease function from known caspases. These unique properties of F-II might be ascribed to a formation of hetero-oligomer consisting of different species of caspase family members. Caspase-like proteases consist of p20 and p10 subunits to form active heterodimer (Thornberry et al, 1992). X-ray crystallography revealed that recombinant caspase-1 protein exists in the form of tetramer consisting of two heterodimers (Walker et al, 1994; Wilson et al, 1994). Because RT-PCR analysis revealed that five different types of caspases are expressed in cultured human keratinocytes, and affinity labeling proved association of several 30 kDa species to form oligomeric F-II, it is reasonable to postulate that multiple species of caspase family members associate each other and form hetero-oligomers. The decrease in affinity to substrates and to inhibitors can be explained by the partial denaturation during the extraction process or the involvement of inactive precursor form (45 kDa) to form hetero-dimerization with different species. Another possibility is a complex formation with unidentified binding protein to regulate caspase activity.

A couple of evidences showed the hetero-oligomerization of caspases could occur, as seen in caspase-3 and caspase-7 (Fernandes-Ahrenmi et al, 1995), caspase-1 and caspase-4 (Gu et al, 1995). Recombinant caspase-1 subunits are in reversible equilibrium to form oligomers in vitro, showing different activity and stability (Talanian et al, 1996). It also reported that C. elegans cell death factor Cед-3 can interact with Cед-4 and Cед-9 (Chinnaiyan et al, 1997; Spector et al, 1997). Recently, human Cед-4 homolog (Apaf-1) has been isolated from cytosolic extract of HeLa cells (Zou et al, 1997). Apaf-1 forms a complex with cytochrome c and caspase-9 (Li et al, 1997). These observations suggest that it is quite possible to associate different caspase family members to form oligomeric protease species like F-II in this study.

RT-PCR analysis demonstrated that at least five caspase mRNA were expressed in cultured human keratinocytes. Expression of caspase-3 and caspase-7 supports the presence of caspase-3-like fraction, F-I, in the cornified cell extract. Expression of multiple caspase family members supports the oligomeric feature of F-II. In addition, our results clearly showed that the production of caspases starts at very early stages when cells are still in log phase proliferation. Although we have no evidence that oligomerization is required for the induction of keratinocyte apoptosis, oligomerization of multiple caspases as seen in F-II seems to be a unique character.

The presence of two distinct fractions may represent an activation process of a caspase cascade in epidermis. It has been shown that caspase-3-like activity is gradually increased depending on the caspase-1-like protease activity in Fas-mediated apoptosis (Enari et al, 1996). This means that caspase-3 is activated downstream of caspase-1 in a sequential caspase cascade. Our results suggest that a similar mechanism may occur in terminal differentiation of keratinocytes. Because YVAD-specific fraction was not extracted from cornified cells, activation of caspase-3 (or caspase-3-like protease) may occur downstream of caspase-1 (or caspase-1-like protease) in terminally differentiated keratinocytes. Concerning the results of biochemical characterization, it might be possible to postulate that F-I is an activated caspase-3-like protease released from F-II, and F-II can be considered as a partially active intermediate complex in the course of the activation process as observed in the case of caspase-3 activation by Apaf complex (Li et al, 1997). It is now evident that the terminal differentiation of keratinocytes involves activation of caspases, in other words, apoptotic processes.
Epidermal apoptosis, however, seems to be much more complicated and likely to involve more factors to form the functional barrier against the environment. Caspases extracted in this study may play important roles in the regulation of epidermal apoptosis and thus for controlling terminal differentiation.

REFERENCES