Processing of native caspase-14 occurs at an atypical cleavage site in normal epidermal differentiation

Andy J. Chien, Richard B. Presland, and Melanie K. Kuechle

Abstract

Caspase-14, a cysteiny1 aspartate-specific protease expressed during epidermal differentiation, is detected exclusively in the cytosolic fraction of epidermis as a complex of procaspase-14 together with caspase-14 large and small subunits. On non-denaturing protein gels, native caspase-14 has a relative electrophoretic mobility of ~80 kDa, which resolves into caspase-14 proform, large and small subunit in SDS-polyacrylamide. Purification of caspase-14 from native skin with subsequent N-terminal sequencing of the small subunit and tryptic digest analysis of the large subunit revealed an atypical processing site between Ile152 and Lys153, which distinguishes it from other caspases described to date that are processed at aspartate residues. Expression of caspase-14 in heterologous systems results in unprocessed procaspase-14 without generation of the large and small subunits that characterize this protein family. However, addition of cellular extracts to purified recombinant human caspase-14 generated immunoreactive peptides indistinguishable from large and small subunits in skin. These data provide evidence for novel processing of caspase-14 suggesting that this enzyme has unique mechanisms of regulation during epidermal differentiation. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Caspase-14; Epidermal differentiation; Heterologous expression; Processing; Sequencing

Caspase-14, the newest member identified in the caspase family of cysteiny1 aspartate-specific proteases, contains all the structural features found in caspase family members, including the catalytic QACRG region and distinct regions containing homology to the large (p20) and small (p10) subunits of other caspase family members (Fig. 1) [1–4]. Perhaps the most consistent and notable observation regarding caspase-14 involves its limited expression to stratifying, cornifying, epithelia [1,5–7]. Immunohistochemical studies reveal localization of caspase-14 to the terminally differentiating, spinous and granular layers of skin, further supporting a role in the process of epidermal ontogenesis [5–7]. Despite these observations, little has been resolved regarding the role of caspase-14 in epidermal physiology, including mechanisms of activation and potential substrates.

Strategies that have previously been utilized to study the activation of other caspases have not been successful with caspase-14, suggesting that activation and processing of caspase-14 may differ from other caspases. In addition, processing of caspase-14 may require other cellular or environmental factors found exclusively in differentiating keratinocytes. The in vivo cleavage site of caspase-14 identified here supports the assertion that caspase-14 processing occurs through a novel mechanism compared to the processing of other caspases described to date. These results will define future directions in the study of caspase-14 function along with the complex interactions and environmental factors that may regulate its function during the terminal differentiation of skin.

Experimental procedures

Purification of native caspase-14. For subcellular fractionation, flash-frozen human foreskins were homogenized in a Tris-EDTA buffer containing 150 mM NaCl using a hand-held homogenizer. The homogenate was first centrifuged at 1000 g resulting in the soluble fraction termed whole-cell lysates. The lysate was subjected to ultracentrifuga-
tion in a Beckman Ty57 rotor at 42,000 rpm for 1 h, and the resultant soluble fraction was considered cytosolic while the pellet was considered the membrane particulate fraction. For large-scale purifications for N-terminal sequencing of the small subunit and mass spectrometer analysis of the large subunit peptide, 8–10 human neonatal foreskins were homogenized in Tris–EDTA buffer containing 2 mM PMSF and 5 mM benzamidine. Samples were then concentrated using a 10,000 Da molecular weight cut off (m.w.c.o.) centrifuge filter (Millipore). Gel filtration was performed using Sephacryl S200 resin from Pharmacia (Piscataway, NJ) with a 50 cm × 1.5 cm column with a flow rate of 23–27 ml/h. The mobile phase was Tris–EDTA with 150 mM NaCl. Fractions of 0.75 ml were collected and alternate fractions were analyzed using SDS–PAGE and immunoblotting. Based on this protocol, the peak of caspase-14 occurred at fraction 23. Fractions 22–24 from the gel filtration column were concentrated using a 10,000 Da m.w.c.o. centrifuge filter prior to ion-exchange chromatography. Ion-exchange was performed using Vivapure Mini H diethylamine anion exchange spin columns from Vivascience/Sartorius (Goettingen, Germany). The resin was washed with 25 mM Tris–HCl, pH 8.0. Elutions were performed using step gradients of increasing NaCl concentration. Eluates were further concentrated using a 10,000 Da m.w.c.o. filter, separated with a 16.5% Tris–tricine gel, and electroblotted onto PVDF using a CAPS-based buffer system. The small subunit was identified by immunoblotting. A parallel sample was stained on the PVDF membrane with Coomassie blue, and the band corresponding to the small subunit was excised. N-terminal sequencing and analysis were performed by the University of California at Davis Molecular Structure Facility (Davis, CA). To confirm the processing site of caspase-14, tryptic digest mapping was performed by mass spectrometer analysis on the large subunit at the University of California at Davis Molecular Structure Facility (Davis, CA).

**Gel electrophoresis and immunoblotting.** Tris–tricine gels (16.5%) and Sequi-Blot PVDF membrane were purchased from BioRad (Hercules, CA). For denaturing gel electrophoresis, proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose membranes, and subsequently blocked with 1% milk–TBS blocking buffer. Antisera to the large and small subunits of caspase-14 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Research Diagnostics (Flanders, NJ). Immune reactive proteins were visualized using either enhanced chemiluminescence (DuPont-NEN, Boston, MA) or the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) using 4-chloro-1-napthol (Pierce Endogen, Rockford, IL) as a substrate. Protein concentrations were determined using a Bradford assay kit from BioRad (Hercules, CA).

Native gel electrophoresis was performed over 20 h at 4 °C using a discontinuous Tris–glycine/Tris–chloride modification of the method of Laemmli [8] in which SDS was omitted. Native gel electrophoresis markers were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Following native gel electrophoresis, the region corresponding to the native caspase-14 complex was excised from the gel, equilibrated overnight in SDS–PAGE sample buffer and subsequently loaded onto a 15% SDS–polyacrylamide gel.

**Generation of human caspase-14 expression constructs.** The coding region of human caspase-14 was generated by polymerase chain reaction (PCR) and subcloned into the pET-15b vector (Novagen, Madison, WI) and a modified version of the pET-22b vector (Novagen, Madison, WI). The pET-15b plasmid encodes an N-terminal hexahistidine tag in series with an engineered thrombin cleavage site for removal of the hexahistidine tag, while the modified pET-22b vector encodes N- and C-terminal hexahistidine tags. The BL21 (DE3) bacterial strain (Novagen, Madison, WI) was utilized for high-level protein expression. The construct for expression of hC14 in HEK293 cells was generated by PCR and subcloning into the adenoenoviral shuttle vector pCMV-Shuttle (Strategene, La Jolla, CA) for expression in mammalian cells. The identity of all plasmid constructs described above was verified by DNA sequencing.

**Expression of recombinant human caspase-14 in heterologous systems.** Recombinant caspase-14 was purified from large-scale bacterial cultures usingBugBuster solution (Novagen, Madison, WI) according to standard protocols. Following centrifugation, the resulting bacterial lysate was then purified with the Clontech (Palo Alto, CA) TALON purification system using manufacturer’s protocols. Purified caspase-14 was then dialyzed using slide dialysis membranes (Pierce Chemical, Rockford, IL) against a dialysis buffer (pH 7.0) of 10 mM HEPES, 100 mM NaCl, and 10% glycerol. Purified protein was stored at –80°C.

HEK293 cells were transfected using Lipofectamine (Invitrogen, San Diego, CA) and harvested at 40–48 h post-transfection for further analysis. Human keratinocytes were cultured using the method of Rheinwald and Green [9].

**In vitro reactions with purified recombinant caspase-14.** Assays were set up with 50 μl total volume in a neutral reaction buffer containing 100 mM HEPES, pH 7.5, 200 mM NaCl, 2 mM MgCl2, 10 mM DTT, 5–10 μg of recombinant caspase-14, and 15–20 μg of membrane particulate fractions isolated from cultured foreskin keratinocytes. Reactions were incubated at 35 °C for 8 h.

**Calpain cleavage assays.** Flash-frozen neonatal foreskins were homogenized in a 50 mM Tris–100 mM NaCl buffer that did not contain EDTA or protease inhibitors. The homogenate was spun at 10,000g for 15 min at 4 °C and the supernatant was used for subsequent reactions. Twenty μg total protein per reaction was incubated in a 0.1% CHAPS–20 mM DTT buffer or a 5 mM CaCl2–20 mM DTT buffer with or without 10 μM human erythrocyte calpain I (Calbiochem, San Diego, CA). Two μg of purified recombinant caspase-14, stored in 10 mM HEPES, 100 mM NaCl, and 10% glycerol, was incubated with calpain I in similar buffer conditions. Reaction mixtures were assayed for caspase-14 cleavage by SDS–PAGE followed by immunoblotting with caspase-14 antibodies.

**Results**

**Caspase-14 and its subunits form a complex and are localized in the cell cytosol.**

Human foreskin extracts were fractionated into membrane particulate and cytosolic fractions, and then analyzed by SDS-PAGE and immunoblotting with different caspase-14 antibodies (Fig. 2A). A polyclonal antibody generated against the large subunit of caspase-14 recognized procaspase-14 at an apparent mass of ~32 kDa in addition to an immunoreactive band with an apparent mass of ~19 kDa, representing the large subunit (Fig. 2A). An antibody generated against the small subunit also recognized procaspase-14 at 32 kDa apparent mass, in addition to detecting the small subunit at ~10 kDa (Fig. 2A). On a 16.5% Tris–tricine gel, procaspase-14 was recognized as an immunoreactive protein with an apparent mass almost identical to its...
predicted molecular weight of 27.7 kDa (data not shown), suggesting that the higher apparent mass of \(\sim 32\) kDa seen on Tris–glycine gels represents an artifact of electrophoresis rather than an increased molecular mass secondary to an event such as post-translational modification. Immunoreactivity was seen exclusively in cytosolic fractions, with no immunoreactivity in the membrane particulate fraction.

Because other caspase family members are known to complex as dimers, non-denaturing gel electrophoresis was employed to further assess the biochemical properties of caspase-14 in human foreskin (Fig. 2B). In non-denaturing gels of human foreskin extracts, immunoblots with caspase-14 antibody revealed an immunoreactive band with a relative electrophoretic mobility of \(\sim 80\) kDa. The corresponding region was excised from identical lanes on the same gel and run on a second dimension of denaturing SDS–PAGE, followed by immunoblotting to confirm the presence of the caspase-14 protein. As shown in Fig. 2B, re-electrophoresis of the band excised from the native gel revealed the presence of pro-enzyme, large subunit and small subunit. A parallel Coomassie-stained gel of the excised band did not reveal any detectable proteins at molecular weights other than those of caspase-14 and its subunits (not shown). When caspase-14 is isolated from native tissue by gel filtration and anion exchange chromatography, the proform, large and small subunits are found in the same fractions (Figs. 2C and D). These results indicate that the processed large and small caspase-14 subunits remain associated with nonprocessed caspase-14, forming a caspase-14 complex.

Identifying the site of in vivo cleavage of caspase-14 from human foreskin

The small subunit was purified from native human neonatal foreskin to identify the site of cleavage and gain insight into the mechanisms responsible for processing caspase-14 into large and small subunits in vivo. N-terminal sequencing of the purified small subunit generated 11 amino acids that aligned perfectly with sequence from human caspase-14 (Fig. 3A). This sequence is located downstream from the conserved

---

**Fig. 2.** Caspase-14 consists of a complex of proform, large and small subunits, and is localized in the cell cytosol. (A) Immunoblots of extracts from human neonatal foreskin reveal that caspase-14 is detected exclusively in cytosolic fractions. Antibodies to caspase-14 recognized a 32 kDa prozymogen in addition to the large and small subunits. L, whole tissue extract; C, cytosolic fraction; M, membrane fraction. (B) Non-denaturing PAGE of human foreskin cytosol was immunoblotted with antibody to either the large or small subunit of caspase-14. (C and D) Gel filtration and anion-exchange chromatography do not separate caspase-14 subunits. Proteins from neonatal foreskin were extracted with Tris–EDTA–150 mM NaCl. Gel filtration was performed using Sephacryl S200 resin from Pharmacia (Piscataway, NJ). The caspase-14 complex is found in fractions 22–26 with a column flow rate of 24 cm/h, and fraction collection every 2.5 min (750 μl). Ion-exchange chromatography was performed using anion exchange columns from Vivascience/Sartorius (Goettingen, Germany). The caspase-14 complex elutes with >150 mM NaCl.
QACRG catalytic core, in a region topologically similar to the processing sites identified in other caspases (Table 1). However, unlike other caspases, the small subunit of caspase-14 results from cleavage between isoleucine and lysine residues rather than at an aspartate residue. The predicted molecular weight of a peptide fragment generated by cleavage at Ile152 and Lys153 is 10,425 Da, which is consistent with the size of the small subunit seen by SDS–PAGE. To verify that cleavage was not occurring upstream, with subsequent N-terminal trimming to Lys153, tryptic digest mapping was performed by mass spectrometry on the large subunit, and a peptide corresponding to a tryptic fragment that terminates at Ile152 was found. Therefore, processing at another site with subsequent modifications of caspase-14 to K153 does not occur in vivo.

A comparison of human and mouse caspase-14 at the processing site shows conservation of the site and surrounding amino acids (Fig. 3B).

Caspase-14 is not processed in cultured keratinocytes, transfected HEK293 cells or transformed bacterial cells.

Extracts from both pre-confluent and post-confluent human keratinocyte cultures were analyzed by SDS–PAGE and immunoblotting (Fig. 4A). Pre-confluent

Table 1

<table>
<thead>
<tr>
<th>Cleavage sites</th>
<th>Reference</th>
<th>Purification/sequenced source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large prodomain caspases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-1 –WFKD</td>
<td>SVGV–FEDD</td>
<td>AIKKAHIEK–</td>
</tr>
<tr>
<td>Caspase-2 –DQQD</td>
<td>GKNH–EESD</td>
<td>AGKEKLPKMRR–</td>
</tr>
<tr>
<td>Caspase-4 –WVRD</td>
<td>SPAS–LEED</td>
<td>AVYKTHVEK</td>
</tr>
<tr>
<td>Caspase-8 –VETD</td>
<td>SEEQ–LEMD</td>
<td>LSSPQTRY–</td>
</tr>
<tr>
<td>Caspase-10 –IEAD</td>
<td>ALNPEQAPT</td>
<td>Site-directed mutagenesis prevented cleavage in vitro</td>
</tr>
<tr>
<td><strong>Small prodomain caspases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3 –IETD</td>
<td>SGVDD–</td>
<td>Purified and sequenced protein from human monocyte line (THP.1)</td>
</tr>
<tr>
<td>Caspase-6 –TEVD</td>
<td>AASYVTL–</td>
<td>Purified and sequenced protein from native hamster liver extracts</td>
</tr>
<tr>
<td>Caspase-7 –NDTD</td>
<td>ANPRYKIP–</td>
<td>Purified and sequenced protein from native hamster liver extracts</td>
</tr>
</tbody>
</table>

The caspase processing sites that have been identified to date are shown with the large subunit, interdomain linker (caspases-1, -2, -4, -8, and -9), and small subunits aligned. The caspases with large cleaved prodomains are grouped first, and the three caspases with small prodomains, caspase-3, -6, and -7 are grouped below. Negatively charged residues in the P2–P4 position of the different cleavage sites are underlined, while residues in the P1 position are in bold.
cultures exhibited no detectable expression of caspase-14. Post-confluent cultures expressed procaspase-14, with no detected processing into the large and small subunits seen in human foreskin extracts despite roughly equivalent amounts of procaspase-14 in the compared samples.

Lysates from transiently transfected HEK293 cells were fractionated and analyzed by SDS-PAGE and immunoblotting (Fig. 4B). Non-transfected and mock-transfected HEK293 cells exhibited no detectable caspase-14 on immunoblots. Transiently transfected HEK293 expressed only procaspase-14 in the cytosolic fraction, with no detectable processing into the large and small subunits. However, when recombinant caspase-14 is incubated with the membrane fraction of epithelial cells, caspase-14 is processed to its large and small subunits (Fig. 4C). This indicates that a factor localized to a specific subcellular compartment is involved in caspase-14 processing.

Human caspase-14 was expressed as a hexahistidine-tagged fusion protein in the protease-deficient BL21 bacterial strain. Immunoblots detected only procaspase-14, with no processing into large and small subunits (data not shown).

Calpain does not process recombinant or tissue caspase-14 in vitro

The identified cleavage site of caspase-14 exhibits several determinants common to calpain substrates, such as large aliphatic side chains (i.e., isoleucine, leucine, or valine) at positions P1 and P2 and a basic residue (lysine) at P0 [10]. Another feature seen in calpain substrates is the presence of PEST domains [11], whose acidic nature may sequester calcium and facilitate the local increases in calcium concentration necessary for activation of calpain [10] (Fig. 3A). To test the possibility that calpain cleaves caspase-14, we first incubated recombinant caspase-14 with calpain I in a variety of buffer conditions, temperatures, and time points. High calcium concentrations (5 mM) resulted in limited proteolysis of caspase-14, but not to fragments of the same size as the large and small subunits of caspase-14 seen in foreskin (not shown). Because the conditions of terminally differentiating epidermis may be hard to recapitulate in vitro, we then incubated epidermal extracts with calpain I using numerous conditions. Caspase-14 proform, large and small subunits were readily detected, but no further processing of caspase-14 was seen with the addition of calpain compared to buffer only controls (not shown). Calpain remains an intriguing possibility as the enzyme responsible for processing caspase-14, but as yet we have not ascertained the correct conditions to reproduce cleavage in vitro.

Discussion

Cleavage and activation of other caspase family members described to date occurs by autoprocessing, cleavage by other caspases, or cleavage by other aspartate proteases such as granzyme B [4]. Some caspases, notably caspase-9, do not require cleavage for activity, but rather require the correct cofactors for optimal en-
zymatic function [12]. Nonetheless, caspase-9 is processed at aspartate residues during apoptotic events, though the implications of processing in vivo are not yet known. We have shown that caspase-14 is processed at Ile152/Lys153 during normal epidermal differentiation, and not at an aspartate residue. Caspase-14, therefore, is not processed by another caspase in vivo. Whether cleavage of caspase-14 at this site is necessary for activity remains to be determined.

Table 1 shows an alignment of the cleavage sites identified in other caspase family members. The sequences for caspase-1, -3, -6, and -7 were identified following purification from native tissue, while the remaining sequences were obtained from studies of recombinant caspases activated in vitro. Interestingly, a survey of previously identified caspase cleavage sites reveals three prominent characteristics. First, all other caspase processing sites identified to date involve cleavage at an aspartate residue. Second, the sites of cleavage that result in small subunit all contain at least one, and in some cases two, negatively charged residues in the P2–P4 positions (underlined in Table 1). Lastly, the residue on the C-terminal end of the cleavage site is almost exclusively a small amino acid (Gly, Ala, Ser, or Leu at P1). The aspartate residues in caspase-14 that could be potential cleavage sites to account for the size of the small subunit (Asp 146, 154, 164 in human caspase-14) do not contain negatively charged residues at P2–P4 nor a Gly, Ala, Ser, or Leu at P1. Our finding that caspase-14 is processed at Ile152/Lys153 and not at a suboptimal aspartate lends additional support to the observation that the P2–P4 and the P1 positions play important roles in determining cleavage specificity and/or activity of the caspases.

Many biochemical events occur during the transition from the living layers of the epidermis to the enucleate, compact, stratum corneum. The limited expression of caspase-14 to cornifying epithelium suggests that it participates in the process of terminal differentiation. In culture systems, caspase-14 proenzyme is expressed only in post-confluent keratinocytes that express markers of differentiation, but is not expressed in rapidly growing keratinocytes with a basal phenotype. Caspase-14 is processed only in culture conditions that simulate cornification (lifted culture system), indicating that processing of caspase-14 is an aspect of terminal differentiation. Identification of the processing site of caspase-14 reported here can further direct efforts towards reconstituting active enzyme in vitro and identifying potential caspase-14 substrates.

One potential candidate for the enzyme involved in caspase-14 cleavage and potential activation is the calcium-activated neutral protease, calpain, which is expressed in the granular layer along with caspase-14 [13]. The high levels of intracellular calcium seen in granular cells could trigger calcium-dependent activation of calpain, providing a mechanism for activation of caspase-14 during terminal differentiation. Calpains have also been shown to activate caspase-12 in a calcium-dependent manner [14], albeit in a way that does not directly generate the typical large and small subunits observed with caspase-14. From both a structural and environmental point of view, caspase-14 has many of the determinants of calpain substrates. However, we have been unable to reproduce this reaction in vitro. This is not surprising in the context of our other finding that recombinant caspase-14 is cleaved in the presence of membrane fractions from epithelial cells. This indicates that caspase-14 likely requires a cofactor that is found in a subcellular, membrane-bound compartment for cleavage.

Because cleavage of caspase-14 at Ile152/Lys153 results in fragments with homology to the p20 and p10 subunits of active caspases, we do not think that this cleavage event inactivates caspase-14. That the generation of the large and small subunits of caspase-14 does not occur via processing at an aspartate residue distinguishes caspase-14 from the other caspases described to date. It is possible that caspase-14 does not require cleavage for activity, but rather functions best in the presence of its required cofactors, analogous to caspase-9. Efforts are ongoing in our laboratory to determine the substrate(s) of caspase-14 and the necessity for cofactors for proper caspase-14 function. Ultimately, these studies will uncover the role of caspase-14 in epidermal ontogenesis.

**Acknowledgments**

The authors thank Dr. Beverly Dale-Crink and Dr. Tanya R. Hathaway for valuable discussions and input throughout the course of this work. We also thank Annalisa Pirrone for technical assistance.

**References**