Activation and Substrate Specificity of Caspase-14[†]

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ABSTRACT: Caspase-14 is a developmentally regulated and tissue restricted member of the caspase family present in mammals. It is mainly found in epidermal keratinocytes and has been hypothesized to be involved in a tissue-specific form of cell senescence, leading to the differentiation of keratinocytes that form the cornified cell layer. However, the substrate specificity, activation mechanism, and function of this caspase have yet to be revealed. We report that caspase-14, in contrast to other caspases, is not produced in active form following expression in *Escherichia coli* but can be activated by high concentrations of kosmotropic salts. Moreover, proteolytic cleavage is also required since the kosmotropic salts were only effective on the cleaved enzyme. We propose that caspase-14 requires proteolytic cleavage within the catalytic domain, followed by dimerization and ordering of mobile active site loops, to generate a competent enzyme. In the presence of kosmotropic salt, we were able to determine the substrate specificities of mouse and human caspase-14. Surprisingly, the substrate preferences for the human and mouse enzyme are dissimilar. The results obtained with human caspase-14 classify this enzyme as a cytokine activator, but the mouse enzyme shows preferences similar to apical apoptotic caspases.

Caspases constitute a family of cysteine proteases with a stringent preference for cleaving their substrates after aspartic acid residues. In humans, caspases-1, -4, and -5 participate in the intracellular activation of the pro-inflammatory cytokines IL1β and IL18, whereas caspases-2, -3, -6, -7, -8, -9, and -10 specialize in the induction and execution of the apoptotic cell death program (reviewed in refs *1* and *2*). All caspases by definition contain a conserved catalytic domain, but they vary significantly in the length and composition of their N-terminal prodomains. Long prodomain caspases have N-terminal protein interaction modules that direct recruitment to multiprotein activation platforms (reviewed in refs *3* and *4*). It is within these platforms that the caspase zymogens undergo proximity-induced activation, upon which they are competent to cleave their target substrates. Current theory suggests that the zymogens of long prodomain caspases are monomeric with dimerization within the activation platforms being the essential event that triggers their latent activity. In contrast to the long prodomain caspases, the zymogens of the short prodomain members of the family (caspases-3, -6, and -7) are already dimeric. These short prodomain, or effector, caspases rely on proteolytic cleavage for their activation (reviewed in refs *3* and *5*).

A third distinct physiologic event in which a mammalian caspase appears to be involved is terminal keratinocyte differentiation (*6, 7*). Formation of the epidermis is a complex, tightly regulated process where the keratinocytes transition from undifferentiated, proliferating cells through the terminally differentiated cells finally resulting in senescence or cell death. Dead keratinocytes (corneocytes) form the outermost layer of the epidermis (*8*). Several reports have indicated that expression and processing of caspase-14, initially identified in mice as a developmentally regulated gene product (*9*−*11*), is involved in this tissue-specific nonapoptotic cell death event (*6, 7, 12*).

On the basis of phylogenetic analysis the catalytic domain of caspase-14 is most closely related to the cytokine activator caspases-1, -4, and -5 (*1*). However, in contrast to these caspases, caspase-14 has a very short prodomain, which is more characteristic of the effector caspases. On these grounds, we hypothesize that caspase-14 has a substrate specificity similar to the cytokine activators, but an activation mechanism similar to the apoptotic effectors, presumably requiring proteolysis for activation. Indeed, cleavage of caspase-14 during keratinocyte differentiation has been observed previously (*6, 7, 12*) and suggested to represent an activation event. To date, however, the substrate specificity and activation mechanism of caspase-14 has yet to be characterized. In this paper, we report the activation mechanism and substrate specificity of human and mouse caspase-14.

EXPERIMENTAL PROCEDURES

Materials. Acetylated (Ac)[1] peptidyl fluorogenic 7-amino-4-trifluoromethyl-coumarin (AFC) caspase substrates (Ac-DEVD-AFC, Ac-IETD-AFC, Ac-LEHD-AFC, and Ac-WEHD-AFC) and biotinyl-VAD(OMe)-fluoromethyl ketone (B-VAD-FMK) were purchased from MP Biomedicals. Polyclonal rabbit anti-hemagglutinin (HA) antibodies were from Santa Cruz. Polyclonal antisera for caspase-14 were

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generated in rabbits using affinity-purified His_6-tagged recombinant human caspase-14 full-length protein as immunogen. Human calpain I was obtained from Calbiochem, and granzyme B was a generous gift from Christopher Froelich (Northwestern University). All other chemicals were from Sigma.

Expression Vectors and Recombinant Proteins. For expression of recombinant protein, full-length human caspase-14 cDNA was cloned into pET-23b(+), and mouse caspase-14 was cloned into a modified pET-15b expression vector. Both proteins were expressed in *Escherichia coli* BL-21(DE3) and purified through Ni^{2+} affinity chromatography. For ectopic expression in mammalian cells, human caspase-14 was cloned into the pC4-Fv1E expression vector (ARIAD Pharmaceuticals, Inc, www.ariad.com). Fv is a FK506 binding protein domain (*13*) with a single amino acid substitution (Phe36Val), which binds with subnanomolar affinity to the synthetic FK506 derivative AP20187 (*14*). Cloning of the human caspase-14 cDNA into pC4-Fv1E resulted in a chimeric protein with the Fv domain at the N-terminus and a HA tag at the C-terminus. For this study, we mutated Asp79 of the Fv domain to glutamate to prevent proteolysis by granzyme B. This construct was subcloned to pET-28a and expressed in *E. coli* as an N-terminal His-tag protein (His-Fv-Casp14-HA). Some constructs were generated where the catalytic cysteine of caspase-14 was replaced with alanine (human Cys132Ala; mouse Cys136Ala). Mutants were generated by using overlap PCR and were verified by sequencing. Recombinant Cys2Ala mutant of baculovirus p35 and mouse Bid proteins were expressed in *E. coli* as described (*15, 16*). The recombinant granzyme B inhibitor anti-GraB was expressed in *E. coli* as described (*17*).

Tissue Culture and Transfection Conditions. Human embryonic kidney 293A cells (QBI-HEK-293, QBiogene, Montreal, Canada) and epidermoid carcinoma A-431 cells were cultured in DME medium, while human breast cancer MCF-7 cells were propagated in RPMI 1640 medium. Media were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 50 μg/mL streptomycin. Cells at 80% confluence were transfected using FuGENE6 (Roche Applied Science) according to manufacturer's instruction. Artificial skin tissue was purchased from MatTek, Corp. mRIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) was used to obtain cell extracts from keratinocytes cells (A-431 cells and skin samples). Extracts of 293A and MCF-7 cells were prepared by swelling cells in ice-cold hypotonic buffer (50 mM Pipes, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT) for 30 min followed by cell disruption and clarification at 15 000 $\times$ *g* for 30 min (*18*). Protein concentration was determined using Coomasie Plus protein assay (Pierce Chem. Co., Rockford, IL).

Gel Filtration. Recombinant proteins and cell lysates were separated using an Amersham-Pharmacia Superdex 200 (300 mm $\times$ 10 mm) gel filtration column in 20 mM Tris, 150 mM NaCl, 5 mM EDTA buffer (pH 8.0). The samples were eluted at a rate of 0.5 mL/min and 0.4 mL fractions were collected for analysis. The column was calibrated prior to each run using gel filtration standards from Bio-Rad.

Gel Electrophoresis. SDS−PAGE was performed with 8−18% acrylamide gels (*19*). For pore-limit PAGE of native proteins, 6−28% polyacrylamide gels were prepared and run at constant voltage for 14 h with buffer recirculation between the reservoirs in 50 mM Tris, 7 mM EDTA, 2 mM boric acid (pH 9.0) (*20*). For immunoblotting, samples were transferred to poly(vinylidene difluoride) (PDVF) membrane (Millipore) after electrophoresis. Membranes were blocked with 1% (w/v) bovine albumin in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl with 0.2% (v/v) Tween 20) for 1 h, immunoblotted with primary antibody overnight at 4 °C in TBS-T, washed for 1 h with TBS-T, probed with goat anti-rabbit Ig HRP (horseradish peroxidase)-conjugated antibody (Pierce) for 1 h at room temperature, washed for 1 h with TBS-T and developed with SuperSignal (Pierce).

Enzymatic Assays. Rates of substrate hydrolysis were monitored using an f-max Molecular Device spectofluorometer (Ex = 405 nm, Em = 510 nm) at 37 °C using Ac-WEHD-AFC (0.1 mM) as a substrate except where otherwise specified. For the preliminary experiments, caspase buffer [10 mM Pipes, 0.1 M NaCl, 0.1 mM EDTA, 10 mM DTT, 10% sucrose, 0.1% CHAPS, pH 7.4 (*21*)] was supplemented with 1.0 M of the indicated salt. Subsequently, human caspase-14 assays were performed with 1.1 M sodium citrate, 100 mM Hepes, 60 mM NaCl, 0.01% CHAPS, 5 mM DTT, (pH 7.0), and the mouse caspase-14 buffer was 1.1 M sodium citrate, 100 mM MES, 60 mM NaCl, 0.01% CHAPS (pH 6.0). We refer to these as kosmotropic buffer, and where appropriate, the final concentration of sodium citrate in the buffer was varied. Prior to assay, samples were incubated for 15 min at 37 °C in the kosmotropic buffer. Specific activities of caspase-14 on individual synthetic substrates were determined as V_{max}/K_m. For Ac-DEVD-AFC, it was not possible to determine K_m. In this case, for [S] $\ll K_m$, the Michaelis−Menten equation,

$$V_0 = V_{max}[S]/(K_m + [S])$$

simplifies to

$$V_0 = (V_{max}/K_m)[S]$$

and the V_{max}/K_m value can be obtained from the slope of linear plots of initial velocity (V_0) versus substrate concentration, [S] (*22*).

Affinity Labeling of Caspase-14. Recombinant caspase-14 was activated in kosmotropic buffer for 15 min at 37 °C, and 100 nM B-VAD-FMK was added for 10 min at 37 °C. Samples were precipitated with 10% trichloroacetic acid for 30 min at 4°C, washed with ice-cold acetone, dissolved in SDS sample buffer, resolved by SDS−PAGE, and blotted to PDVF membrane. Labeled proteins were visualized with streptavidin-HRP (10 ng/mL, Sigma).

N-Terminal Sequencing of Protein Samples. Protein samples were resolved by SDS−PAGE and transferred to poly-(vinylidene difluoride) membrane by electroblotting. The membrane was briefly stained in Coomassie Brilliant Blue

[1] Abbreviations: Ac, acetyl; AFC, 7-amino-4-trifluoromethyl-coumarin; B-VAD-FMK, biotinyl-VAD(OMe)-fluoromethyl ketone; AMC, 7-amino-4-methyl-coumarin; HA, hemagglutinin epitope tag; PIPES, 1,4-piperazinediethanesulfonic acid; PDVF, poly(vinylidene difluoride); CHAPS, 3-[(3-cholamido-propyl)dimethylammoniol]-1-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; HRP, horseradish peroxidase; RFU, relative fluorescence units; TCA, trichloroacetic acid.

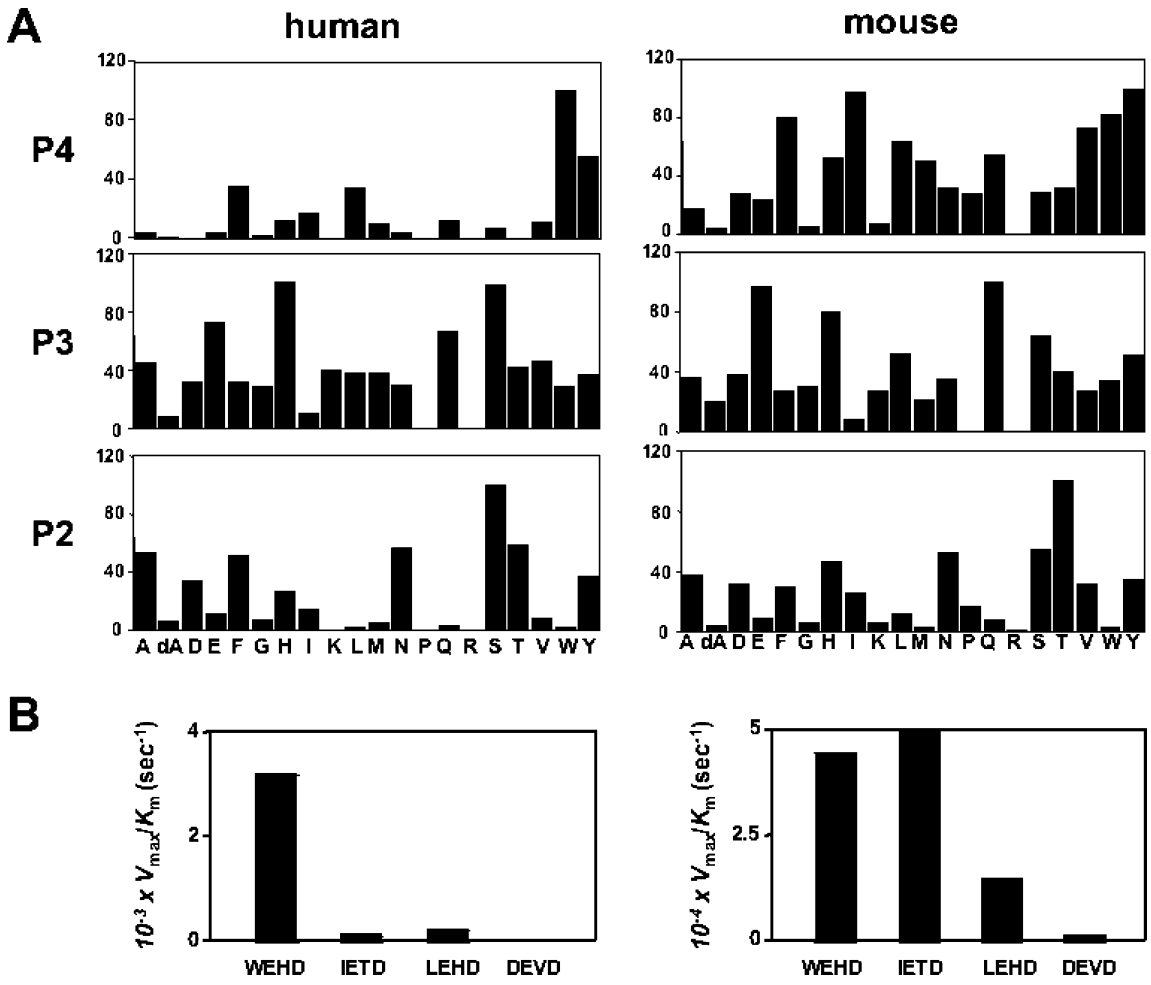

FIGURE 1: Substrate specificities of caspase-14. In part A, Recombinant caspase-14 was incubated in the presence of 1.1 M sodium citrate and assayed with a positional scanning substrate library with P1 fixed at aspartic acid. The *y* axis is the hydrolysis rate presented as a percentage of the maximal rate observed. The *x* axis provides the positionally defined L-amino acid (single letter code, dA is D-alanine). In part B, V_{max}/K_m values were obtained for mouse and human caspase-14 with indicated synthetic fluorogenic substrates.

R250. Appropriate bands were excised and sequenced by Edman degradation at the Eastern Quebec Proteomics Core Facility.

RESULTS

Kosmotropic Salts Activate Recombinant Caspase-14. Upon expression of catalytic domains in *E. coli*, all other human caspases that we have analyzed (caspases-3, -6, -7, -8, -9, and -10) undergo autolytic processing to generate large and small subunits of the catalytic unit (*21, 23, 24*). Their autolytic processing is a signature of proteolytic activity that occurs under the unusually high concentrations of expression in *E. coli*. Unlike these caspases, recombinant human and mouse caspase-14 is obtained as a full-length protein irrespective of expression conditions (Figure 4A). The purified protein has no protease activity in the caspase assay buffer tested against variety of synthetic fluorogenic caspase substrates or other substrates containing arginine, lysine, or leucine in the P1 position (not shown). The absence of processing or activity of caspase-14 indicates that this protease zymogen is not amenable to proximity induced activation and that activation of its latent form is stringently controlled.

It has been reported that protease activity can be significantly enhanced in the presence of salts that tend to order protein structures—kosmotropic salts (*25*−*29*). We tested a variety of salts to determine whether this phenomenon would also apply to caspase-14. Indeed, caspase-14 activity was induced in the presence of high concentrations of sodium citrate, sodium sulfate, ammonium citrate, and ammonium sulfate. Highest activity was seen in sodium citrate, and a preliminary titration revealed that maximum activity was achieved above 1.0 M. Consequently, in characterizing caspase-14, we utilized 1.1 M sodium citrate unless otherwise specified. Enzyme activity was inhibited by Z-VAD-FMK, and recombinant caspase-14 catalytic mutant (human Cys132Ala and mouse Cys136Ala) was not active in the presence of kosmotropic salts.

Determination of Caspase-14 Specificity. To examine substrate specificity of caspase-14 systematically, we employed a positional scanning substrate library with the general structure Ac-X-X-X-Asp-AMC (where X is a mixture of amino acids) that explores preferences in the S2, S3, and S4 extended subsites (*26, 30*). Significantly, the substrate preferences for human and mouse caspase-14 were not identical (Figure 1A). Subsites S2 and S3 appeared similar, but the S4 subsite differed substantially between the two enzymes. While the human enzyme has a requirement for tryptophan or tyrosine, the mouse enzyme is more broadly tolerant with almost equal preferences toward β-branched and aromatic amino acids.

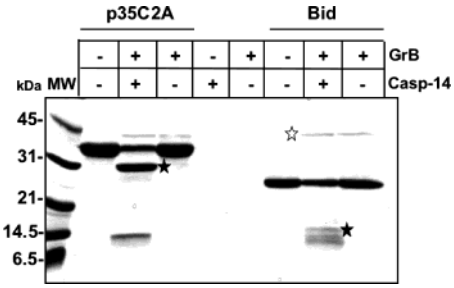

FIGURE 2: Caspase-14 is an endopeptidase. Two protein substrates, p35 Cys2Ala (3.0 μM) and Bid (5.0 μM), were subjected to cleavage by caspase-14 (0.09 μM) in 1.0 M sodium citrate for 1 h at 37 °C. All reactions contained an equivalent amount of granzyme B. To ensure that processing is solely due to caspase-14, reactions were supplemented with granzyme B inhibitor (anti-GraB, white star) at a concentration of 0.4 μM. Reactions were stopped by precipitation with TCA; products were separated by SDS−PAGE and visualized by staining with Coomasie Blue. Cleavage products indicated by a black star were submitted to N-terminal sequence analysis.

The mixture of sequences in individual assay wells of the positional scanning library precludes accurate calculations of catalytic rates. Consequently, we verified the apparent distinctions between the mouse and human enzymes by comparing their relative specificities on commercial substrates that most closely matched the library consensuses (Figure 1B). In agreement with the substrate library, human caspase-14 was most efficient on Ac-WEHD-AFC substrate, while the mouse enzyme had an equal preference for Ac-IETD-AFC and -WEHD-AFC (Figure 1B). Ac-DEVD-AFC, an executioner caspases optimal recognition motif, was a very poor substrate for both enzymes, in contrast to a previous study (*10*). Taken together, these data suggest that human caspase-14 falls into the Thornberry substrate classification group I (*30*), which includes the cytokine activator caspases-1, -4, and -5, but the mouse enzyme seems to fall into group III, which includes the apoptotic initiator caspases-8 and -9. Positional scanning substrate libraries are useful in revealing intrinsic subsite preferences in proteases, but they do not indicate whether the enzyme has endopeptidase activity. To test this, we examined cleavage of two well-established caspase substrates, baculovirus p35 and mouse Bid (Figure 2), by caspase-14 that had been processed by granzyme B (for a description of the granzyme B cleavage, see later). The former is a caspase inhibitor, and to convert it to a pure substrate, we generated the mutant Cys2Ala (*15*). This mutant was a substrate for caspase-14 with cleavage occurring at the canonical Asp87 (*24*). Similarly, Bid was cleaved at Asp59, the caspase-8 cleavage site (*31*). These data confirm that caspase-14 is an aspartate-specific endopeptidase, a property that it shares with other caspases. Granzyme B activity in the reactions was controlled for by addition of recombinant anti-GraB, a potent and specific granzyme B inhibitor (*17*).

Caspase-14 Is Primarily a Monomer in Solution, and the Active Form Is a Dimer. The enzymatically active form of all caspases so far examined is a dimer of catalytic domains (reviewed in ref *32*), and their zymogens are monomeric or dimeric depending on type. We employed size-exclusion chromatography and pore-limit (native) PAGE to assess whether casapse-14 is a monomer or dimer. Recombinant protein eluted with a major peak corresponding to a monomer

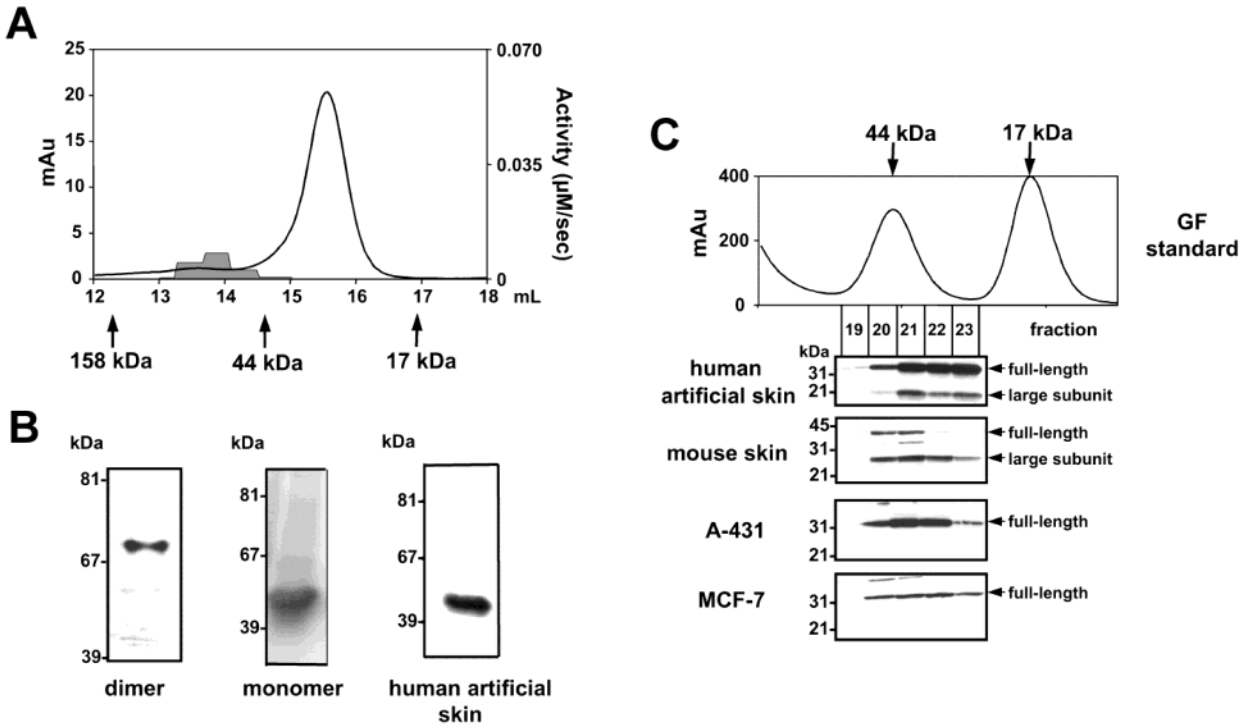

FIGURE 3: Caspase-14 is a monomer. Part A presents the gel filtration analysis of full-length recombinant human caspase-14. The smooth curve shows the elution profiles (left ordinate, mAU = milli-absorbance units at 280 nm), and the shaded area represents the activity assayed in each fraction (right ordinate). In part B, dimer and monomer species of recombinant caspase-14 isolated by gel filtration and mRIPA extract of human artificial skin were prepared and electrophoresed in native pore-limit PAGE under nondenaturing conditions. Dimeric and endogenous caspase-14 were immunoblotted with caspase-14 antibody, and monomeric caspase-14 was visualized by GelCode Blue staining. Part C presents gel filtration of endogenous caspase-14 from different sources. The upper panel shows the elution profile of gel filtration standards. Fractions were TCA precipitated and immunoblotted with caspase-14 antibody (lower panel).

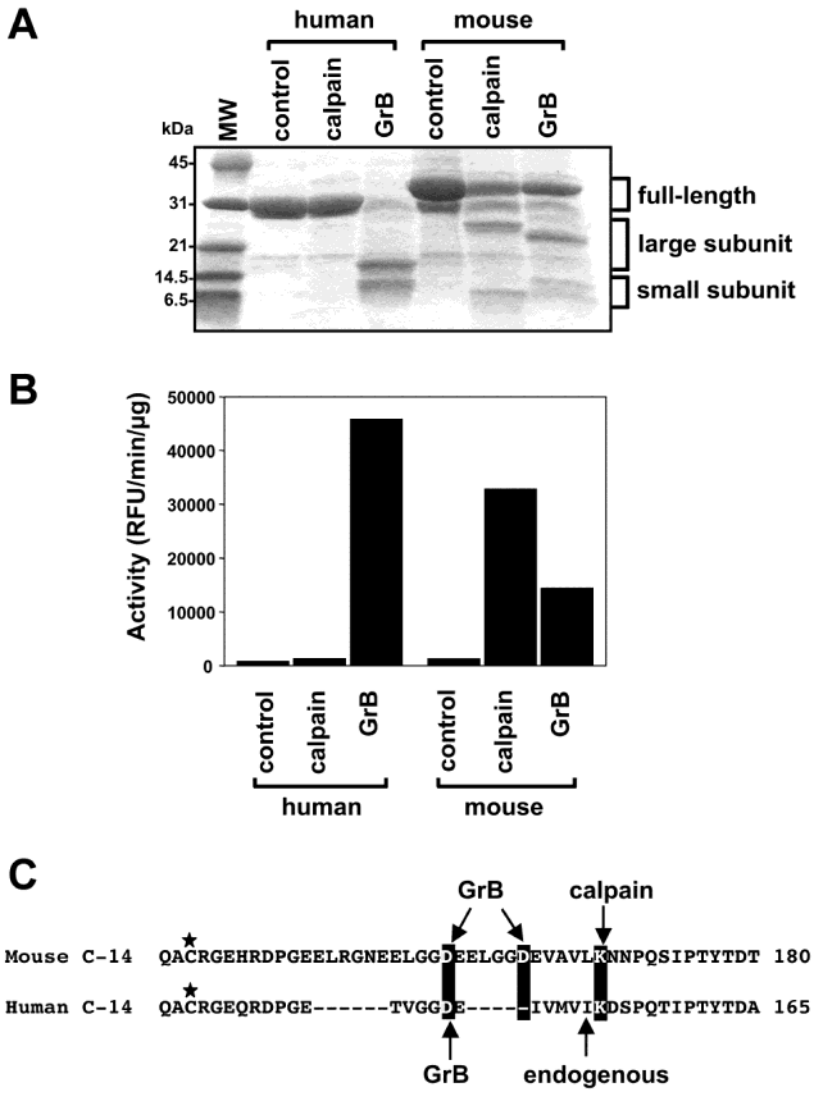

FIGURE 4: In vitro processing of caspase-14. Recombinant caspase-14 (1.2 mg/mL) was incubated with granzyme B (GrB) (0.01 mg/mL) or calpain I (0.06 mg/mL) for 3 h at 37 °C, and cleavage products were analyzed. Part A presents SDS−PAGE analysis; full-length enzyme and processed fragments are indicated to the right. In part B, a portion of the cleavage mixture was assayed for enzymatic activity (hydrolysis of Ac-WEHD-AFC) in the presence of 1.1 M sodium citrate. In part C, the sequence of the interchain linker regions of human and mouse protein is presented with the processing sites identified, compared with the endogenous site from a previous study (*12*). The catalytic cysteine is denoted by a star.

of approximately 29 kDa. Significantly, we did not detect any activity in the fractions corresponding to the monomeric peak in the presence or absence of 1.1 M citrate (Figure 3A). However, trace amounts of protein that eluted with a size equivalent to dimer of approximately 57 kDa contained the citrate-dependent enzymatic activity. The dimer and monomer status was confirmed by native PAGE (Figure 3B). Importantly, endogenous caspase-14 from human artificial skin or present in human keratinocyte A-431 cells and MCF-7 breast carcinoma cells was also determined to be a monomer by gel filtration (Figure 3C). Pore-limit PAGE analysis of artificial skin confirmed the gel filtration results (Figure 3B). These data are consistent with a model in which the zymogen form of caspase-14 is a monomer that requires dimerization for activity.

Caspase-14 Requires Proteolytic Cleavage To Become Active. Proteolytic cleavage at a position separating the large and small subunit within the catalytic domain is a mechanism typical of the activation of effector caspases. Indeed, caspase-14 has previously been demonstrated to be cleaved during keratinocyte differentiation at a position within the interdomain linker that separates these subunits (*12*). It has also been reported that the cytotoxic cell serine protease granzyme B and caspases-8 and -10 can process mouse caspase-14 (*11*). Unfortunately, we were unable to replicate the cleavage of caspase-14 by caspases-8 or -10. Therefore we used two proteases to process caspase-14, granzyme B and calpain I, since the former is able to activate executioner caspases (*17*, *33*−*36*) and the latter has been proposed to activate caspase-14 (*12*). We emphasize that granzyme B probably is not a physiologic processing enzyme for caspase-14 but is used here as a tool to examine the importance of cleavage in caspases activation. Cleavage products were analyzed by SDS−PAGE and Edman degradation (Figure 4A) and tested for Ac-WEHD-AFC activity (Figure 4B). Calpain I processed mouse caspase-14; however, it was unable to process the human protein, in agreement with previous findings (*12*). Granzyme B efficiently cleaved human caspase-14, and processing was also observed with mouse caspase-14, again in accordance with previous findings (*11*). Two identical

cleavage sites for granzyme B are present in mouse enzyme. As a result, small subunits with different N-terminal sequences were generated (Figure 4A,C). Calpain only processed mouse caspase-14. Although lysine is conserved in the human protein, the P1′ and P2 positions are not conserved, and this may explain the lack of the cleavage in the human protein by calpain I (Figure 4C).

We next tested whether cleavage would result in a change in enzyme activity. Significantly, processed caspase-14 did not show any activity when tested in the absence of kosmotropic salt; however, in the presence of 1.1 M citrate, we observed a 50−80-fold increase in enzymatic activity (Figure 4B). None of the proteases tested showed any detectable activity against Ac-WEHD-AFC in the presence of kosmotropic salt, so all increases in substrate hydrolysis are due to activation of caspase-14.

In a second set of experiments, caspase-14 was first cleaved with granzyme B and then separated by gel filtration (Figure 5A). Again the majority of the protein eluted as a monomer, suggesting that cleavage does not induce dimerization. In contrast to the uncleaved enzyme, kosmotrope-dependent activity was found in monomeric fractions. However, this does not imply that caspase-14 is active as a monomer, because without kosmotrope no activity was detected. To determine whether monomeric or dimeric caspase-14 served as the substrate for cleavage-induced activation, we separated them by gel filtration as described in Figure 3 and subjected each to cleavage by granzyme B (Figure 5B). Cleavage of the dimer resulted in a decrease of activity (Figure 5C), presumably due to degradation of the small subunit (Figure 5B). On the other hand, processing of the monomer transformed the inactive enzyme into an active protease (Figure 5C). Taken together, these results suggest that cleavage is required but not sufficient to activate caspase-14. Significantly, cleavage of caspase-14 did not change the order of preference for the synthetic substrates.

Caspase-14 Is Active as a Dimer. Kosmotropic salts can have two main effects in activating proteins: overcoming kinetic barriers to dimerization (*25*, *26*, *29*) and ordering active site loops (*27*). To distinguish these events in the activation of caspase-14, we examined the role of dimerization by employing a system composed of caspase-14 fused to a domain derived from FK506 binding protein. The construct is composed of the N-terminal dimerization domain fused to human caspase-14 that contains a C-terminal HA epitope tag for identification purposes, designated His-Fv-Casp-14-HA (Figure 6A). In the presence of the synthetic dimerizing compound AP20187, constructs containing the binding domain dimerize (*14*). Control experiments demonstrated that only in the presence of AP20187 is the Fv-HA domain copurified with chimeric caspase-14. Kosmotrope did not alter the ability of the synthetic dimerizer to bind the Fv domain (Figure 6B). His-Fv-Casp-14-HA recombinant protein was separated by gel filtration to isolate trace amounts of spontaneously occurring dimer. Additionally, a portion of the purified monomer was selectively cleaved by granzyme B to yield large subunit fused to Fv domain and HA tagged small subunit. The artificial dimerizer is only able to induce activity in the presence of kosmotrope and cleavage of caspase-14 (Figure 6C). As anticipated, activity of the spontaneous dimer was not enhanced in the presence of dimerizer (data not shown).

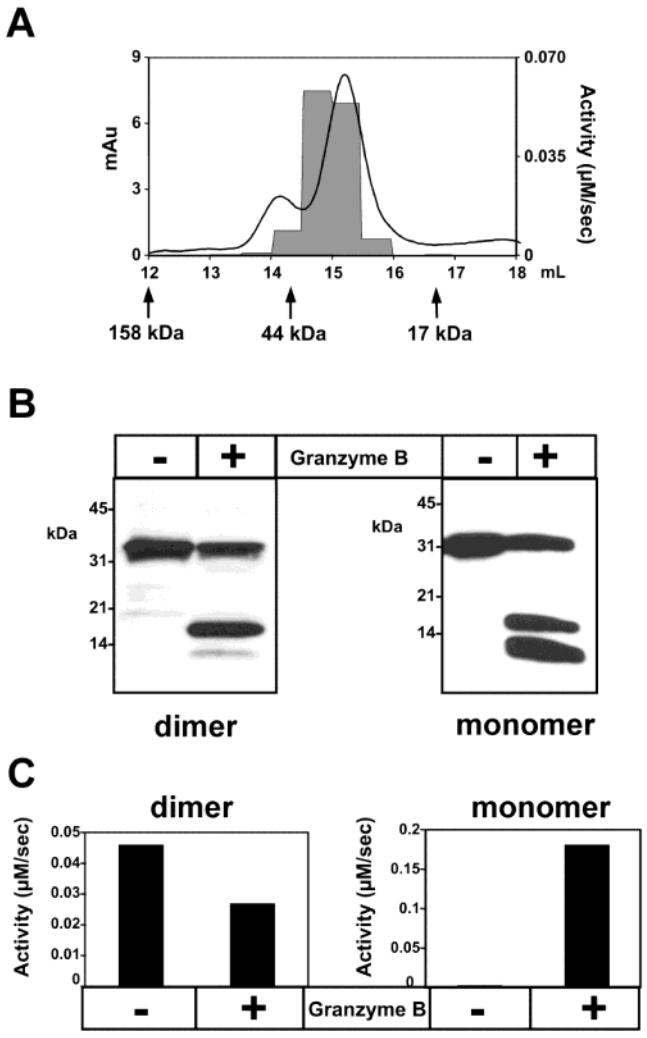

FIGURE 5: Cleavage of caspase-14 is required for activity. Part A presents gel filtration analysis of recombinant human caspase-14 cleaved by granzyme B. The smooth curve shows the elution profile (left ordinate, mAU = milli-absorbance units at 280 nm), and the shaded area represents the activity assayed in each fraction (right ordinate). Recombinant human caspase-14 was fractionated by gel filtration to separate spontaneous dimer from monomer. A portion of each form (500 μL) was subjected to cleavage by granzyme B (0.5 μg) and examined by (B) Western blot using polyclonal anti-caspase-14 antibody and (C) enzyme activity against Ac-WEHD-AFC in the presence of 1.1 M sodium citrate. Granzyme B under these conditions does not hydrolyze Ac-WEHD-AFC.

These findings suggest that the increase in activity of a cleaved monomer in the presence of AP20187 is due to dimer formation or stabilization of existing dimers formed in the presence of kosmotrope. To interpret the enzymatic analysis, we examined the ability of caspase-14 to incorporate the biotynylated inhibitor B-VAD-FMK in the presence or absence of AP20187. In agreement with enzymatic analysis, Figure 6D illustrates that only the processed enzyme is active because full-length monomer was not able to bind the inhibitor. Moreover, as expected, the large subunit, which contains the active site cysteine, can incorporate the probe. Consistent with the activity assay, addition of AP20187 in the presence of kosmotrope activated the enzyme much more than kosmotrope alone, as manifested by a much stronger band observed on the Western blot (Figure 6D). Finally, we analyzed the effect of kosmotrope concentration on caspase-14 activity (Figure 7). To reach maximum enzyme activity,

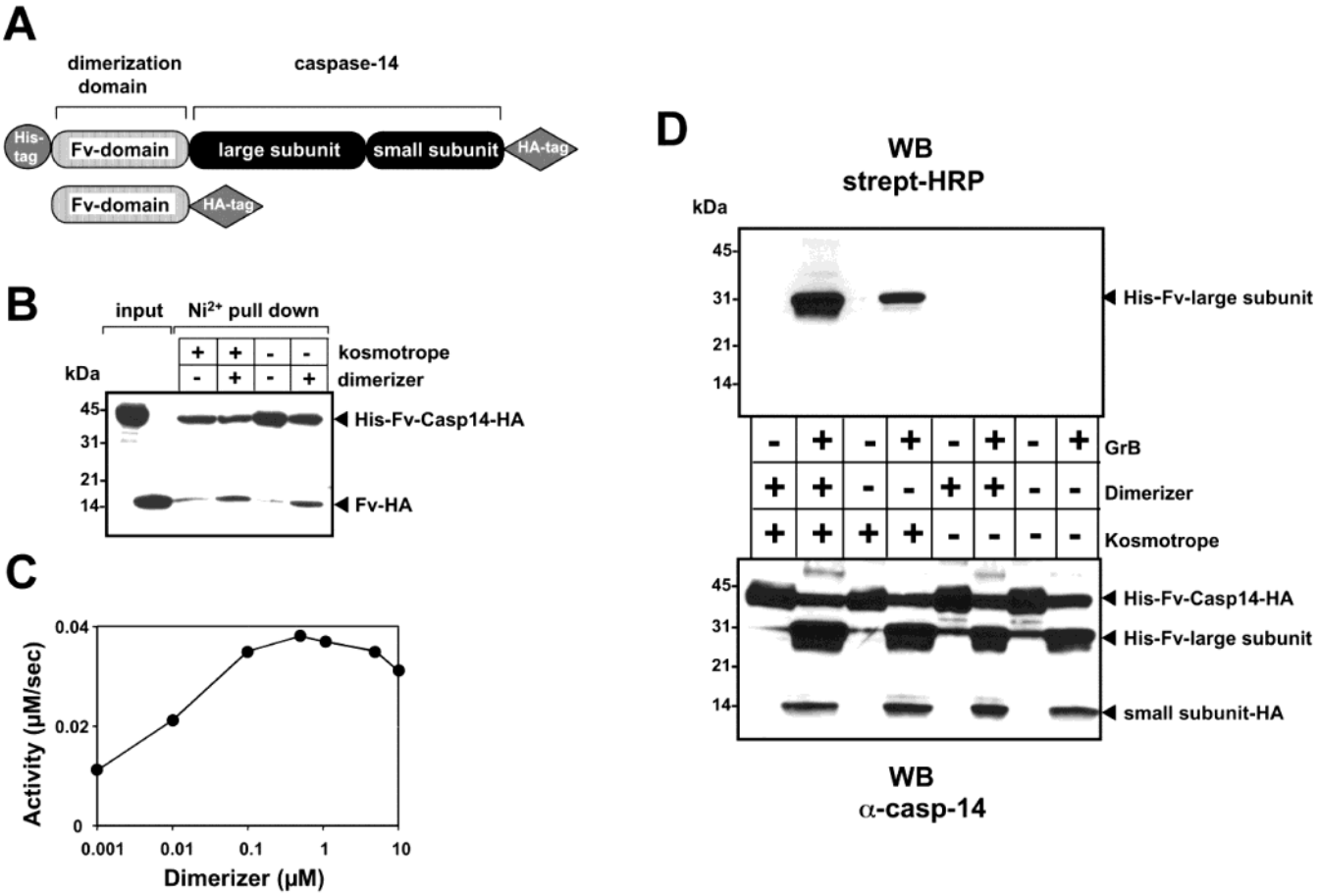

FIGURE 6: Induced dimerization of caspase-14. Part A presents a schematic representation of chimeric caspase-14. In part B, hypotonic extract from 293A cells transfected with empty pC4-Fv1E vector was used as a source of Fv-HA domain. Recombinant His-Fv-Casp14-HA protein and Fv-HA were incubated together in the presence or absence of 0.9 M sodium citrate with or without 500 nM synthetic dimerizer AP20187. Recombinant caspase-14 from the mixture was purified by Ni-chelate chromatography and immunoblotted with anti-HA polyclonal antibodies. In part C, His-Fv-Casp14-HA recombinant chimeric protein was separated by gel filtration, and the monomeric form was cleaved by granzyme B followed by assay in the presence of 0.9 M sodium citrate with the indicated concentrations of AP20187. In part D, full-length or granzyme B-processed monomeric His-Fv-Casp14-HA were subjected to affinity labeling with biotynylated inhibitor (B-VAD-FMK) under the indicated conditions. Labeled proteins were visualized with streptavidin-HRP (upper panel) and then reprobed with antibody against caspase-14 (lower panel).

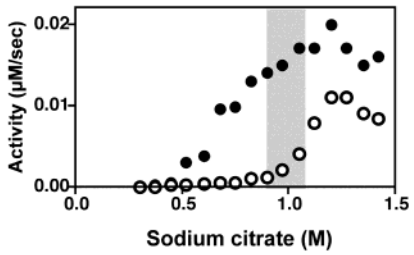

FIGURE 7: Effect of kosmotrope concentration on caspase-14 activity. Activity of His-Fv-Casp-14-HA processed by granzyme B was assayed with various concentrations of sodium citrate in the absence (○) or presence (●) of 500 nM AP20187.

we had to supplement buffer with 1.3 M sodium citrate when the assay was performed without the dimerizer. The effect of AP20187 in activating caspase-14 was clearly evident at lower (0.5−1.0 M) kosmotrope concentration, demonstrating dimerization lowers the requirement for kosmotrope to induce maximum activity.

DISCUSSION

Caspase-14 is the most recent addition to the mammalian caspase family. In many aspects, this protein is different than others caspases: (i) it is expressed in limited tissues, mainly in the epidermis (*6*, *7*, *10*, *37*, *38*); (ii) overexpression in mammalian cells does not lead to apoptosis (*11*, *12*); (iii) recombinant protein expressed in *E. coli* remains as a full-length protein (*11*, *12*); (iv) although all the residues important for substrate catalysis are conserved, caspase-14 is not active in standard caspase buffer (*9*). We were able to induce activity of this enzyme by using a high concentration of kosmotropic salts. In general, the activation of caspase-14 follows the Hofmeister series and is strongly anion-dependent.

Using kosmotrope-driven enzyme activation, we were able to start characterizing the substrate specificity of this member of the caspase family for the first time by means of a positional scanning substrate library. It is likely that the preferences disclosed by the library reflect the true caspase-14 specificity because similar treatment of related caspases-3, -8, and -9 has been shown not to alter their inherent substrate preferences (*26*). Human caspase-14 matches the specificity of the cytokine activator caspases-1, -4, and -5, while the mouse enzyme shows more similarity toward apical caspases-8 and -9 (*30*). Conservation of residues involved in the S4 preference of caspase-1 for aromatic residues,

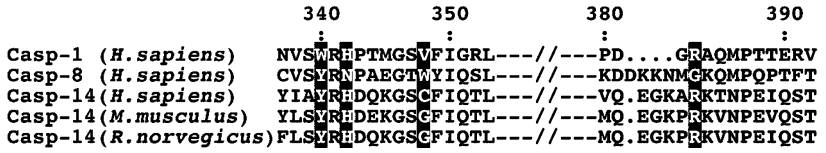

FIGURE 8: Comparison of caspases-14 with residues determining the S4 specificity of caspases-1 and -8. The crystal structures of caspase-1 (*43, 44*) and caspase-8 (*45, 46*) are used as a reference to compare the likely equivalent S4-determining residues in caspases-14 from three species. Residues interacting with the P4 residue in caspases-8 and -1 are highlighted.

particularly His342, accounts for the similarity of human caspase-14 (Figure 8). However, there is no clear explanation of the broader S4 preference of mouse caspase-14. Possibly the differences at position 348 could explain this (Figure 8), since the glycine residue found in mouse caspase-14 may alter the conformation of the main chain sufficiently to broaden the tolerance of residues in the S4 pocket, but such speculation requires the 3-D structure of the caspase for confirmation. Regardless, the specificity of human caspase-14 raises the possibility that its natural substrate may be a pro-inflammatory cytokine. Among these, we tested whether caspase-14 is able to cleave pro-interleukin-18, which has been demonstrated to be a substrate of caspase-1 (*39*). Significantly, no cleavage of pro-interleukin-18 was observed (not shown) under conditions where the same amount of caspase-14 efficiently cleaved the protein substrates p35 Cys2Ala and Bid (Figure 2). Thus the natural substrate(s) of caspase-14 remains elusive.

All known active caspases are composed of a dimer of catalytic domains (reviewed in ref *3*). A current hypothesis suggests the zymogens of the long prodomain apical caspases are monomers that are activated by dimerization when brought into proximity via their recruitment domains. Executioner caspases, on the other hand, preexist as inactive dimers, and proteolytic processing is required for their activation (reviewed in ref *32*). Full-length caspase-14 was only active in the presence of high concentrations of kosmotrope, which has been shown to enforce a monomer−dimer transition in apical caspases-8 and -9 (*26*). On the basis of this finding, we initially concluded that caspase-14 activation may be similar to these apical caspases. However, detailed analysis of recombinant protein showed two forms of caspase-14. The majority of protein was present as inactive full-length monomer, whereas all the activity was associated with a small amount of a species corresponding to a dimer. Interestingly, similar dimeric species were observed in recombinant preparations of caspases-8 and -9, where the majority of protein is monomeric but the bulk of activity was found in the small amount of dimeric species (*26*). However, the caspase-14 dimer was active only in the presence of kosmotropic salt. As previously pointed out, this kinetically trapped dimer may form as an artifact of high concentration during expression in *E. coli* (*32, 40*). The monomeric form of caspase-14 required proteolytic processing for its activation, but again kosmotrope was necessary to detect enzyme activity, indicating that cleavage is necessary but not sufficient to activate caspase-14.

It is not completely clear how activity is generated in caspase-14 in the presence of kosmotrope, but there seem to be two possibilities. Previous studies reported kosmotrope-induced dimerization leading to the activation of herpes virus type 1 protease (*25, 29*) and caspases-8 and -9 (*26*). Indeed this kosmotrope-induced dimerization is not restricted to proteases since glucose oxidase can also be activated in this manner (*41*). Kosmotropes are thought to allow monomers to surmount an entropic barrier that, at least in the cases of caspases-8 and -9, is normally overcome by recruitment to molecular dimerization platforms in vivo (*4, 5*). Recruitment to the platforms is achieved by binding of the N-terminal prodomains of caspases-8 and -9 to homotypic interaction domains present in the recruitment platform. The second type of kosmotrope-induced activity is generated by the ordering of the active site, as demonstrated for the protease prostate specific antigen (*27*). In the latter case, it seems that general desolvation of the protein drives a more compact, catalytically active protease.

We believe that we are observing both effects in caspase-14. For the trapped (spontaneous) dimer, kosmotrope is probably ordering surface loops at the active site, which facilitates substrate binding and catalysis. This is aided by the high mobility, relative to other proteases, of loops that contain the substrate binding and catalytic residues (reviewed in ref *1*). However, in the case of the cleaved monomer, kosmotrope causes dimer formation and ordering of active site loops, which explains why a much higher salt concentration is required to activate monomeric caspase-14. Consistent with this is the effect of the synthetic dimerizer on the activation of a dimerizable caspase-14 construct. Addition of dimerizer to monomeric caspase-14 enhanced activity, and this effect was maximal at low (nonsaturated) kosmotrope concentration. Moreover, in the absence of dimerizer, the response of activity to kosmotrope concentration displayed a sigmoidal dependence (Figure 7), which is diagnostic of a bimolecular (dimerization) response rather than just a unimolecular loop ordering. We propose that when caspase-14 is chemically dimerized, kosmotrope orders the conformation of the active site to permit substrate binding in a manner similar to the effect of kosmotrope on the trapped dimer.

Most importantly, cleavage in the interdomain region is required for activation of caspase-14. Granted, we see kosmotrope-induced activity in the uncleaved recombinant trapped (spontaneous) dimer, but we consider that this may represent an artifactual condition of material overexpressed in *E. coli*. Since endogenous caspases-14 from a variety of natural sources is monomeric, we consider this to be the normal condition of the protein that will undergo activation. Indeed, proteolytic cleavage of caspase-14 is observed during keratinocyte maturation (*6, 7*), and the site has been mapped in endogenous human protein (*12*) to the sequence preceding the calpain cleavage site (Figure 4C).

In conclusion, we propose a two-step mechanism for caspase-14 activation. Proteolytic cleavage by a yet to be identified cellular protease (*12*) generates a species that can undergo dimerization and ordering of the active site. It is likely that proteolysis and dimerization are insufficient for activity because our cleaved chemically induced dimer still

requires kosmotrope for activation in vitro. This is in contrast to other caspases, where chemical dimerization by itself is often sufficient for activation (reviewed in ref *42*). How dimerization of caspase-14 is executed in vivo is unclear given that it does not have any recognizable recruitment domain of the type required for dimerization of caspases-8 and -9 in vivo. One possibility is that during terminal keratinocyte differentiation, during cornification where caspase-14 is thought to be active (*6*, *7*), the cellular environment somehow changes to favor activation by a desolvation mechanism. Indeed, a related possibility has been raised to explain the activation of prostate specific antigen in prostatic fluid, where the citrate concentration is 150−1000-fold higher that the concentration found in prostatic stromal tissue (*27*).

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REFERENCES

1. Denault, J. B., and Salvesen, G. S. (2002) Caspases: keys in the ignition of cell death, *Chem. Rev. 102*, 4489−4500.
2. Thornberry, N. A., and Lazebnik, Y. (1998) Caspases: enemies within, *Science 281*, 1312−1316.
3. Shi, Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis, *Mol. Cell 9*, 459−470.
4. Tschopp, J., Martinon, F., and Burns, K. (2003) NALPs: a novel protein family involved in inflammation, *Nat. Rev. Mol. Cell. Biol. 4*, 95−104.
5. Boatright, K. M., and Salvesen, G. S. (2003) Caspase activation, *Biochem. Soc. Symp.*, 233−242.
6. Lippens, S., Kockx, M., Knaapen, M., Mortier, L., Polakowska, R., Verheyen, A., Garmyn, M., Zwijsen, A., Formstecher, P., Huylebroeck, D., Vandenabeele, P., and Declercq, W. (2000) Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing, *Cell Death Differ. 7*, 1218−1224.
7. Eckhart, L., Declercq, W., Ban, J., Rendl, M., Lengauer, B., Mayer, C., Lippens, S., Vandenabeele, P., and Tschachler, E. (2000) Terminal differentiation of human keratinocytes and stratum corneum formation is associated with caspase-14 activation, *J. Invest. Dermatol. 115*, 1148−1151.
8. Haake, A. R., and Holbrook, K. (1999) in *Dermatology in General Medicine* (Freedberg, I. M., Eisen, A. Z., Wolff, K., Austen, K. F., Goldsmith, L. A., Katz, S. I., and Fitzpatrik, T. B., Eds.) pp 70−114, McGraw Hill, New York.
9. Van de Craen, M., Van Loo, G., Pype, S., Van Criekinge, W., Van den brande, I., Molemans, F., Fiers, W., Declercq, W., and Vandenabeele, P. (1998) Identification of a new caspase homologue: caspase-14, *Cell Death Differ. 5*, 838−846.
10. Hu, S., Snipas, S. J., Vincenz, C., Salvesen, G., and Dixit, V. M. (1998) Caspase-14 is a novel developmentally regulated protease, *J. Biol. Chem. 273*, 29648−29653.
11. Ahmad, M., Srinivasula, S. M., Hegde, R., Mukattash, R., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) Identification and characterization of murine caspase-14, a new member of the caspase family, *Cancer Res. 58*, 5201−5205.
12. Chien, A. J., Presland, R. B., and Kuechle, M. K. (2002) Processing of native caspase-14 occurs at an atypical cleavage site in normal epidermal differentiation, *Biochem. Biophys. Res. Commun. 296*, 911−917.
13. Clackson, T., Yang, W., Rozamus, L. W., Hatada, M., Amara, J. F., Rollins, C. T., Stevenson, L. F., Magari, S. R., Wood, S. A., Courage, N. L., Lu, X., Cerasoli, F., Jr., Gilman, M., and Holt, D. A. (1998) Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity, *Proc. Natl. Acad. Sci. U.S.A. 95*, 10437−10442.
14. Xie, X., Zhao, X., Liu, Y., Zhang, J., Matusik, R. J., Slawin, K. M., and Spencer, D. M. (2001) Adenovirus-mediated tissue-targeted expression of a caspase-9-based artificial death switch for the treatment of prostate cancer, *Cancer Res. 61*, 6795−6804.
15. Riedl, S. J., Renatus, M., Snipas, S. J., and Salvesen, G. S. (2001) Mechanism based inactivation of caspases by the apoptotic suppressor p35, *Biochemistry 40*, 13274−13280.
16. Schendel, S. L., Azimov, R., Pawlowski, K., Godzik, A., Kagan, B. L., and Reed, J. C. (1999) Ion channel activity of the BH3 only Bcl-2 family member, BID, *J. Biol. Chem. 274*, 21932−21936.
17. Quan, L. T., Tewari, M., O'Rourke, K., Dixit, V., Snipas, S. J., Poirier, G. G., Ray, C., Pickup, D. J., and Salvesen, G. (1996) Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B, *Proc. Natl. Acad. Sci. U.S.A. 93*, 1972−1976.
18. Ellerby, H. M., Martin, S. J., Ellerby, L. M., Naiem, S. S., Rabizadeh, S., Salvesen, G. S., Casiano, C. A., Cashman, N. R., Green, D. R., and Bredesen, D. E. (1997) Establishment of a cell-free system of neuronal apoptosis: comparison of premitochondrial, mitochondrial, and postmitochondrial phases, *J. Neurosci. 17*, 6165−6178.
19. Bury, A. (1981) Analysis of protein and peptide mixtures: Evaluationof three sodium dodecyl sulphate-polyacrylamide gel electrophoresis buffer systems, *J. Chromatogr. 213*, 491−500.
20. Barrett, A. J., Brown, M. A., and Sayers, C. A. (1979) The electrophoretically 'slow' and 'fast' forms of the α_2-macroglobulin molecule, *Biochem. J. 181*, 401−418.
21. Stennicke, H. R., and Salvesen, G. S. (1997) Biochemical characteristics of caspases-3, -6, -7, and -8, *J. Biol. Chem. 272*, 25719−25723.
22. Coombs, G. S., Bergstrom, R. C., Madison, E. L., and Corey, D. R. (1998) Directing sequence-specific proteolysis to new targets. The influence of loop size and target sequence on selective proteolysis by tissue-type plasminogen activator and urokinase-type plasminogen activator, *J. Biol. Chem. 273*, 4323−4328.
23. Stennicke, H. R., Deveraux, Q. L., Humke, E. W., Reed, J. C., Dixit, V. M., and Salvesen, G. S. (1999) Caspase-9 can be activated without proteolytic processing, *J. Biol. Chem. 274*, 8359−8362.
24. Zhou, Q., Krebs, J. F., Snipas, S. J., Price, A., Alnemri, E. S., Tomaselli, K. J., and Salvesen, G. S. (1998) Interaction of the baculovirus anti-apoptotic protein p35 with caspases: specificity, kinetics, and characterization of the caspase/p35 complex, *Biochemistry 37*, 10757−10765.
25. Schmidt, U., and Darke, P. L. (1997) Dimerization and activation of the herpes simplex virus type 1 protease, *J. Biol. Chem. 272*, 7732−7735.
26. Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I., Ricci, J.-E., Edris, W. A., Sutherlin, D. P., Green, D. R., and Salvesen, G. S. (2003) A Unified Model for Apical Caspase Activation, *Mol. Cell 11*, 529−541.
27. Huang, X., Knoell, C. T., Frey, G., Hazegh-Azam, M., Tashjian, A. H., Jr., Hedstrom, L., Abeles, R. H., and Timasheff, S. N. (2001) Modulation of recombinant human prostate-specific antigen: activation by Hofmeister salts and inhibition by azapeptides. Appendix: thermodynamic interpretation of the activation by concentrated salts, *Biochemistry 40*, 11734−11741.
28. Yamanaka, G., DiIanni, C. L., O'Boyle, D. R., 2nd, Stevens, J., Weinheimer, S. P., Deckman, I. C., Matusick-Kumar, L., and Colonno, R. J. (1995) Stimulation of the herpes simplex virus type I protease by antichaeotrophic salts, *J. Biol. Chem. 270*, 30168−30172.
29. Khayat, R., Batra, R., Bebernitz, G. A., Olson, M. W., and Tong, L. (2004) Characterization of the monomer−dimer equilibrium of human cytomegalovirus protease by kinetic methods, *Biochemistry 43*, 316−322.
30. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis, *J. Biol. Chem. 272*, 17907−17911.
31. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis, *Cell 94*, 491−501.

32. Boatright, K. M., and Salvesen, G. S. (2003) Mechanisms of caspase activation, *Curr. Opin. Cell Biol. 15*, 725−731.
33. Duan, H., Orth, K., Chinnaiyan, A. M., Poirier, G. G., Froelich, C. J., He, W.-W., and Dixit, V. M. (1996) ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B, *J. Biol. Chem. 271*, 16720−16724.
34. Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B, *Nature 377*, 446−448.
35. Martin, S. J., Amarante-Mendez, G. P., Shi, L., Chuang, T.-S., Casiano, C. A., O'Brien, G. A., Fitzgerald, P., Tan, E. M., Bokoch, G. M., Greenberg, A. H., and Green, D. R. (1996) The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE, Ced3 family protease, CPP32, via a novel two-step mechanism, *EMBO J. 15*, 2407−2416.
36. Zhou, Q., and Salvesen, G. S. (1997) Activation of pro-caspase-7 by serine proteases includes a noncanonical specificity, *Biochem. J. 324*, 361−364.
37. Pistritto, G., Jost, M., Srinivasula, S. M., Baffa, R., Poyet, J. L., Kari, C., Lazebnik, Y., Rodeck, U., and Alnemri, E. S. (2002) Expression and transcriptional regulation of caspase-14 in simple and complex epithelia, *Cell Death Differ. 9*, 995−1006.
38. Lippens, S., VandenBroecke, C., Van Damme, E., Tschachler, E., Vandenabeele, P., and Declercq, W. (2003) Caspase-14 is expressed in the epidermis, the choroid plexus, the retinal pigment epithelium and thymic Hassall's bodies, *Cell Death Differ. 10*, 257−259.
39. Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M. A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R. A., Sato, V., Harding, M. W., Livingston, D. J., and Su, M. S. (1997) Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme, *Science 275*, 206−209.
40. Renatus, M., Stennicke, H. R., Scott, F. L., Liddington, R. C., and Salvesen, G. S. (2001) Dimer formation drives the activation of the cell death protease caspase 9, *Proc. Natl. Acad. Sci. U.S.A. 98*, 14250−14255.
41. Ahmad, A., Akhtar, M. S., and Bhakuni, V. (2001) Monovalent cation-induced conformational change in glucose oxidase leading to stabilization of the enzyme, *Biochemistry 40*, 1945−1955.
42. Steller, H. (1998) Artificial death switches: induction of apoptosis by chemically induced caspase multimerization, *Proc. Natl. Acad. Sci. U.S.A. 95*, 5421−5422.
43. Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J. A., McGuiness, L., Orlewicz, E., Paskind, M., Pratt, C. A., Reis, P., Summani, A., Terranova, M., Welch, J. P., Xiong, L., and Möller, A. (1994) Crystal structure of the cysteine protease interleukin-1beta-converting enzyme: A (p20/p10)2 homodimer, *Cell 78*, 343−352.
44. Wilson, K. P., Black, J. A., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Structure and mechanism of interleukin-1 beta converting enzyme, *Nature 370*, 270−275.
45. Blanchard, H., Kodandapani, L., Mittl, P. R. E., Di Marco, S., Krebs, J. F., Wu, J. C., Tomaselli, K. J., and Grütter, M. G. (1999) The three-dimensional structure of caspase-8: an initiator enzyme in apoptosis, *Structure 27*, 1125−1133.
46. Watt, W., Koeplinger, K. A., Mildner, A. M., Heinrikson, R. L., Tomasselli, G., and Watenpaugh, K. D. (1999) The atomic resolution structure of human caspase-8, a key activator of apoptosis, *Structure 27*, 1135−1143.

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