Expression and characterization of constitutively active human caspase-14

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Abstract

Caspase-14 is a cysteine endoproteinase that is expressed in the epidermis and a limited number of other tissues. It is activated during keratinocyte differentiation by zymogen processing, but its precise function is unknown. To obtain caspase-14 for functional studies, we engineered and expressed a constitutively active form of human caspase-14 (Rev-hC14) in Escherichia coli and cultured mammalian cells. Rev-hC14 required no proteolytic processing for activity, showed strong activity against the caspase substrate WEHD, and was inhibited by the pan-caspase inhibitor zVAD-fmk. Mammalian cells that expressed active caspase-14 showed normal cell adherence and morphology. Using positional scanning of synthetic tetrapeptide libraries, we determined the substrate preference of human caspase-14 to be W (or Y)-X-X-D. These studies affirm that caspase-14 has a substrate specificity similar to the group I caspases, and demonstrate that it functions in a distinct manner from executioner caspases to carry out specific proteolytic events during keratinocyte differentiation.

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Caspases are an evolutionarily conserved family of cysteine endoproteinases that cleave their substrates after aspartic acid (D) residues and function primarily in apoptosis (programmed cell death) or inflammation. Most caspases are expressed as inactivezymogens termed pro-caspases, that consist of an N-terminal prodomain of variable length followed by a large and small subunit [1,2]. Caspase activation generally involves proteolytic processing of the zymogen (procaspase) to generate the large and small subunits, which subsequently associate to form the active enzyme. To date, 15 caspases have been identified, which are generally divided into three groups based on structure and function [3–5]. These include caspase-1, -4, and -5, which process and activate pro-inflammatory cytokines including IL-1β and IL-18 during the inflammatory response; caspase-8, -9, and -10, which function as the upstream or initiator caspases of apoptosis; and caspase-3, -6, and -7 which function as the downstream or executioner caspases.

Caspase-15, a recently described caspase that is present in some mammals but not humans or mice, shares many properties of executioner caspases [5]. In most instances, the initiator caspases cleave and activate other pro-caspases such as caspase-3, while the downstream caspases cleave cellular protein substrates including cytoskeletal proteins, transcription factors, and enzymes, resulting in cell death. Other endoproteinases such as granzyme B and the calpains can also cleave caspases, which can result in their activation or inactivation [6,7].
Caspase-14 is a recently described member of the caspase family that is abundantly expressed in a variety of epithelial tissues such as the epidermis, oral mucosa, retinal pigment epithelium, and the epithelial cells of the choroid plexus [8–12]. In mice, caspase-14 is expressed coincident with the stratification and formation of the epidermal barrier at embryonic day 16–18, suggesting an important role in epidermal maturation [8]. It also has been reported in some non-epithelial tissues including ischemic canine brain [13] and in the trophoblast cells of the developing human placenta [14]. In addition to its somewhat restricted tissue distribution, caspase-14 exhibits a number of other unusual features including: (1) its inability to self-cleave into large and small subunits when overexpressed in Escherichia coli [15,16]; (2) its inability to induce apoptosis when overexpressed in mammalian cells [17]; and (3) its processing to large and small subunits at a site distinct from other caspases [18]. Mouse procaspase-14 is processed by calpain I and II in vitro [(16); M. Kuechle et al., unpublished results]. Immunolocalization studies have demonstrated that caspase-14 is present in both the cytoplasm and nucleus of epidermal granular cells [12] and cultured keratinocytes [11], suggesting that caspase-14 cleaves protein substrates in different intracellular compartments during keratinocyte differentiation.

A major hurdle to the study of caspase-14 activity and substrate specificity has been an inability to obtain highly active caspase-14 for in vitro studies. In this study, we utilized a reverse engineering approach in which the order of the large and small subunits was reversed in the polypeptide chain. This strategy was first applied to produce active caspase-3 and -6 from E. coli [19]. The reverse-engineered form of caspase-14 is constitutively active as a single, uncleaved polypeptide chain both in vitro and when transiently expressed in mammalian cells. We determined the substrate specificity of caspase-14 by profiling a completely randomized tetrapeptide library, which affirms its close structural similarity and substrate specificity to the group I caspases that function in the inflammatory response.

Materials and methods

Materials and antibodies. Acetylated (Ac) peptidyl fluorogenic 7-amino-4-trifluoromethyl-coumarin (AFC) caspase substrates (Ac-YVAD-AFC, Ac-DEVD-AFC, Ac-WEHD-AFC, and Ac-LEHD-AFC) and the pan-caspase inhibitor z-VAD-fmk were purchased from Biovision (Mountain View, CA). Commercial caspase enzymes (caspase-1, -3, -5, and -9) were purchased from Biovision. IPTG was from Gold Biotechnology (St. Louis, MO). Mouse anti-hexahistidine (His6) antibody was from Covance (Berkeley, CA). Two polyclonal antibodies to human caspase-14 were used in this study: MK94 and H-99 (Santa Cruz Biotechnology, Santa Cruz, CA). MK94 was generated against the C-terminus of the large subunit of human caspase-14 (GETVGGDEIVMVI). The 13 residue peptide was conjugated to keyhole limpet hemocyanin prior to injection into New Zealand white rabbits (Genemed Synthesis, South San Francisco, CA). The serum was affinity purified prior to use. Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham Pharmaica Biotech (Piscataway, NJ) or Pierce (Rockford, IL). All other chemicals were of analytical grade.

Construction of reverse-engineered human caspase 14 cDNAs in pET expression plasmid. CDNA constructs were generated that encoded two forms of reverse-engineered caspase-14 that differed at the N-terminus (Rev-hC14, Fig. 1 and Table 1). The large and small subunits of human caspase-14 were amplified with the primers indicated in Table 1, using human caspase-14 cDNA [18] as template. Each fragment contained restriction sites to facilitate cloning into pET-15b (underlined in Table 1). cDNA constructs containing two different N-termini were generated, one containing the amino acid sequence VMVI at the N-terminus, and one lacking, and the results compared. These caspase-14 proteins were termed the V and K forms, respectively. VMVI is the last four amino acids of the large subunit of caspase-14 [18].

PCR was carried out in 50 μl reactions using the Herculase Hotstart DNA polymerase (Stratagene, La Jolla, CA) as recommended by the manufacturer. PCR was performed in an Eppendorf thermocycler by heating at 92 °C for 2 min followed by 10 cycles of 10 s at 92 °C, 30 s at 65 °C, 3 min at 68 °C, followed by a further 15 cycles of 15 s at 92 °C, 50 s at 80 °C, and 3 min at 68 °C, with a final incubation at 68 °C for 10 min. The PCR products comprising large and small subunits (450 and 270 bp, respectively) were purified by agarose gel electrophoresis using standard procedures [20] and cloned into pET-15b (Novagen, Madison, WI) using a two-step cloning procedure, with the cloning of the small subunit (the N-terminal portion) being performed first. The small subunits were cloned into pET-15b vector using the restriction enzymes Xdel and Xhol, and the large subunit subsequently added by cloning using the enzymes XhoI and BamHI. The identities of all constructs were verified by DNA sequencing.

Expression of reverse caspase-14 cDNAs in E. coli and purification by nickel-chelate affinity chromatography. The pET15b-caspase-14 constructs were transformed into E. coli BL21 (DE3) or Origami (DE3) competent cells (Novagen) and expression induced by growth in the presence of 1–2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) essentially as described previously [21]. Briefly, a single colony from an L-agar plate was grown overnight in 5 ml of L-broth containing 50 μg/ml ampicillin. This culture was diluted 1:50 into fresh L-broth and the bacteria were grown at 37 °C until the A600 reached OD 0.6–0.8. Expression of histidine-tagged caspase-14 was induced by addition of 1–2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by growth for an additional 5 h at 30 °C. The cells (50–200 ml) were harvested by centrifugation at 10,000g for 10 min.

Soluble recombinant protein was isolated from E. coli using the BugBuster system (Novagen) with the addition of lysozyme and Benzonase nuclease (Novagen) essentially as recommended by the manufacturer. Protein concentration of total soluble lysates was determined using the BioRad Protein Assay kit with Bradford reagent (BioRad, Hercules, CA). His Bind resin (Novagen) was prepared in a small column as described by the manufacturer. Protein lysate (15 ml or 2–2.5 g of bacterial cell pellet) was generally loaded onto 2.5 ml of nickel-charged His Bind resin pre-equilibrated with binding buffer. The column was washed under non-denaturing conditions with column wash buffer and bound proteins were eluted with a buffer containing 50–500 mM imidazole. Fractions (0.1–0.5 ml) were collected at each imidazole concentration and low molecular

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**Fig. 1. Construction of recombinant reverse-engineered human caspase-14 proteins.** Reverse-engineered human caspase-14 (Rev-hC14) was engineered by PCR and the cDNAs cloned into pET-15b to generate caspase-14 recombinant protein with an N-terminal hexahistidine (His6) tag. The normal, endogenous procaspase-14 zymogen is shown at top for comparison. The position of the small 13-amino acid propeptide (Pro) in the normal and reverse-engineered proteins is shown. There were two versions of Rev-hC14 produced, the V- and K-variations. The V-version contained 4 additional amino acids (VMVI) at the N-terminus of the protein (see Table 1). LS, large subunit; SS, small subunit.
contaminants removed by centrifugation in a Sorvall SS-34 centrifuge using Centriplus 10/30/60 columns from Millipore (Bedford, MA). Generally, a second round of nickel affinity chromatography was carried out to remove contaminating bacterial proteins. Recombinant enzyme preparations were further purified and desalted using Vivapure anionic exchange spin column from Vivascience (Goettingen, Germany). Purified recombinant enzyme was aliquoted and stored at −20 °C, or at −80 °C for long-term storage.

**SDS/PAGE and Western blots.** Protein samples were separated by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to either nitrocellulose (Schleicher and Schuell, Keene, NH) or PVDF membrane (Millipore). SDS/polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 or by silver staining with the Silver Snap kit (Pierce, Rockford, IL). Western blots were probed with primary antibody, e.g., anti-hexahistidine antibody, as recommended by the manufacturer, and immunoreactive proteins visualized with ECL (Amersham Pharmacia Biotech).

**In vitro caspase assays.** Activity of Rev-hC14 preparations was assessed using the fluorogenic AFC substrates (Biovision). Reactions (100 µl) were incubated at 37 °C and contained 50 µM fluorogenic peptide substrate, 300–400 ng of recombinant protein (final concentration, 100–140 nM), and two different buffers. A high salt-citrate buffer (“kosmotrophic buffer”) was generally used to assay for caspase-14 activity [16]. Some assays of reverse caspase-14 activity were carried out in a universal caspase buffer (50 mM Hepes, 0.5% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid (Chaps), 0.1 M NaCl, 1 mM EDTA, and 10 mM DTT, and 10% glycerol, pH 7.4; modified from [23]). For the high salt-citrate buffer, reaction mixtures were pre-incubated for 15 min at 37 °C in the absence of substrate as recommended by Mikolajczyk et al. [16]. Caspase-1, -3, -5, and -9 (Biovision, 1 U enzyme/assay) were used as positive controls for assays with each tetrapeptide substrate (YVAD-AFC, DEVD-AFC, WEHD-AFC, and LEHD-AFC, respectively). Caspase-1, -5, and -9 were assayed in a buffer containing 0.1 M Hepes, pH 7.5, 10% sucrose, 0.1% Chaps, and 10 mM DTT [23]. Caspase-5 was assayed in a buffer containing 50 mM Hepes, pH 7.0, 0.1 M NaCl, 0.5% Chaps, 10 mM DTT, 1 mM EDTA, and 10% glycerol. The pan-caspase inhibitor zVAD-fmk was used at a final concentration of 100 µM. Activity was measured using a Tecan Genios spectrophotometer (excitation at 405 nm, emission at 505 nm) and data imported into an Excel spreadsheet for analysis.

**Determination of caspase-14 substrate specificity by positional scanning of peptide libraries.** The substrate specificity of the S1–S4 subsites of reverse caspase-14 was determined by positional scanning of synthetic combinatorial library (abbreviated PS-SCL) as described by Choe et al. [23] except that assays were performed at 37 °C in a buffer containing 100 mM Hepes, pH 7.0, 1 M sodium citrate, 60 mM NaCl, 0.01% Chaps, 5 mM DTT, and 1% DMSO (from substrates). To determine P1 specificity, a P1-diverse library consisting of 20 sublibraries was used [24]. In each sublibrary, the P1 position contained one native amino acid (cysteine was omitted and methionine was replaced with norleucine), and the P2–P4 positions were randomized with equimolar mixtures of amino acids, for a total of 6859 tetrapeptide substrates per sublibrary. Aliquots (1 µl) from each sublibrary were added to 20 wells of a 96-well Microfluor-1 U-bot-tomed plate (Dynex Technologies) for a final concentration of 7.3 nM of each compound per well.

To determine specificity at the other positions (P2–P4), a library with aspartic acid (D) fixed at the P1 position was used. In the P2–P4 positions, all amino acids were randomized, except that cysteine was omitted and methionine was replaced with norleucine. Aliquots (1 µl) of each sublibrary were added to 57 wells (361 compounds/well; final concentration, 277 nM of each compound/well).

For all PS-SCL experiments, duplicate hydrolysis reactions were initiated by the addition of preactivated recombinant reverse caspase-14 (21–42 nM final concentration) and the activity monitored fluorometrically using a SpectraMax Gemini fluorescence spectrometer (Molecular Devices), with excitation at 380 nm, emission at 460 nm, and cutoff at 435 nm [23]. Fluorescence readings from the cleavage of each peptide substrate (relative fluorescence units/s) were converted to moles of peptide cleaved/s. The mean data set was reported as percent of the maximum hydrolysis observed for each substrate. Both the V-form and K-forms of the reverse caspase-14 showed very similar substrate specificity by PS-SCL.

**Cell culture and transfections.** V and K versions of reverse caspase-14 were cloned into pcDNA3 (Invitrogen, Carlsbad, CA) using a PCR approach. Constructs were verified by DNA sequencing, and plasmid DNA was isolated and purified using the maxiprep kit from Marligien Biosciences (Ijamsville, MD).

**COS-7 cells** were grown as described previously in six-well plates [25,26] and transfected at 60–80% confluency with Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN) essentially as described by the manufacturer. Replicate wells were transfected with 1 µg Rev-hC14 plasmids or control pcDNA3 plasmid. Cells were harvested 48 h after transfection in TBS (0.05 M Tris/HCl, 0.15 M NaCl, pH 7.4) for caspase assays, and for SDS/PAGE and immunoblot. Protein concentrations were determined using the Protein Assay Kit (BioRad).

Caspase activity was measured in COS-7 cell extracts using the substrate WEHD-AFC. Assays (100 µl) were performed in high salt/citrate buffer using 10 µl of cell lysate, as described above. Caspase-5 (Biovision, 1 U/assay) was used as a positive control for the assay. Reverse caspase-14 expression in the transfected COS cells was assessed by SDS/PAGE and immunoblotting with MK94 antibody. For immunofluorescence studies, COS-7 cells were transfected with caspase-14 (V- or K-form) plasmids, or pcDNA3, and the cells plated on glass coverslips at a density of 4–8 × 10^4 cells per well in a 12-well plate. Cells were fixed and immunolabeled with MK94 as described previously [26,27]. Coverslips were counterstained with 0.001% 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize nuclei and mounted in Prolong mounting medium (Invitrogen) to minimize fading. Image acquisition was carried out with a Nikon Microphot-SA microscope equipped with a CCD camera (Photometrics, Tucson, AZ).

**Cleavage assays of potential keratinocyte substrates.** Protein substrates for cleavage assays were prepared by in vitro expression using the TnT system (Promega, Madison, WI). TnT assays were performed in the presence of 25 µCi [35S]methionine (Rediue, >1000 Ci/mmol; Amersham Biosciences, Piscataway, NJ) using a plasmid encoding the C-terminal
domain of desmoglein-1 (gift of Dr. K. Green, Northwestern University School of Medicine [28]) and human lamin B2 (full-length cDNA clone obtained from Open Biosystems, Huntsville, AL) as recommended by the manufacturer. The caspase-3 substrate vimentin was also expressed in vitro and utilized as a positive control for the in vitro cleavage assay [29]. Cleavage assays were carried out for 3 hr at 37°C in 50 μl in the presence or absence of caspase-3 enzyme (1 U/assay) or recombinant caspase-14 (300 ng/assay) in caspase buffer or citrate buffer, as described above. Cleavage products were separated by SDS/PAGE, and the gels washed, dried, and autoradiographed.

Results

Expression and purification of reverse human caspase-14 from E. coli

We initially attempted to generate active caspase-14 by overexpression of the complete polypeptide chain, i.e., the normal zymogen procaspase, in E. coli. Procaspase-14 showed little or no processing to large and small subunits in E. coli, and purified protein did not cleave synthetic caspase substrates (data not shown). Similar findings have been reported by others [15,16]. Therefore, we adopted the reverse-engineering approach to produce active caspase-14, a method that was previously used to generate active forms of other short prodomain caspases [19,30].

Two versions of reverse human caspase-14, denoted the V-form and K-form, were constructed and expressed in E. coli. We engineered these two forms in order to reproduce the strategy of the earlier study with caspase-3 [19]. The V-form contains an additional four amino acids, VMVI, at the N-terminus (Table 1). This sequence constitutes the last four amino acids of the large subunit and was added to mimic the proform and/or enhance the stability of the enzyme. The recombinant proteins were purified from E. coli by two rounds of nickel chelate affinity chromatography, followed by anion exchange chromatography on Vivapure ion exchange columns. The resulting enzyme was ≥80% pure, as judged by visualization of proteins after SDS/PAGE, and immunoblot analysis with antibodies directed against the hexahistidine tag (Fig. 2). For most caspase assays, recombinant protein eluting with 75–100 mM imidazole was used, as this concentration gave a good yield of hRev-C14 of high purity.

Determination of substrate specificity of caspase-14 by positional scanning of tetrapeptide libraries

In initial studies we determined that the V- and K-forms of reverse caspase-14 cleaved commercial tetrapeptide substrates, in particular WEHD, which is a model substrate for group I caspases, e.g., caspase-5 (Fig. 4). To examine the precise substrate specificity of reverse human caspase-14, PS-SCL (positional scanning of synthetic combinatorial libraries) was utilized [23,24,31]. Two different tetrapeptide libraries were profiled; one to determine the P1 specificity and a second one to determine the P2–P4 subsite specificities (see Materials and methods). The P1 position adjacent to the fluorogenic leaving group showed a relatively strict requirement for aspartic acid (D), as expected for a caspase (Fig. 3). A small amount of cleavage was seen when a glutamic acid (E) was at the P1 position. The P2 and P3 position displayed a much broader preference, with the P3 position preferring a charged residue (e.g., serine, alanine, glutamic, and aspartic acid) to a hydrophobic residue. The P4 position exhibited a preference for aromatic or aliphatic amino acids, e.g., tryptophan and tyrosine, with weaker activity shown with leucine and isoleucine (Fig. 3). Overall, a consensus consisting of W/Y-X-X-D approximates the cleavage specificity of the reverse caspase-14. Both the V-form (data shown in Fig. 3) and K-form (that lacks the VMVI tetrapeptide at the N-terminus) showed the same pattern of substrate preference in PS-SCL experiments (data not shown).

To confirm the results obtained with the positional scanning experiments, we performed a series of cleavage assays in high salt/citrate (“kosmotrophic”) buffer using the synthetic caspase substrates YVAD, DEVD, and WEHD, and LEHD. The highest cleavage activity was seen with WEHD, consistent with the PS-SCL results.
The WEHD’ase activity could be blocked with the pan-caspase inhibitor zVAD-fmk. Lower activities were seen with YVAD and LEHD (22% and 11.8% of the relative activity seen with WEHD), while almost no cleavage activity was seen using the caspase-3 substrate DEVD. Reverse caspase-14 exhibited no detectable activity against these substrates in a universal caspase buffer, demonstrating a requirement for kosmotropic (high salt) conditions for enzymatic activity, as reported previously [16].

**The reverse caspase-14 is active in transfected mammalian epithelial cells**

To determine whether reverse caspase-14 was stable and active in mammalian cells, we transfected COS-7 cells with hRev-C14 cDNAs and measured caspase cleavage activity using the substrate WEHD. Cells transfected with the V- or K-forms of reverse caspase-14 showed similar levels of WEHD’ase activity, and this cleavage activity could be inhibited in vitro using zVAD-fmk (Fig. 5A). Western analysis confirmed the presence of reverse caspase-14 in cell lysates, with no evidence of proteolytic processing (Fig. 5B). These results show that the reverse caspase-14 is active when expressed in cultured mammalian cells. Immunofluorescence staining demonstrated that the reverse caspase-14 protein was present in both the cytoplasm and nucleus of hRev-C14 expressing cells. Further, cells expressing the active caspase-14 enzyme showed normal cell adherence to the substratum, and normal cell shape and nuclear integrity (data not shown). This is in accordance with studies demonstrating that caspase-14 expression and activation in stratified epithelia is not associated with apoptosis, as measured by caspase-3 activation or TUNEL assay [11,32,33]. We attempted to perform similar assays of transfected mammalian cells using YVAD-AFC as a substrate. However, both COS-7 cells and rat epidermal keratinocytes exhibited high levels of endogenous YVAD’ase activity, and therefore we were unable to carry out these studies. The endogenous YVAD’ase present in these cells was not inhibited by zVAD-fmk, and hence may represent a cathepsin-like activity [34].

**Discussion**

Caspase-14 is a member of the caspase family of endoproteinases and shows strong expression in epithelial...
keratinocytes and a small number of other cell types (reviewed in [10,12]). Evidence from several laboratories has demonstrated that caspase-14 activation is associated with differentiation or terminal differentiation, rather than apoptosis [8,11,32,33]. However, in vitro studies of caspase-14 activity and function have been hampered by the difficulty of obtaining active caspase-14 by normal recombinant approaches. In this study, we used a reverse engineering approach to produce a constitutively active form of human caspase-14 (W/Y-X-X-D; [16]). In this previous study, procaspase-14 purified from E. coli was activated by proteolytic cleavage with granzyme B or calpain I followed by assay of the purified subunits in a kosmotrophic high salt buffer containing 1.1 M sodium citrate. Unlike the previous study, the reverse-engineered enzyme utilized here did not require any in vitro cleavage for activity and exhibited a similar substrate preference. Caspase-14 extracted from the stratum corneum of human epidermis also shows a strong preference for WEHD, with a lower amount of cleavage seen with YVAD, and negligible activity against DEVD [35]. These results using enzyme isolated from human epidermis parallel our own with recombinant human caspase-14 enzyme.

Our combined studies demonstrate that caspase-14 has a substrate preference similar to the group I caspases, that include caspase-1, -4, and -5, which cleave and activate cytokines including IL-1β and IL-18 [3]. Caspase-14 showed the highest activity against the synthetic commercial substrate WEHD, which is a preferred substrate for caspase-1 and -5. A robust WEHD'ase activity is also observed when the reverse caspase-14 was expressed in mammalian cells (Fig. 5). This activity is attributable to the exogenous caspase-14, as only a low level of endogenous WEHD'ase activity is observed in control transfected cells, and the WEHD'ase activity observed in cells transfected with caspase-14 was inhibited with zVAD-fmk.

At present, the in vivo substrate(s) of caspase-14 is unknown. The pro-apoptotic Bcl-2 protein Bid, a normal substrate of caspase-8, is a substrate of caspase-14 in vitro [16], but this substrate has not been verified in vivo. The apoptosis-inducing caspase, caspase-15, also cleaves Bid in vitro [5]. In this study, we examined a number of keratinocyte proteins as potential caspase-14 substrates. These candidate substrates were either recombinant proteins produced in E. coli or expressed from a sequence-validated cDNA construct using the TnT in vitro transcription/translation system. These candidate substrates included desmoglein 1, which is cleaved in the C-terminal keratin-binding region by caspase-3 [28] and two forms of recombinant periplakin purified from bacteria. Periplakin is cleaved by caspases during keratinocyte apoptosis [36]. Neither desmoglein 1 nor periplakin were cleaved by reverse caspase-14 in vitro (37 and data not shown). We also tested vimentin tetramers, assembled vimentin IF, keratin 8/18, keratin 5/14, and lamin B2, all of which are known to be caspase substrates [29,38,39]. In each case, the intermediate filament proteins were produced in E. coli and purified [40] prior to carrying out the cleavage assays with reverse caspase-14.

substrate with the consensus sequence W (or Y)-X (P3)-X (P2)-D. The P1 position shows a relatively strict requirement for aspartic acid (D), with other positions showing more flexibility. The P4 position exhibited a preference for aromatic or aliphatic amino acids, e.g., tryptophan and tyrosine, with weaker activity shown with leucine and isoleucine (Fig. 3). Our results are in agreement with the findings of a previous report on the substrate preference of human caspase-14 (W/Y-X-X-D; [16]). In this previous study, procaspase-14 purified from E. coli was activated by proteolytic cleavage with granzyme B or calpain I followed by assay of the purified subunits in a kosmotrophic high salt buffer containing 1.1 M sodium citrate. Unlike the previous study, the reverse-engineered enzyme utilized here did not require any in vitro cleavage for activity and exhibited a similar substrate preference. Caspase-14 extracted from the stratum corneum of human epidermis also shows a strong preference for WEHD, with a lower amount of cleavage seen with YVAD, and negligible activity against DEVD [35]. These results using enzyme isolated from human epidermis parallel our own with recombinant human caspase-14 enzyme.

Our combined studies demonstrate that caspase-14 has a substrate preference similar to the group I caspases, that include caspase-1, -4, and -5, which cleave and activate cytokines including IL-1β and IL-18 [3]. Caspase-14 showed the highest activity against the synthetic commercial substrate WEHD, which is a preferred substrate for caspase-1 and -5. A robust WEHD'ase activity is also observed when the reverse caspase-14 was expressed in mammalian cells (Fig. 5). This activity is attributable to the exogenous caspase-14, as only a low level of endogenous WEHD'ase activity is observed in control transfected cells, and the WEHD'ase activity observed in cells transfected with caspase-14 was inhibited with zVAD-fmk.
None of these intermediate filament proteins, in either the assembled or unassembled form, showed any detectable cleavage by caspase-14 under several different assay conditions (A. Kalinin et al., unpublished results). Lamin B2, a known substrate of executioner caspases during apoptosis [39], also was not cleaved by reverse caspase-14. Thus, the substrate(s) of caspase-14 remain to be identified. That reverse caspase-14 does not cleave structural elements of the cell including keratins, laminas, and some cell adhesion components (desmoglein 1, periplakin) is consistent with our observation that cultured cells expressing active caspase-14 showed normal cell adherence and morphology. These findings suggest that this protease participates in very specific proteolytic events in keratinocytes, rather than acting as a more general initiator or promoter of the terminal differentiation/cell death pathway in stratified epithelia.

In summary, we have utilized a reverse engineering approach to produce human caspase-14 that is active without proteolytic processing. The recombinant enzyme shows many of the properties of caspase-14 produced by other methods [16], including a similar substrate preference and inhibition by the pan-caspase inhibitor zVAD-fmk. The reverse-engineered enzyme can be stably expressed in mammalian COS cells and cleaves synthetic caspase substrates. The reverse engineering approach should facilitate the search for caspase-14 substrates using expression cloning strategies with cDNA libraries [29] or other methods.

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