Short Communication

Characteristics of the caspase-like catalytic domain of human paracaspase

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Abstract

Human paracaspase has been predicted to be a member of the protein structural fold that encompasses protease clan CD. To determine whether paracaspase has catalytic activity we have expressed the region corresponding to the catalytic domain and used protease activity-based chemical probes to profile the putative active site. A leucine-based acyloxymethyl ketone probe that covalently labels cysteine proteases discloses a hydrophobic P_1 preference in the putative active site. The probe covalently labels Cys539, which is not the predicted catalytic site based on structural and sequence comparisons with other clan CD proteases. Using a combinatorial peptide substrate library approach we have been unable to detect amidolytic activity of paracaspase, implying that if it is a protease it must be very specific. We suggest a switch in the use of catalytic residues to generate an enzyme overlapping the canonical clan CD protease active site.

Keywords: activity profiling; catalytic site; protease.

Proteolytic enzymes that utilize a cysteine residue as the catalytic nucleophile belong to one of several clans that have separate evolutionary origins (Barrett and Rawlings, 2001). The characteristics of each clan derive from diverse structural solutions to the problem of hydrolyzing peptide bonds, different organization of catalytic residues, and distinct ways of binding substrates. Prominent among the cysteine proteases are members of clan CA, containing the lysosomal cysteine proteases, the calpains, some families of deubiquitinating proteases, and plant papain-like proteases; clan CE containing adeno-

virus and orthopoxvirus processing proteases and a family of de-sumoylating proteases; and clan CD encompassing the five families, caspases, legumains, gingipains, clostripain, and separase. Recently, bioinformatics approaches have identified two further families within this clan: the paracaspases and the metacaspases (Uren et al., 2000). Each shares predicted structural elements, with the fundamental catalytic domain of clan CD proteases most closely related, though still quite distantly, to caspases.

Given the conservation of putative structural elements and conservation of the key residues His and Cys that define the catalytic activity of other clan CD members, it is likely that paracaspases and metacaspases are proteolytic enzymes. Indeed, metacaspases in plants and fungi have been reported to have catalytic activity on protease substrates, though there is disagreement regarding the specificity (Madeo et al., 2002; Vercammen et al., 2004). In contrast, paracaspases, which are found in animals, have yet to be investigated in this respect. Genetic ablation studies failed to demonstrate a role for *Dictyostelium* paracaspase (Roisin-Bouffay et al., 2004), but ablation of the mouse paracaspase gene revealed a role in NF-κB activation following T-cell receptor ligation (Ruefli-Brasse et al., 2003; Ruland et al., 2003; Che et al., 2004). Significantly, mouse paracaspase, and presumably also its ortholog in other mammals, participates in a signaling cascade by acting as a ubiquitin E3 ligase or E3 adapter for the modification of NEMO/IKKγ (Sun et al., 2004; Zhou et al., 2004). To test the hypothesis that human paracaspase is a protease, we examined typical proteolytic properties of the caspase-like domain.

Attempts to express full-length paracaspase, or the hybrid oncogenic forms of MALT (Dierlamm et al., 1999) as soluble proteins in *E. coli* were unsuccessful. Consequently, we dissected the protein by expressing different-length constructs encompassing the catalytic domain (Figure 1). Several constructs were readily expressed in *E. coli*, but as insoluble inclusion bodies. Eventually, we were able to express a construct containing residues 329-566 in soluble form (Figure 1).

A distinguishing characteristic of clan CD proteases is that all of its known members have a strict preference for specific amino-acid side chains in the S1 subsite, and this clearly distinguishes them from other cysteine protease clans, which usually possess much broader specificity. Thus, caspases are selective for Asp, legumains for Asn, K-gingipains for Lys and R-gingipain for Arg, and separase for Arg. Like many other cysteine and serine proteases, the catalytic site can be characterized by the use of activity-based probes that covalently label the catalytic residues. We took advantage of these two prop-
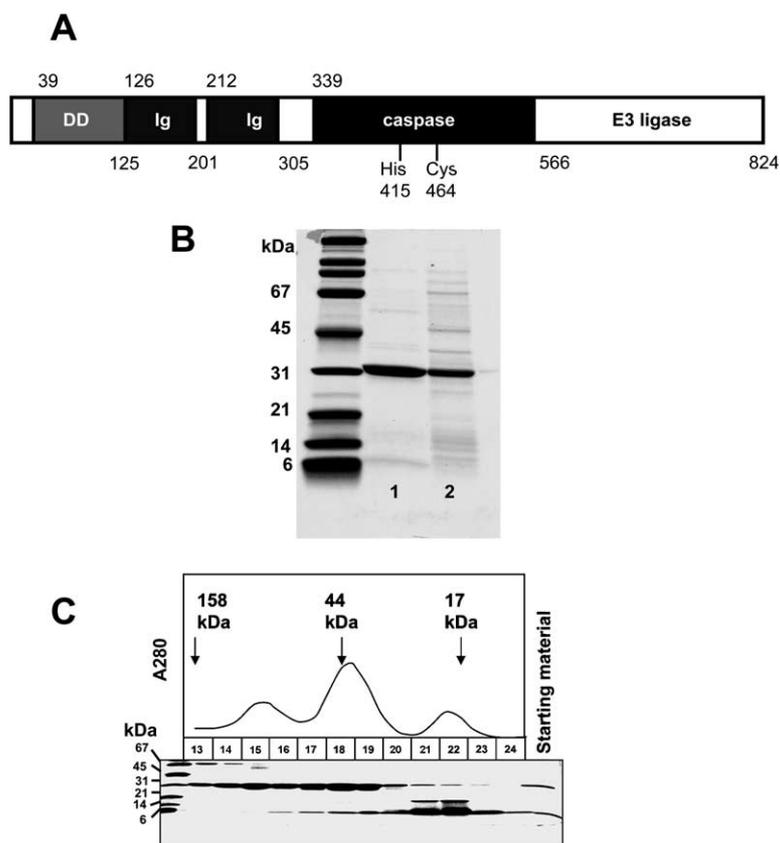
Figure 1 Recombinant human paracaspase.
(A) Schematic diagram of the deduced protein domains in human paracaspase. The numbers indicate the start and end of the domains, and the predicted catalytic residues of the caspase domain are arrowed. (B) Lane 1 shows soluble paracaspase 329-566 and lane 2 shows insoluble material purified in the presence of 8 M urea. (C) Soluble paracaspase was run on a Superdex 200 column and fractions 13–24 (0.5 ml) were analyzed by SDS-PAGE. Gel filtration revealed peaks of ∼84 kDa (paracaspase dimer) ∼41 kDa (paracaspase monomer) and ∼17 kDa (C-terminal cleavage fragment of paracaspase caused by *E. coli* proteolysis). Several truncated versions of the cDNA encoding human paracaspase and a C-terminal His_6 purification tag were cloned into the expression vector pET21b(+) (Novagen, Madison, USA) and expressed in *E. coli* strain BL21(DE3). Soluble and insoluble proteins from a construct encoding residues 329-566 were purified by affinity chromatography using chelating Sepharose (Pharmacia, Piscataway, USA) charged with $NiSO_4$ according to the manufacturer's instructions. Eluted proteins were analyzed by SDS-PAGE. An 8–18% linear acrylamide gradient PAGE with a 2-amino-2-methyl-1,3-propanediol/glycine/HCl system with SDS was used for resolving proteins (Bury, 1981), and gels were stained with Gelcode (Pierce, Rockford, USA). All experiments were conducted with material prepared within 1 week, and 5 μl of 25 μM stocks were loaded.

erties to examine the specificity of the putative catalytic function of paracaspase. Activity probes containing the general structure acetyl-biotinyl-Lys-6-aminohexanoic-Xaa-acyloxymethylketone (where Xaa is an amino acid) were synthesized, purified, and characterized as described for the Arg derivative (Mikolajczyk et al., 2003). These probes contain the covalently reacting acyloxy-methylketone moiety that reacts with the catalytic nucleophile of cysteine proteases (Pliura et al., 1992; Brady et al., 1999) with Arg, Asp, Asn, Leu or Gly in the P1 position to define specificity, and biotinylated for detection. None of these probes were able to label paracaspase under low salt conditions, but by increasing the ammonium citrate concentration to 1.0 M we were able to label paracaspase specifically with the Leu probe, but not with the others (Figure 2). This may be attributed to the ordering of the active site, as observed in other proteases, such as caspases, in the presence of high salt concentrations (Boatright and Salvesen, 2003). The ability of the Leu probe to label paracaspase signifies the presence of a specific catalytic site, and to characterize this we carried out competition analysis using covalently acting chloro-

methylketone (CK) and fluoromethylketone (FMK) probes. Significantly, a Tos-Phe-CK, but not Tos-Lys-CK or Z-VAD-FMK, was able to block binding of the Leu probe, indicating hydrophobic specificity within the catalytic site.

Interestingly, Leu probe binding was also blocked by 3,4-dichloroisocoumarin (3,4-DCI), a hydrophobic compound that is generally used as a serine protease inhibitor (Figure 2D). We doubt that 3,4-DCI could react covalently with paracaspase, and it most likely blocks Leu probe binding in a reversible competitive manner. More importantly, the Leu probe had the same properties in labeling the C464A mutant of paracaspase, in which the presumptive catalytic Cys residue is mutated. Moreover, the probe equally labeled the H415Q mutant, in which the presumptive catalytic His is ablated, as well as the double mutant H415Q/C464A. These data imply that probe labeling is not at the expected catalytic residues.

To determine the location of the probe, labeled paracaspase was purified by virtue of the attached biotinyl group using avidin agarose, digested with trypsin and analyzed by matrix-assisted laser desorption/ionization
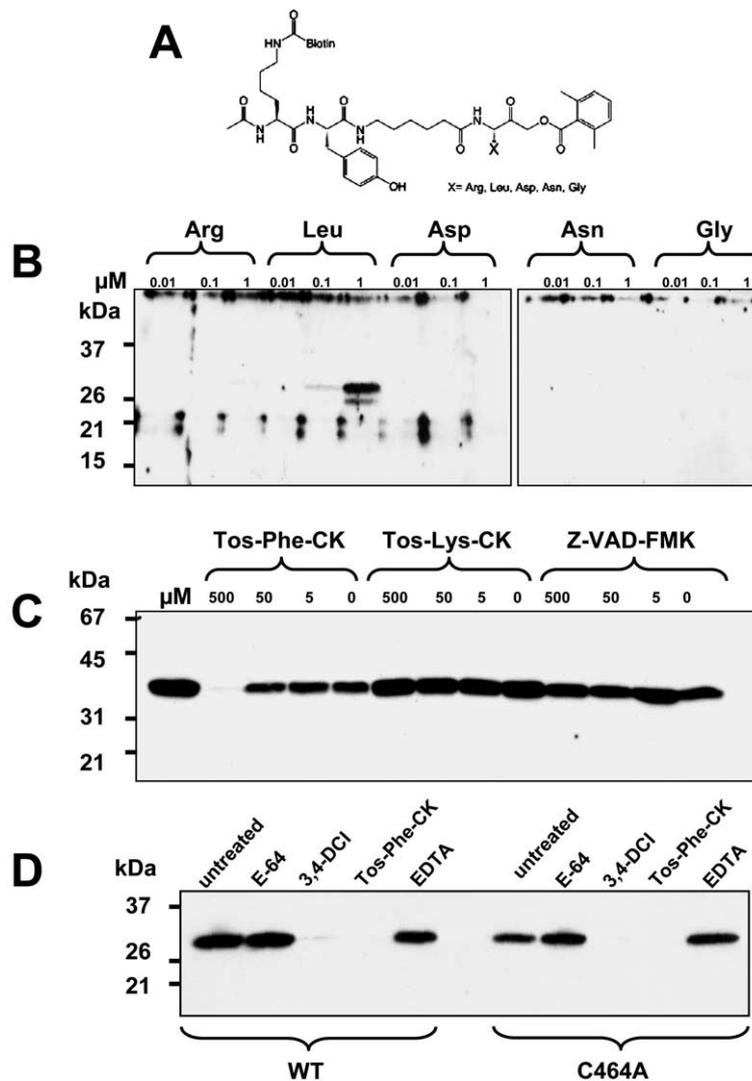
Figure 2 Labeling with biotinylated active site probes.
(A) Structure of the acyloxymethyl ketone probes. (B) Soluble paracaspase 329-566 was treated with 0.01–1 μM biotinylated acyl-oxymethyl ketone probes containing Arg, Leu, Asp, Asn or Gly in the P1 position. (C) Competition of chloromethylketone or fluoro-methylketone probes for the binding of the Leu probe identified in panel B (Z-VAD is benzoxycarbonyl-Val-Ala-Asp). (D) Similar competition for probe labeling, using both the wild-type 329-566 paracaspase and the Cys464Ala mutant to ablate the putative catalytic nucleophile, and using additional competitors. All samples were labeled in 1.0 M ammonium citrate, pH 7.4 with 10 mM dithiothreitol (DTT) for 30 min at 37°C, followed by trichloroacetic acid precipitation and separation by SDS-PAGE, transferred to a PVDF membrane and detected with horseradish peroxidase-conjugated streptavidin (Sigma, St. Louis, USA).

time-of-flight (MALDI-TOF) mass spectroscopy to identify modified peptide(s). Peptide mapping identified a novel peptide signal in the bio-Leu-aomk-labeled paracaspase sample at 1425.4784 m/z (expected: 1424.7652 m/z) that represented the Na+-adducted peptide sequence bio-Leu-C[539]HLTK[543] (Figure 3). We mutated Cys539 to Ala and compared Leu probe labeling with the mutant and wild-type proteins (Figure 3B). In confirmation of our peptide mapping analysis, the C539A mutant was not labeled with the probe, verifying that Cys539 is the site of labeling and is part of a presumptive catalytic site.

Having identified a probable catalytic residue, and conditions under which specific labeling occurs, we next attempted to characterize the catalytic activity in terms of hydrolysis of protease substrates. Given the predicted specificity for Leu or hydrophobic side-chains in S1, we used succinyl-Ala-Ala-Pro-Leu-p-nitroanilide and succinyl-Ala-Ala-Phe-p-nitroanilide to probe for endopepti-

dase activity, and Leu-7-amido-4-fluoromethylcourmarin to probe for aminopeptidase activity. No activity was detected using these substrates, in the presence or absence of 1.0 M ammonium citrate. Since these specific substrates may not satisfy the extended subsite preferences of paracaspase, we employed a positional scanning substrate library in which the P1 position is fixed as one of 18 amino acids contained in proteins (with the exception of Met and Cys). The P2, P3 and P4 positions are evenly represented by these 18 amino acids, so that almost all possible P1–P4 combinations are available.

To validate the library we examined trypsin and caspase-3. At low concentration, trypsin only cleaved peptides containing Lys and Arg in P1, in keeping with its known specificity (Figure 4). At higher concentrations the chymotrypsin contaminant present in commercial trypsin was readily detectable by cleavage of Tyr, Phe and Trp. As expected, caspase-3 was specific for Asp. In con-
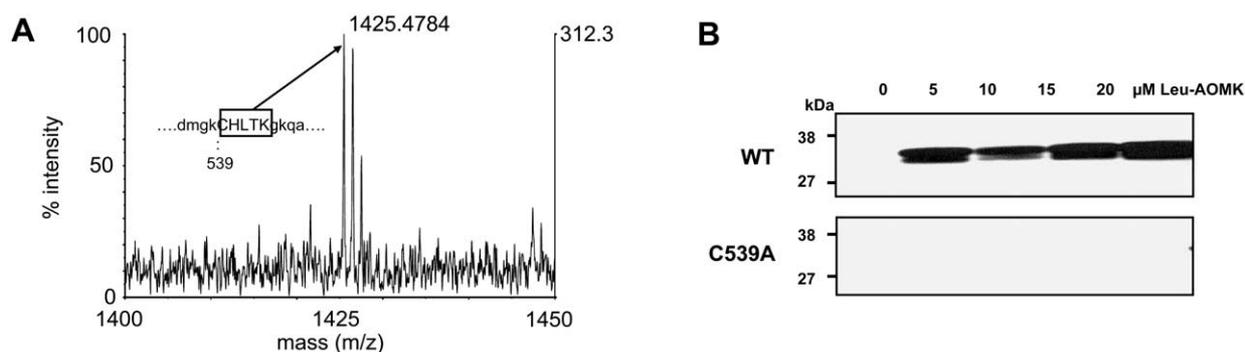
Figure 3   Identification of the probe-reacting residue.
A sample of biotin-Leu-acyloxymethylketone-labeled paracaspase was digested with trypsin. (A) Blow-up of the mass spectrum identifying the novel peptide with the probe adduct, containing Cys529. (B) Streptavidin probe of the SDS-PAGE blot of the result of labeling wild-type paracaspase 329-566 compared to labeling a Cys539Ala mutant under the same conditions, confirming that Cys539 is the site of probe labeling. For mass analysis a 10-μl aliquot of the derivatized digest was desalted using C_{18} ZipTip (Millipore, Bedford, USA) following the manufacturer's recommended protocol. The sample was eluted from the ZipTip to the MALDI target in 2 μl of 20 mg/ml α-cyano-4-hydroxycinnamic acid in 75% acetonitrile/0.1% trifluoroacetic acid. Mass spectra were collected using an Applied Biosystems (Foster City, USA) Voyager DE Pro MALDI-TOF instrument.

trast, even at 6 μM, paracaspase failed to cleave any of the peptides represented in the library. Taken together, the results for the peptide library suggest that paracaspase has undetectable activity on simple synthetic substrates, despite having an enzyme active site.

It is possible that the probe labeling is somehow an artifact in terms of predicting an active site. Moreover, the probe unexpectedly labeled Cys539, a residue distant in the primary sequence from the predicted Cys nucleophile of a clan CD protease. Interestingly, in an alignment with caspases (not shown) Cys539 maps to the position of the 381 loop, a structural unit juxtaposed to the catalytic site of caspases. Thus, it is possible that a catalytic site switch has occurred. This is rare, but has
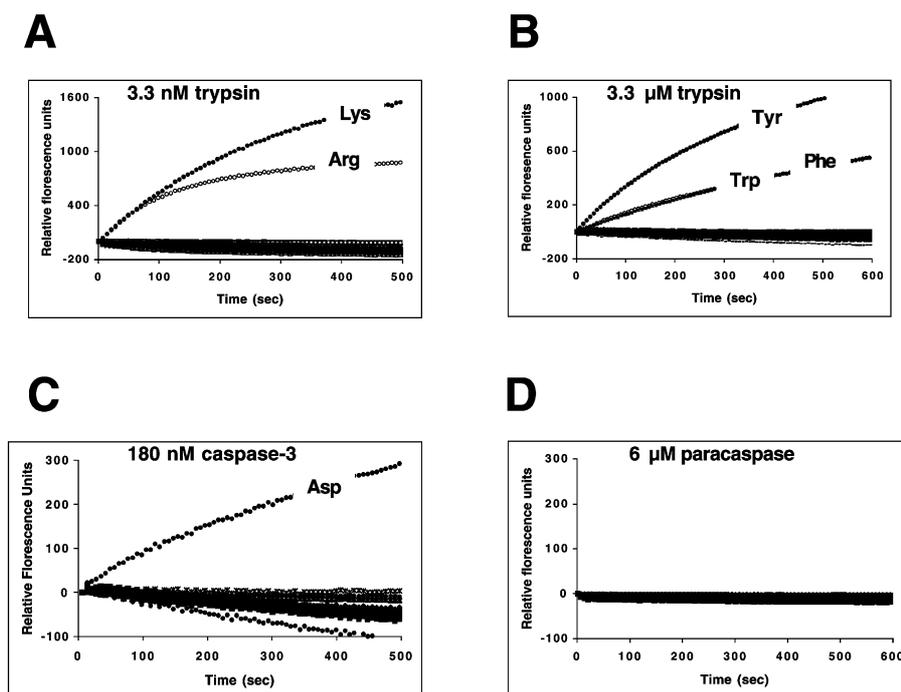

Figure 4   Paracaspase does not cleave tetrapeptide substrates.
(A–C) Test proteases used to validate the library, using the proteases indicated. The identified amino acids constitute the P1 residue. For 3.3 μM trypsin (panel B) cleavage of the Lys and Arg peptides was so fast that the points were off-scale before the first reading could be taken. All reactions were carried out in 100 μl in a 96-well plate containing: trypsin: 50 mM Tris, 100 mM NaCl, 5 mM CaCl$_2$, pH 8.0; caspase-3: 20 mM PIPES, 10% sucrose, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10 mM DTT, pH 7.2; paracaspase: 50 mM Tris, 100 mM NaCl, 1 M ammonium citrate, 10 mM DTT, pH 7.5; and substrate library concentration in the range 5–10 μM at 37°C, and were analyzed using an f_{max} spectrofluorimetric plate reader with excitation at 320 nm and emission at 460 nm. In addition to the amino acids indicated, the library also included P1 Ala, Val, Pro, Ile, Leu, Glu, Ser, Thr, His, Asn, Gln, and Gly. The fluorogenic 7-amino-4-methylcoumarin peptide substrate library was synthesized by solid phase on an Fmoc-7-amino-4-methylcoumarin resin essentially as previously described (Maly et al., 2002). Selected peptide pools were subjected to amino acid analysis to verify library composition and concentration, and the concentration of each remaining peptide pool was determined by absorbance measurement at 320 nm.

been proposed in sortases, clan CA proteins from Gram-positive bacteria responsible for covalently attaching surface proteins to the cell wall envelope (Zong et al., 2004a,b). In the sortases the canonical His that is part of the catalytic dyad of clan CA proteases has been replaced by an Arg residue on an adjacent structural element. Though still peptidases, sortases also act as transamidases, and this switch in catalytic residues has presumably aided their dual function (Zong et al., 2004a,b). We speculate that an analogous event may have occurred in paracaspase.

It is somewhat unusual for a protein that specifically labels with an activity-based protease active site probe to show no activity on synthetic substrates. For example, the activity-based approach has been utilized to characterize the active forms of many lysosomal cathepsins (Bogyo et al., 2000; Joyce, et al., 2004), calpains (Baruch et al., 2001), parasite cysteine proteases (Sajid et al., 2003), plant cysteine proteases (Van Der Hoorn et al., 2004), bacterial cysteine proteases (Mikolajczyk et al., 2003; Oleksy, 2004), and human caspases (Lazebnik et al., 1995; Boatright and Salvesen, 2003). The method has been proposed as a way to rapidly profile enzyme active sites *ex vivo* (Liu et al., 1999) and analyze the activity status of a proteasome (Bogyo et al., 1997). Presumably, the activity of paracaspase, which seems to be cryptic in the sense that it is revealed at high salt concentrations (1.0 M ammonium citrate), relies on a much more specific substrate. For example, deubiquitinating enzymes are detectable by the activity-based probe approach (Borodovsky et al., 2002), but they are very specific and do not cleave simple peptide substrates. In this context it may be significant that paracaspase can act as a ubiquitin E3 ligase or ligase adapter (Zhou et al., 2004), with the region C-terminal to the caspase domain (see Figure 1) containing the docking site (Sun et al., 2004). Conceivably the caspase domain, which as we have demonstrated has the properties of a catalytic unit, participates in proteolytic modulation of the ubiquitination/deubiquitination of the target NEMO/IKKγ, although it is clear that more detailed studies will be needed to address this.

Acknowledgments

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