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Activation mechanism and substrate specificity of the *Drosophila* initiator caspase DRONC

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Drosophila **Nedd2-like caspase (DRONC), an initiator caspase in *Drosophila melanogaster* and ortholog of human caspase-9, is cleaved during its activation *in vitro* and *in vivo*. We show that, in contrast to conclusions from previous studies, cleavage is neither necessary nor sufficient for DRONC activation. Instead, our data suggest that DRONC is activated by dimerization, a mechanism used by its counterparts in humans. Subsequent cleavage at Glu352 stabilizes the active dimer. Since cleavage is at a Glu residue, it has been proposed that DRONC is a dual Asp- and Glu-specific caspase. We used positional-scanning peptide libraries to define the P1–P4 peptide sequence preferences of DRONC, and show that it is indeed equally active on optimized tetrapeptides containing either Asp or Glu in P1. Furthermore, mutagenesis reveals that Asp and Glu residues are equally tolerated at the primary autoprocessing site of DRONC itself. However, when its specificity is tested on a natural substrate, the *Drosophila* executioner caspase DRICE, a clear preference for Asp emerges. The formerly proposed Glu preference is thus incorrect. DRONC does not differentiate between Asp and Glu in poor substrates, but prefers Asp when tested on a good substrate.**

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Apoptosis is a highly regulated mechanism used by multicellular organisms to eliminate unneeded or damaged cells. Important in the apoptotic demise of these cells are a family of proteases, the caspases, that initiate and execute the apoptotic program.[1] In human cell lines, where caspase mechanisms have been most extensively studied, initiator caspases integrate the several cell death signals that begin the apoptotic program, and pass on the signal by directly activating executioner caspases.[2,3] The activated executioners in turn cleave a number of proteins to drive forward the apoptotic phenotype.

To date, seven caspases have been identified in *Drosophila melanogaster*. DRONC, Dredd and Strica are predicted to be initiator caspases and *Drosophila* interleukin-1B-converting enzyme (DRICE), death caspase-1 (DCP-1), DAMM and DECAY are predicted to be executioner caspases.[4] Like the mammalian initiator caspase-9, DRONC possesses a long prodomain that includes a caspase recruitment domain (CARD). In human caspase-9, the CARD allows recruitment of the caspase zymogen to its activator apoptosis protease-activating factor-1 (Apaf-1), which serves as the caspase-9 activator, or apoptosome.[5] Similarly, the CARD of DRONC is thought to direct this initiator caspase to *Drosophila* Apaf-1-related killer (DARK), the *Drosophila* counterpart of Apaf-1, forming the *Drosophila* apoptosome.[6,7]

Despite the evident similarities between the human and *Drosophila* apoptosomes both in composition and structure,[8,9] there are important distinctions between them. Specifically, formation of the *Drosophila* apoptosome is independent of cytochrome *c*, at least in some cells.[10–12] Moreover, unlike human caspase-9, which is activated by dimerization within the apoptosome,[13] DRONC is reported to require proteolytic autoprocessing for activation,[14] although this has recently been disputed.[15] Finally, in normal living S2 cells, DRONC is processed between the large and small subunits at Glu352, implying that DRONC can cleave C-terminally to a Glu residue.[14,16] The requirement for autoprocessing and specificity for Glu residues thus places DRONC in a separate category mechanistically from its human ortholog caspase-9, and thereby violates the principle of conservation of mechanism.

To test the hypothesis that DRONC requires autoprocessing for its activation, we obtained uncleavable mutants, analyzed their mechanism of activation and used a specific protease that cleaves within the inter-chain linker segment that defines the autoprocessing site. We also evaluated the cleavage specificity of DRONC by using positional-scanning peptide libraries, and importantly, also targeted mutational analysis of its natural substrate, the *Drospohila* executioner caspase DRICE.[17]

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Abbreviations: AFC, amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; Apaf-1, apoptosis protease-activating factor-1; CARD, caspase recruitment domain; DARK, *Drosophila* Apaf-1-related killer; DCP-1, death caspase-1; DRICE, *Drosophila* interleukin-1B-converting enzyme; DRONC, *Drosophila* Nedd2-like caspase; NaCit, sodium citrate
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Results

Activation mechanism of DRONC. We first sought to determine which of the two mechanisms of activation, dimerization or cleavage, is required for DRONC activation. Full-length DRONC containing a C-terminal His_6 tag was expressed in *Escherichia coli* and purified essentially as described[18] (Figure 1). Material was recovered as the two-chain form, and Edman degradation and mass spectrometry analysis demonstrated the expected cleavage at Glu352 to separate the large and small subunits of the catalytic domain, as well as cleavage at Asp135 to remove the CARD. As previously demonstrated, *E. coli* expression of the Glu352Ala mutant resulted in material that was primarily full length when expressed in *E. coli*,[14] although there was partial CARD removal (Figure 1b).

Human initiator caspases can be activated by high concentrations of sodium citrate (NaCit), probably via dimerization.[19] We tested NaCit for its ability to activate DRONC and found that the extent of activation depended on both the concentration of DRONC and that of NaCit (data not shown). Maximum activation was at 1.4 M NaCit, and DRONC Glu352Ala activity was enhanced by up to 1000-fold. However, for some experiments we were not able to use 1.4 M NaCit due to protein concentration issues, and so in this study we used 0.7, 1.0 or 1.4 M NaCit as appropriate for the experimental conditions. Each concentration substantially activated DRONC.

We incubated the Glu352Ala mutant for 30 min at 37°C with 1.0 M NaCit, which resulted in complete processing within the inter-chain linker and quantitative removal of the CARD (Figure 1b). Sequence analysis and mass spectroscopy revealed that the site of cleavage had been shifted to Glu344. To prevent any cleavage in the linker, we mutated the Glu residues at positions 343, 344 and 352 to Ala, and expressed and purified the material from *E. coli*. When the triple mutant was incubated with 1.0 M NaCit, as before no inter-chain cleavage could be observed (Figure 1b).

Now that we had intact full-length material that could not be processed, we tested whether active DRONC is a monomer or dimer, and whether it can be activated by proteolysis. When analyzed by size exclusion on Superose 200, two-chain DRONC eluted with an apparent size of 75.6 kDa, which, given that the monomeric size of this material that has lost its CARD is about 38 kDa, is consistent with the size of a dimer (Figure 2a). We tested a number of commercially available caspase substrates and found that acetyl-Ile–Glu–Thr–Asp–7-amino-4-trifluoromethylcoumarin (Ac-IETD-AFC) was the most effective for measuring DRONC. Interestingly, the bulk of activity against Ac-IETD-AFC was associated with fractions corresponding to the leading edge of the peak when measured in caspase buffer (see Materials and Methods), but the activity mirrored the main part of the protein peak when measured in buffer containing 1.0 M NaCit. The Glu352Ala mutant eluted as a bimodal peak, with the first part of the peak at 70.8 kDa corresponding to material that was full length, and a shoulder at 40 kDa corresponds to material that had lost its CARD (Figure 2b). The elution profile of the 40-kDa CARD-deleted material is consistent with that of a monomer (predicted size 45 kDa), and the 70.8-kDa elution position of the full-length material is between a monomer (predicted size 48 kDa) and a dimer (predicted size 96 kDa). It most likely represents a monomer, and the larger than expected size in size-exclusion chromatography is probably the result of

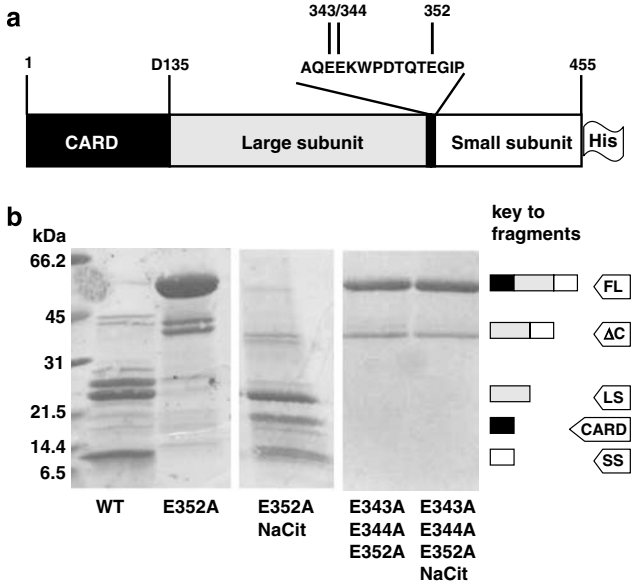

Figure 1 Constructs and proteins used in the study. (**a**) DRONC is composed of two domains, the CARD and the catalytic domain. The catalytic domain itself is composed of a large and a small subunit joined by the inter-domain linker segment that is a substrate for autolytic cleavage. (**b**) Expression of full-length DRONC in *E. coli* leads to processing that removes the CARD and generates large and small subunits of the catalytic domain. MALDI-TOF and N-terminal sequence analysis revealed that the double bands corresponding to the wt DRONC large subunit are a mixture of cleavage at Glu343 and Glu352. Mutation at Glu352 produces a single-chain enzyme that removes its CARD and undergoes processing at Glu344 when incubated in 1.0 M NaCit. The triple Glu/Ala mutant has a small amount of CARD removal, but is prevented from further autoprocessing in the presence of 1.0 M NaCit. FL, full length; ΔC, CARD removed; LS, large subunit; SS, small subunit

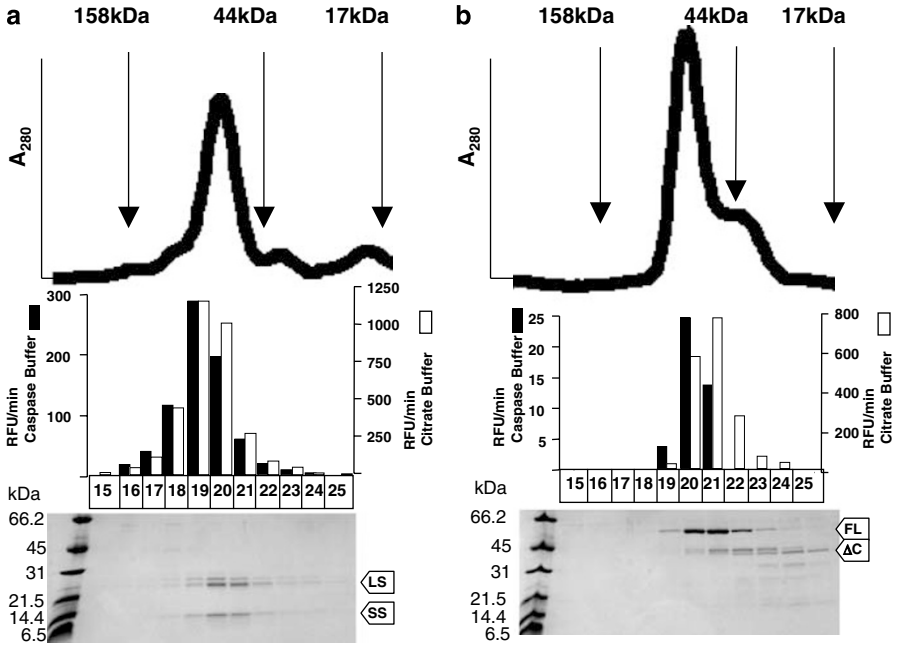

Figure 2 Size-exclusion chromatography of wt DRONC and DRONC Glu352Ala mutant. Purified wt DRONC (11 μM) (**a**) or DRONC Glu352Ala mutant (**b**) were analyzed by size exclusion on Superose 200 (upper traces). Fractions were diluted twofold into caspase buffer (black bars) or 1.0 M NaCit buffer (white bars) and assayed on Ac-IETD-AFC (middle panels). Samples from each fraction were also analyzed by SDS-PAGE (lower panels). Activity is expressed as RFU/min, relative fluorescence units per minute

an asymmetric Stoke's radius due to the non-ideal behavior of a protein composed of two globular domains (caspase domain plus CARD). Consequently, in contrast to the two-chain form, the majority of single-chain protein, whether on not it contained a CARD, appeared to be monomeric. Again, the bulk of activity associated with fractions corresponding to the leading edge of the peak when measured in caspase buffer, but the activity mirrored the main part of the protein peak when measured in NaCit.

From this we conclude that, when expressed and purified from *E. coli*, the two-chain form of DRONC (cleaved at Glu352 in the inter-chain linker) has lost its CARD and is primarily dimeric. In contrast, the single-chain form (mutated from Glu to Ala at residue 352) is primarily monomeric, irrespective of whether it retains the CARD. Thus, cleavage in the inter-domain region stabilizes dimer formation and this dimerization is apparently independent of the CARD.

Cleavage does not activate DRONC. Because two-chain DRONC demonstrated higher activity than the single-chain enzyme, we investigated whether simply cleaving the linker region of a single-chain form was the mechanism of activation. For this we utilized the Glu352Ala mutant to obtain single-chain material, and analyzed several endopeptidases that could in principle cleave in the linker region to generate the two-chain form. Of those tested, GluC protease from *Streptomyces griseus*, commonly used in protein fragmentation strategies, cleaved primarily in the selected region, specifically at Glu344, the same position cleaved during DRONC-mediated auto-cleavage of the Glu352Ala mutant (Figure 3a). GluC also removed the CARD, and therefore its activity essentially mimicked NaCit-mediated auto-proteolysis of the Glu352Ala mutant. However, we observed no increase in activity above background following cleavage, and in comparison 1.4 M NaCit activated this material about 50-fold (Figure 3b). We can therefore conclude that cleavage in the inter-chain linker segment is, in itself, insufficient to activate DRONC.

Activity of single-chain *versus* two-chain DRONC. The activity of two-chain DRONC increased by about fourfold, and that of the Glu352Ala single-chain form by about 32-fold, when measured using Ac-IETD-AFC in buffer containing 1.0 M NaCit (Figure 2). However, Ac-IETD-AFC was a relatively poor substrate for all three forms of DRONC, and we decided to identify a better substrate that would allow more accurate comparison of the activities of the differentially processed forms of the enzyme. Because DRONC has been reported to possess activity against Asp and Glu in P1, we first determined the preferred P1 residue by positional-scanning (Figure 4a). DRONC and human caspase-9 were assayed for activity on 18 individual substrate pools containing P1 residues fixed as one of the 20 natural amino acids (with the absence of Cys and Met), and each pool contains a mixture of these 18 amino acids in the P2, P3 and P4 positions. This allowed us to determine the optimal P1 position for each protease, and we found that DRONC had appreciable activity in pools containing a P1 Glu (as did caspase-9), but substantially more activity on pools containing a P1 Asp, indicating a P1 preference for Asp.

We next optimized the P4–P2 preference by using a second positional-scanning library where these positions are varied, with P1 fixed at Asp, as previously described.[19] DRONC showed surprisingly little selectivity in P2 and P4, although there was some preference for hydrophobes in P4, but with a stricter preference in P2 for hydrophobes (Figure 4b). On the

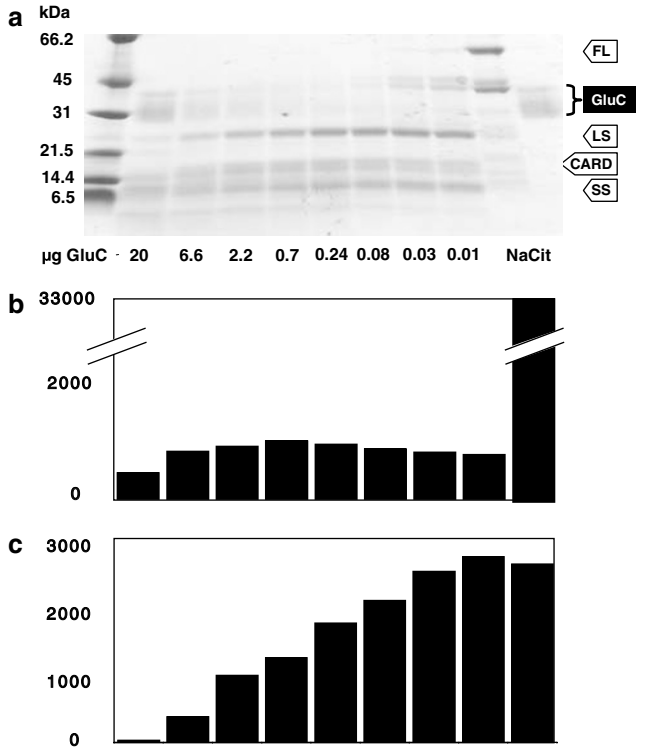

Figure 3 Endoproteinase Glu-C cleavage of DRONC Glu352Ala. DRONC (4 μM) Glu352Ala was cleaved with the indicated range of endoproteinase Glu-C concentrations and activity assessed on Ac-IETD-AFC. Samples were analyzed by SDS-PAGE (**a**) and the activity of DRONC Glu352Ala from which the background of Glu-C activity has been subtracted was determined (**b**). The NaCit control represents maximum activity in the presence of 1.4 M NaCit. (**c**) Upon completion of Glu-C cleavage, one-tenth of each sample was assayed again in the presence of 1.4 M NaCit to determine whether endoproteinase Glu-C inactivates DRONC. The result reveals that endoproteinase Glu-C above 0.24 μg inactivate DRONC, but below this there is little inactivation but almost complete processing of DRONC. The fraction containing 0.03 μg of Glu-C was analyzed by MALDI/TOF, which identified fragments corresponding to the CARD, and large and small subunits of DRONC Glu352Ala, cleaved at Glu344

basis of these two libraries, the sequence Leu–Ala–Leu–Asp (P4–P1) was judged to represent the optimal substrate. This result is very close to a similar approach used by Hawkins *et al*.[16] who determined the optimal P2–P4 sequence as Thr–Ala–Thr, and inspection of Figure 4b reveals that this sequence is also close to optimal.

Armed with this information, we synthesized two substrates, Ac-LALD-AFC and Ac-LALE-AFC, with which we characterized the forms of DRONC. Even though we used the optimal tetrapeptide sequence, we could not achieve saturating conditions for any form of DRONC with Ac-LALD-AFC, preventing us from determining individual k_{cat} and K_M values. Consequently, we used the linear portion of a substrate/velocity plot to determine the ratio of k_{cat}/K_M, which defines the specificity of each form for the substrate (see Materials and Methods; Equation (1)). Although we could determine k_{cat}/K_M for wild-type (wt) DRONC in caspase buffer, we were unable to determine any reliable values for the Glu352Ala and E343/344/352A mutants in the absence of NaCit (Table 1). However, in the presence of 1.4 M NaCit, we obtained catalytic parameters on both substrates within a factor of 3 for all forms of DRONC. Interestingly, we obtained almost identical k_{cat}/K_M values for Ac-LALD-AFC and Ac-LALE-AFC, indicating that in the context of the optimal P4–P2 subsite occupancies, no forms of DRONC could distinguish between Asp or Glu in P1, at least by using these tetrapeptide substrates.

The k_{cat}/K_M values we determined were surprisingly low, with no form of DRONC exceeding $5.5\,M^{-1}\,s^{-1}$(Table 1). These rates are of the orders of magnitude below the k_{cat}/K_M values of other caspases on their optimal peptidyl substrates. For example, caspase-3 has a k_{cat}/K_M of $1.3 \times 10^6\,M^{-1}\,s^{-1}$ on Ac-DEVD-pNA,[20] and caspase-9 has a k_{cat}/K_M of $1.9 \times 10^4\,M^{-1}\,s^{-1}$ on Ac-LEHD-AFC.[21] Clearly, we had to employ a better substrate with which to explore the specificity and activity of DRONC, and to this end, we chose to work with the natural substrate DRICE, an executioner caspase and downstream target of DRONC.[16,17]

Kinetics and specificity of DRICE cleavage by DRONC and DRONC autoproteolysis. DRICE is cleaved at Asp230 during its activation by DRONC,[14,16] and DRONC cleaves itself at Glu352 during its autoactivation, and so clearly in the context of natural protein substrates, DRONC can cleave after Asp or Glu. To determine how efficient DRONC is in cleaving a natural (protein) *versus* synthetic (tetrapeptide) substrate, we incubated a constant concentration of the DRICE precursor with increasing concentrations of DRONC. The DRICE precursor contained a Cys211Ala catalytic mutation to prevent autoprocessing during expression in *E. coli*. We also replaced the natural Asp230 cleavage site with Glu in the background of the DRICE Cys211Ala mutant, and we compared the efficiency of DRICE processing of the wild type and the Glu mutant. The objective was to determine whether DRONC preferred Asp or Glu in P1 in the context of this natural substrate. Figure 5 reveals that a lower concentration of DRONC is required to cleave wt DRICE than the Asp230Glu mutant, indicating that Asp at position 230 is preferred over Glu. To quantitate the difference we determined the concentration of DRONC required to cleave 50% of the DRICE precursor, and applied this concentration to equation (2) described under Materials and Methods, allowing us to determine the k_{cat}/K_M value for each protein substrate as previously described.[18] We were not able to determine cleavage of DRICE by NaCit-treated DRONC, since high concentrations of the salt tended to precipitate DRICE, but wt two-chain DRONC in caspase buffer cleaved wt DRICE with k_{cat}/K_M of $1.3 \times 10^3\,M^{-1}\,s^{-1}$ and the Asp230Glu mutant with k_{cat}/K_M of $1.6 \times 10^2\,M^{-1}\,s^{-1}$ (Table 1). Consequently, in the context of a natural substrate, DRONC prefers Asp to Glu by a factor of about 8.

In a related experiment, we analyzed the cleavage of a protein substrate that naturally contains a Glu at the DRONC cleavage site – DRONC itself. Because cleavage of DRONC by itself is very inefficient *in vitro*, we compared autoprocessing during expression in *E. coli*, where very high protein levels promote autoproteolysis. Figure 6 reveals that wt DRONC (Glu352) is processed to roughly the same extent as the Glu352Asp mutant. Thus, in the context of a natural, but

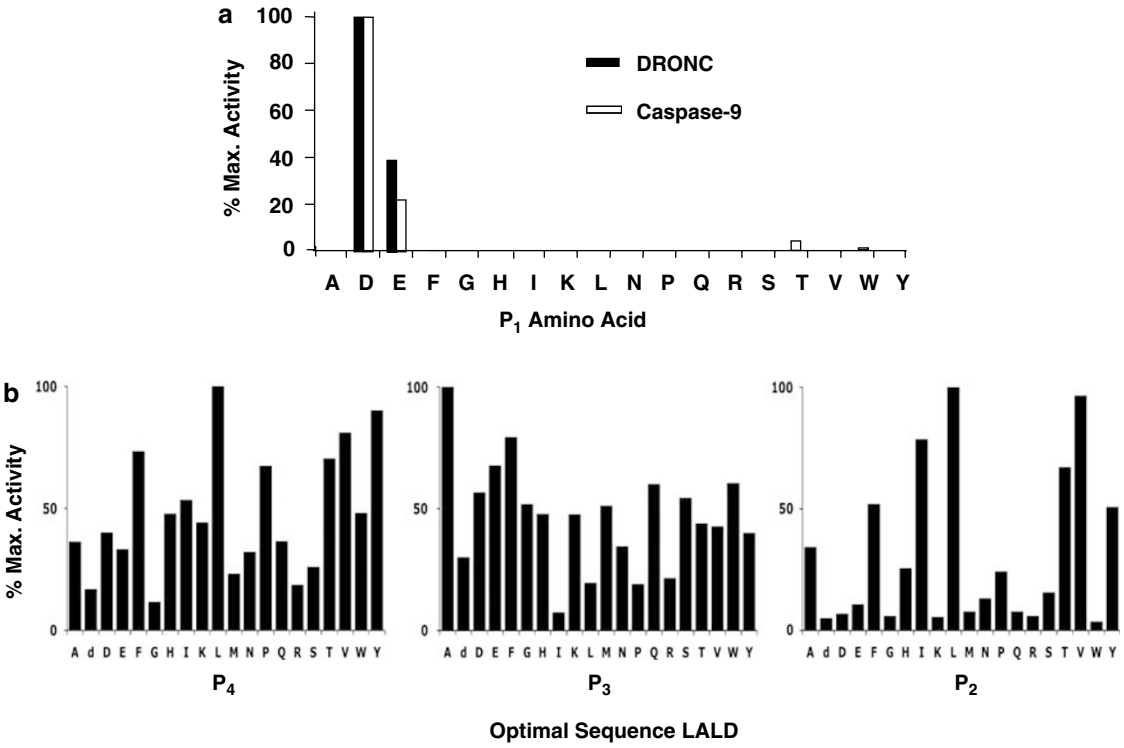

Figure 4 Substrate specificity of wt DRONC. (**a**) wt DRONC was added to a final concentration 26 μM in 0.7 M NaCit buffer pH 7.4 incubated at 37°C for 1 h and assayed for 2 h on a fluorimeter using excitation 320 emission 460 filter pairs. Caspase-9 was added to a final concentration of 7 μM in 0.7 M NaCit buffer pH 7.4 incubated at 37°C for 30 min and assayed for 15 min. Recombinant wt DRONC and delta CARD caspase-9 were assayed for activity on a P1 substrate scanning library, where P1 is fixed and P2, P3 and P4 are a mixture of 18 amino acids. (**b**) wt DRONC was incubated at a final concentration of 19 μM in the presence of 1.4 M NaCit and assayed with a positional-scanning substrate library with P1 fixed at aspartic acid. The *y*-axis represents 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, d is D-alanine)

Table 1 k_{cat}/K_M determination ($M^{-1}\,s^{-1}$) of DRONC and DRONC mutants on LALD and LALE-based synthetic substrates

	Ac-LALD-AFC		Ac-LALE-AFC	
	Caspase buffer	**Citrate buffer**	**Caspase buffer**	**Citrate buffer**
wt DRONC (3.7 μM)	0.25	5.2 ($\pm$3)	0.23	5.5 ($\pm$2.3)
DRONC Glu352Ala (1.6 μM)	N/D	2.65 ($\pm$0.6)	N/D	2.35 ($\pm$0.32)
DRONC Glu352/343/344A (2.8 μM)	N/D	1.6	N/D	2.04
Caspase-9 (2.9 μM)	N/D	30.3 ($\pm$6)	N/D	8.75 ($\pm$1.25)
	wt DRICE		**Asp230Glu DRICE**	
wt DRONC (12.5 μM)	1300	N/D	160	N/D

Abbreviations: Ac, acetyl; AFC, amino-4-trifluoromethylcoumarin; DRICE, *Drosophila* interleukin-1B-converting enzyme; DRONC, *Drosophila* Nedd2-like caspase; wt, wild type. wt DRONC, DRONC Glu352Ala and DRONC Glu352/343/344Ala were tested for activity on Ac-LALD-AFC and Ac-LALE-AFC in caspase buffer and 1 M NaCit buffer. N/D, no activity was found for the DRONC mutants unless assayed in NaCit. For comparison on the peptidyl substrates, the activities of caspase-9 are shown, and the lower row shows the k_{cat}/K_M values for wt DRONC on the natural substrate DRICE

rather poor substrate, there is no substantial preference of DRONC for Glu or Asp.

Discussion

The general mechanism of caspase activation requires transition of loops carrying the catalytic and specificity-determining residues from and exposed to a constrained conformation, allowing the enzyme to accept substrate and enhance catalysis.[2,3] This is achieved for the human initiator caspase-8 and caspase-9 by dimerization, and for the human executioner caspase-3 and caspase-7 by cleavage in the inter-chain linker of already assembled dimers.[22] The distinction in mechanism is presumably due to the position of a caspase in a pathway, such that executioners are downstream of initiators, and therefore can be activated through proteolysis by the initiator, but initiators have no protease above them, and have to employ a non-proteolytic mechanism for their activation. Consequently, it came as somewhat of a surprise that the *Drosophila* initiator caspase DRONC, the

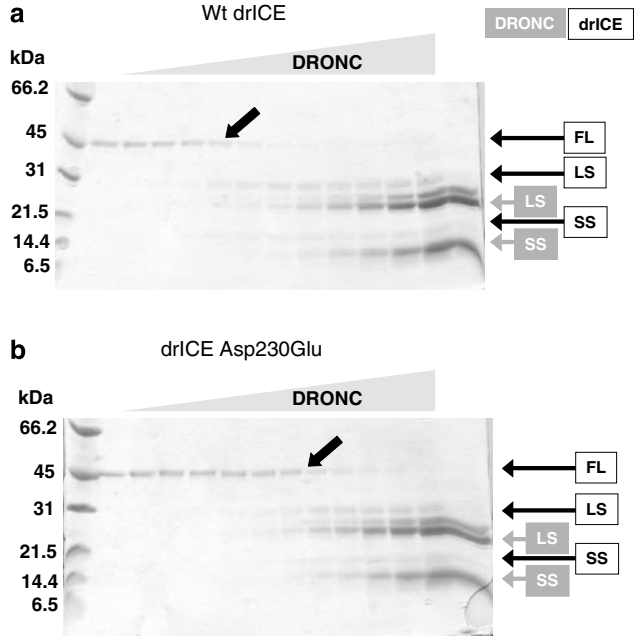

Figure 5 DRONC prefers Asp over Glu in the context of a natural substrate. Catalytic Cys/Ala mutants of wt DRICE (**a**) and an Asp230Glu mutant (**b**) were expressed and purified from *E. coli* and used as substrates for cleavage by wt DRONC in caspase buffer. A standard concentration of DRICE wt or mutant (0.85 μM) was incubated with increasing concentrations (125 nM to12.5 μM) of DRONC (left to right) for 90 min at 37°C. The arrow indicates the concentration of DRONC required to cleave 50% of the respective DRICE precursor

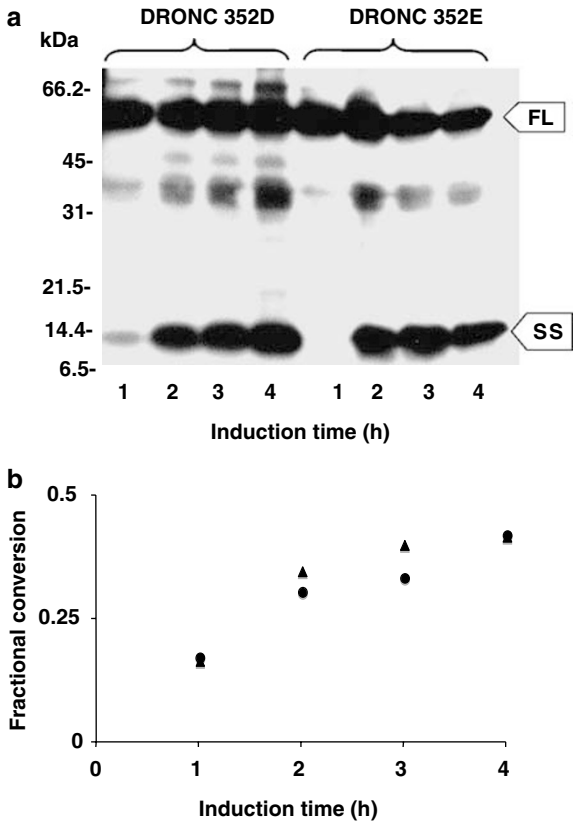

Figure 6 Specificity of DRONC autolytic processing. (**a**) Recombinant DRONC Glu352Asp and wt DRONC with a C-terminal His tag were expressed in *E. coli*. After induction with 0.1 mM IPTG, samples were taken from the culture at the indicated induction times. *E. coli* lysates from each time point were then analyzed by western blotting for the presence of a His tag. Cleavage of full-length DRONC (FL) to generate the small subunit (SS) at 11 kDa indicates cleavage at residue 352. (**b**) Quantitative image analysis of the same samples using a CCD camera from the developing PVDF membrane; ●, wt and ▲, Glu352Asp mutant. Cleavage is expressed as fractional conversion of the immunoreactivity in the SS divided by the sum of total immunoreactivity in the FL plus SS

ortholog of human caspase-9, was reported to be activated by proteolysis,[14] since this would enforce a revision of the general mechanism of initiator caspase activation. We demonstrate that direct cleavage of single-chain DRONC, using endoprotease GluC, does not activate the enzyme. In contrast, incubation of the same protein, Glu352Ala DRONC, in 1.0 M NaCit activated the enzyme by more than two orders of magnitude. Since NaCit activates the human initiator caspase-8 and caspase-9 by dimerization,[19] with a similar gain in activity, it is likely that the same mechanism is in play for DRONC. Our data support recent work questioning the role of autoproteolysis in DRONC activation.[15]

Cleavage in the inter-chain linker clearly has a role to play in the regulation of DRONC activity. In caspase buffer, single-chain DRONC has almost no activity on synthetic substrates when compared with the two-chain enzyme, and it was this observation that led to the suggestion that cleavage in the inter-chain linker was the mechanism of activation.[14] However, the two-chain protein is dimeric, but the single-chain protein is monomeric, and it is this difference that accounts for the increase in activity. The simplest explanation is that cleavage in the inter-chain linker stabilizes dimerization of DRONC, very much as is seen with the human initiator caspase-8.[23,24] But first the protein must be dimerized, which can be achieved *in vitro* with high concentrations of NaCit, and presumably *in vivo* following recruitment to the *Drosophila* apoptosome.[9] This scenario is fully consistent with the process that activates the human initiator caspase-9, occurring through apoptosome-driven dimerization of monomers to induce the active conformation of this ortholog of DRONC.[13]

A property of DRONC that distinguishes it from other caspases is its acceptance of Glu in the primary specificity pocket P1. This has been demonstrated previously on synthetic peptidyl substrates,[16] and also in terms of its autolytic cleavage and cleavage of *Drosophila* IAP1.[14,16,25] Interestingly, as shown in Figure 4a, caspase-9 also tolerates Glu in its primary specificity pocket, and can cleave its own inter-chain segment following a Glu residue, although this cleavage is subservient to the natural Asp cleavage sites in the caspase-9 linker.[26,27] We demonstrate, using a matched set of substrates, that DRONC may have almost equal activity on tetrapeptides containing Asp and Glu in P1, but this does not hold for a downstream substrate. DRONC cleaves itself with approximately equal preference for Asp and Glu, but for the natural substrate DRICE, Asp is substantially favored over Glu in P1. This apparent contradiction relates to the absolute values of the second-order rate of proteolysis, k_{cat}/K_M, for it appears that in the context of an optimal tetrapeptide substrate (Ac-LALD-AFC), or autoproteolysis, there is little discrimination at P1, but in the context of a good protein

substrate (DRICE), discrimination between Asp and Glu is readily apparent.

The concept that DRONC is equally active on Asp and Glu substrates is inaccurate because in the context of a substrate that is cleaved more rapidly (DRICE), Asp dominates the P1 preference. But why is DRONC such a poor enzyme on itself, and also on tetrapeptides compared with a downstream natural protein substrate? After all, LALD is the optimal tetrapeptide sequence in our hands, very similar to the TATD sequence found previously by a similar combinatorial technique,[16] in which study it was shown that DRONC had k_{cat}/K_M values of 2.73 M^{-1} s^{-1} for Ac-TQTE-AFC and 3.36 M^{-1} s^{-1} for Ac-TQTD-AFC. When another residue is added to the P5 position, DRONC catalysis increases but only by twofold,[16] suggesting that the enhancement in catalysis on DRICE is largely due to interactions outside of the catalytic cleft. Such 'exosite' interactions are important in enhancing the catalysis of inherently poor enzymes, including metalloproteases[28] and coagulation proteases.[29,30]

Since DRONC has no intrinsic preference for Glu over Asp, why is the primary autolytic cleavage site at position 352 conserved as Glu in both *D. melanogaster* and *Drosophila pseudoobscura*? To answer this will require some sophisticated experiments, but it could simply be to prevent other caspases, such as DCP-1 and DRICE with the canonical Asp specificity,[16] from adventitiously cleaving DRONC. Such cleavage may be associated with premature removal of DRONC via the ubiquitin system before its activation.[31] Our profiling of DRONC's primary substrate specificity, and demonstration that cleavage of DRONC is neither necessary nor sufficient for its activation, are, of necessity, founded on *in vitro* observations. Assembling a *Drosophila* apoptosome that efficiently cleaves peptide substrates is not trivial. Indeed, in a recent paper,[15] the only assay possible for ARK activated DRONC was to amplify the signal and measure downstream DEVD-ase activity. Unfortunately this cannot tell us about DRONC specificity on synthetic substrates because the signal needed executioner caspase activation.

On the basis of our observations, one could envision different scenarios for how DRONC activity and specificity are enhanced *in vivo*. Although cleavage does not activate DRONC, our data suggest that the stability of the active dimer is substantially enhanced by cleavage. This may be important if DRONC dissociates from the apoptosome following dimerization and cleavage, where it would be stable for long enough to cleave good targets such as DRICE. Indeed, stabilization may explain why, although cleavage of DRONC is not required for activity *in vitro*, it may be required for cell death *in vivo*.[14] Association of DRONC and DARK may result in an improved ability to bind non-optimal substrates, such as DRONC itself and DCP-1 (the other proposed executioner caspase targeted by DRONC). When compared with DRICE, DCP-1 is reportedly a much poorer substrate of recombinant DRONC,[16] and may require exosite interactions provided by the full apoptosome. Obviously, a combination of these options could be envisioned, but our data firmly suggest that the activity of DRONC is not only dependent on the formation of a correct dimer, but perhaps to a greater extent on interactions distant from the active site.

Materials and Methods

Materials. Ac-IETD-AFC was from MP Biomedicals (Irvine, CA, USA). The vector pET23b was from EMD Biosciences (San Diego, CA, USA). *E. coli* strain BL21pLysS(DE3) and QuickChange site-directed mutagenesis kit were from Stratagene (La Jolla, CA, USA). Caspase buffer contains 20 mM Pipes, 10% sucrose, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA and 10 mM dithiothreitol (DTT), pH 7.2. NaCit buffer contains 50 mM Tris, 100 mM sodium chloride, pH 7.4, containing NaCit in the range of 0.7 to 1.4 M, depending on experimental conditions.

Proteins. The plasmid pET23b encoding *Drosophila* wt DRONC and Glu352Ala DRONC plus a C-terminal His_6 purification tag was a generous gift from Dr Rollie Clem. These plasmids were transformed into *E. coli* strain BL21pLysS(DE3) expressed and purified by a Ni-chelate affinity method.[18] Expressed proteins were tested within 2 days of purification. QuickChange site-directed mutagenesis kits (Stratagene) were used to substitute Glu343 and Glu344 of DRONC with an Ala residue, and to create a Glu352Asp mutant.

The cDNA encoding *Drosophila* wt DRICE plus a C-terminal His_6 purification tag was a generous gift from Dr Pascal Meier. This cDNA was inserted via *Nde*I/*Xho*I into pET23b, transformed into *E. coli* strain BL21pLysS(DE3) expressed and purified by a Ni-chelate affinity method.[18] Expressed proteins were tested within 2 days of purification. QuickChange site-directed mutagenesis kit (Stratagene) was used to generate Glu230Asp and Cys211Ala mutants of DRICE. All plasmids were sequenced to verify the success of the mutagenesis and the absence of other mutations.

Positional-scanning substrate libraries. We employed two positional-scanning libraries to define the P1–P4 subsite preferences of DRONC. The first library was used to define the preferred P1, and contained 18 pools in which the P1 position is fixed as one of the 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 in each pool.[32] This fluorogenic 7-amino-4-methylcoumarin (AMC) peptide substrate library was synthesized by solid-phase chemistry on an Fmoc-AMC resin essentially as previously described.[33] Selected peptide pools were subjected to amino-acid analysis to verify library composition and concentration, and the concentration of each remaining peptide pools was determined by absorbance at 320 nm. All reactions were carried out in 100 μl in a 96-well plate containing and substrate library concentration in the range 5–10 μM at 37°C, and analyzed with excitation at 320 nm and emission at 460 nm.

The second library was used to define the P4, P3 and P2 subsite preferences of DRONC, and we employed a positional-scanning substrate library with the general structure Ac-OXX-Asp-AMC for P4 scanning, Ac-XOX-Asp-AMC for P3 scanning and Ac-XXO-Asp-AMC for P2 scanning (where O is the positionally specified residue and X signifies the mixture of the 20 natural amino acids omitting Cys, and including D-Ala). Standard peptide synthesis was used to prepare the library, as previously described.[19] The variable amino-acid pools were prepared as an isokinetic mixture with all 20 amino acids.

Synthesis of the Ac-LALD-AFC and Ac-LALE-AFC. Fmoc-Asp(OtBu)-AFC and Fmoc-Glu(OtBu)-AFC were synthesized as described[34] and subsequently the Fmoc group was deprotected using four equivalents of diethylamine in *N,N'*-dimethylformamide for 1 h. Ac-LAL-COOH was synthesized by classic solution-phase peptide methods, and coupled to the deprotected Asp- or Glu-AFC.

Enzyme kinetics. Assays using fluorogenic AFC substrates (excitation wavelength 405 nm and emission wavelength 510 nm) were carried out on an fMax Fluorescence Microplate reader (Molecular Devices) operating in the kinetic mode. Reactions of 100 μl contained enzyme and substrate (final concentration 100 μM) in appropriate buffer. To determine the rates of synthetic substrate cleavage, we measured the release of the AFC fluorophore continuously using an fMax Fluorescence Microplate reader (Molecular Devices) operating in the kinetic mode. To determine the catalytic efficiency of the enzyme, initial velocities were measured as a function of substrate $[S_0]$. When $[S_0] \ll K_M$, the plot of velocity *versus* $[S_0]$ is a straight line with a slope of V_{max}/K_M. The k_{cat}/K_M values were calculated using the following expression.

$$k_{cat}/K_M = \text{slope}/[E] \quad (1)$$

To determine the rate of DRICE cleavage by DRONC, we calculated the enzyme concentration [E] required to decrease 50% (EC_{50}) of precursor in time *t* from the

respective SDS gels, and the catalytic efficiency was expressed as k_{obs}. When the reaction is carried out below K_M for protein substrate (which is probably true for the cases examined in our study), k_{obs} becomes the kinetic constant k_{cat}/K_M.[35]

$$k_{cat}/K_M = \ln 2/[E]\,t \qquad (2)$$

Enzyme concentrations [E] were calculated on the basis of total protein concentration estimated from the absorbance at 280 nm.[36]

SDS-PAGE and N-terminal sequencing. An 8–18% linear acrylamide gradient SDS gel run in a 2-amino-2-methyl-1,3-proanediol/glycine/HCL system was used for resolving proteins.[37] Samples were boiled in SDS sample buffer containing 50 mM DTT for 5 min and then loaded on the stacking gel. For N-terminal sequencing, protein samples were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) by electroblotting. The membrane was briefly stained with Coomassie Brilliant Blue R-250, destained and washed with water. Appropriate bands were excised and sequenced by Edman degradation on a 492 protein sequencer (Applied Biosystems, Foster City, CA, USA).[38]

Size-exclusion chromatography. Experiments were performed using a Pharmacia AKTA purifier system equipped with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) with a flow rate of 0.4 ml/min, and 0.4 ml fractions were collected for analysis. The buffer used for size-exclusion chromatography was 20 mM Tris (pH8.0)/100 mM NaCl. The column was calibrated using protein standards from Bio-Rad.

Mass spectrometry. Mass spectra of Endoproteinase Glu-C-digested fragments were collected using an Applied Biosystems Voyager DE Pro MALDI-TOF instrument with the help of the Burnham Institute Proteomics Facility.

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