Molecular determinants involved in activation of caspase 7

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SYNOPSIS

During apoptosis, initiator caspases (8, 9, and 10) activate downstream executioner caspases (3, 6, and 7) by cleaving the interdomain connector (IDC) at two sites. Here, we demonstrate that both activation sites, site 1 and site 2, of caspase 7 are suboptimal for activation by initiator caspases 8 and 9 *in cellulo*, and *in vitro* using recombinant proteins and activation kinetics. Indeed, when both sites are replaced with the preferred motifs recognized by either caspases 8 and 9, we measured improvement in activation of up to 36 folds. Moreover, cleavage at site 1 is preferred over site 2 because of its location within the IDC since swapping sites does not lead to a more efficient activation. We also demonstrate the paramount role of Ile₁₉₅ of site 1 involved in maintaining a network of contacts that preserves the proper conformation of the active enzyme. Finally, we show that the length of the IDC plays a crucial role in maintaining the need for proteolysis for activation. In fact, although we were unable to generate a caspase 7 that does not require proteolysis for activity, shortening the IDC of the initiator caspase 8 by four residues was sufficient to confer a requirement for proteolysis, a key feature of executioner caspases. Altogether, the results demonstrate the critical role of the primary structure of caspase 7's IDC for its activation and proteolytic activity.

Keywords

apoptosis; caspase; protease; activation mechanism; caspase 7

INTRODUCTION

Proteases play crucial roles in regulating a wide range of events such as, digestion, coagulation, development, and elimination of superfluous and damaged cells. In animals, the latter two events essentially occur by apoptosis, an inflammation-free process in which cellular functions are halted, and in which the cell is packaged into smaller bodies ready for efficient phagocytosis by professional macrophages or neighboring cells. Apoptosis is driven by a family of ubiquitously expressed cytosolic cysteine peptidases called caspases.

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Although the primary role of caspases is to cause cellular demise [1], they are also necessary for some non-lethal processes [2, 3].

Apoptotic caspases are divided in two groups namely initiator (caspases 8, 9, and 10) and executioner caspases (caspases 3, 6, and 7). They are all present in cells as latent zymogens but the mechanism by which they are activated differs. Initiator caspases are activated by dimerization on macromolecular platforms and they are further stabilized by cleavage of the interdomain connector (IDC) that separates the large and small subunits of the catalytic domain [4–7]. Therefore, cleavage of the IDC is not essential for the proteolytic activity of initiator caspases. Conversely, the executioner caspases are always dimeric and activated solely by processing of the IDC by initiator caspases or other peptidases able to cleave the IDC [8–10]. Cleavage of the IDC, usually at two sites close in the primary structure, exposes the C-terminus of the large subunit and the N-terminus of the small subunit, which is sufficient for executioner caspase activation [11]. The processed neo-N-terminus of the small subunit is also key in the interaction of caspases 3, 7, and 9 with the endogenous inhibition X-linked inhibitor of apoptosis protein (XIAP) [12–15], and protein stability of caspases 3 and 7 [16]. Based on structural and biochemical analyses, the IDC is a highly flexible segment and is therefore very sensitive to proteolysis [8, 17].

To study the relevance of the IDC primary sequence in the activation process of caspases, we chose caspase 7 as our model of executioner caspase. Our work is facilitated by the availability of several X-ray structures of the active and the zymogen forms of caspase 7 that has allowed us to make structure-based predictions. Like all apoptotic caspases, caspase 7 possesses two cleavage sites in the IDC. Site 1 is always cleaved before site 2 (and refer to site 1 and site 2 throughout the text) *in cellulo*, in *in vitro* assays by initiator caspases and during bacterial protein expression in which caspases self-activate [18, 19]. Notably, both cleavage sites are suboptimal for processing by initiator caspases and also by self-processing because the preferred recognition sequences of caspases 7, 8, and 9 do not match the sequence of the cleavage sites. Moreover, initiator caspase IDCs are longer than that of most executioners supporting the notion that initiator caspases must have enough leeway to become active without cleavage. This is necessary because there is no upstream protease in their respective pathway to activate them. Here we have focused on the primary structure of the two activation sites of caspase 7, and how these sequences influence the activity and activation of the enzyme by the initiator caspases. We also show that the location of the cleavage site is paramount in dictating activation efficacy, which also suggests the contribution of other factors beyond the primary sequence. We also test the hypothesis that the IDC's length constrains the caspase by requiring cleavage to become active. Our data demonstrate the importance of the primary structure of the interdomain connector on the activity and activation of caspase 7.

EXPERIMENTAL

Reagents

All caspase substrates were obtained from BIOMOL (Plymouth Meeting, PA, U.S.A.), and were calibrated using the molar extinction coefficient of free pNA (*p*-nitroanilide)/afc [7-amino-4-(trifluoromethyl)coumarin] after full hydrolysis using excess caspase 3. The

irreversible caspase inhibitor Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) used to active site-titrate caspases was purchased from MP Biomedicals (Solon, OH, U.S.A.). Other chemicals were from Sigma-Aldrich (St-Louis, MI, U.S.A.) or Laboratoire MAT (Beauport, QC, Canada).

Plasmids, recombinant protein expression and purification

Site-directed mutagenesis was performed using overlapping PCR, and ensuing cDNAs were sequenced to ensure their integrity. Full-length caspase 7 cDNA subcloned into pET-23b(+) (Novagen, Madison, WI, U.S.A.) [10], and DEDs (death effector domain)-caspase 8 cDNA in pET-15b (Novagen) [20] were used for all constructs. Full-length caspase 9 and caspase 9 lacking the caspase recruitment domain (CARD, caspase recruitment domain) were described elsewhere [6]. Caspases, CrmA (cytokine response modifier protein), and p35 were produced in the BL21(DE3)*pLysS* (Novagen) *E. coli* strain as described previously [19, 21, 22]. Procaspase 7 mutants were produced following short expression times (25 min). In some instances, cleavage site mutants were activated by co-purification with untagged caspase 8 catalytic domain to cleave the caspase 7 zymogens that do not self-activate in *E. coli*. Remaining active caspase 8 was then neutralized with the poxvirus CrmA protein, which does not inhibit caspase 7 [22]. Protein concentrations of inactive or of caspases that cannot be active site-titrated were determined using a Coomassie protein assays and the Edelhoch relationship on purified material [23]. For mammalian expression, all cDNAs were subcloned by PCR into an engineered pcDNA3 plasmid that encodes a C-terminus flag epitope (LEDYKDDDDK).

Cell culture, transfection, and apoptosis stimuli

The human embryonic kidney 293Ad cells (MP Biomedicals, Solon, OH, U.S.A.) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Wisent, St-Bruno QC), 2 mM L-glutamine (Wisent), and antibiotics. For caspase 8 directed apoptosis, cells grown in 100-mm plates were transfected using Eugene 6 (Roche Diagnostics, Mannheim, Germany) and 6 µg of the indicated plasmid DNA then splitted into 6-well plates after 24 hrs. After another 24 hrs, cells were left untreated or treated with the indicated concentration of recombinant human soluble KillerTRAIL (Enzolife Sciences, Farmingdale NY). Detergent extracts (mRIPA buffer with protease inhibitors) were prepared and analyzed as described [18] after 12 hrs of treatment. For caspase 9-directed caspase activation, cells grown in 150-mm plates were transfected using Eugene 6 (Roche) and 9 µg of the indicated plasmid DNA then split into three 150-mm plates after 24 hrs. After another 24 hrs, hypotonic extract were prepared as described [24] in the presence of 30 µg/mL RNase A, and programmed to activate caspases using 1 µM horse cytochrome c and 1 mM dATP (both Sigma-Aldrich) for the indicated period of time. Reactions were stopped with SDS gel loading buffer. In both cases, samples were analyzed by immunoblotting as previously described [25] using M2 (0.5 µg/mL; F1804; Sigma-Aldrich), p23 (1:2,000; MA3-414; Pierce Chem Co., Rockland IL), or anti-hsp90 (0.05 µg/mL; 610419; BD Biosciences) antibodies.

Biochemical characterization

All caspases were active site-titrated as previously described using Z-VAD-fmk or p35 (a pan-caspase inhibitor from baculovirus) as titrant, and AcDEVD-afc (for caspase 7), AcIETD-afc (caspase 8), or AcLEHD-afc (caspase 9) as substrate [19]. Kinetic parameters were determined by non-linear regression at 37°C using the preferred substrate of each caspase [26] in standard caspase buffer [20 mM Pipes pH 7.2, 200 mM NaCl, 20% (w/v) sucrose, 0.2% (w/v) CHAPS, 2 mM EDTA, and 20 mM dithiothreitol (DTT)] for executioner, or high salt buffer [50 mM Hepes pH 7.4, 1 M sodium citrate, 50 mM NaCl, 0.01% (w/v) Chaps, and 10 mM DTT] for initiator caspases [27]. In some instances, parameters were measured in pseudo-first order kinetic conditions ($[S] \ll K_m$) when K_m values were out of the workable substrate range ($K_m > 200 \mu\text{M}$). In all cases, initiator caspases at concentration above 100 nM were preincubated for 1 hour in high salt buffer at 37°C to promote dimerization and maximal activity of the peptidases. Fluorescence was acquired using a M1000 Infinite platereader (Tecan, Switzerland) or a FLX800 (Bio-Tek Instruments Inc., Winooski, VT, U.S.A.). Results were analyzed with GraphPad Prism 5 software.

Caspase cleavage assay by initiator caspases

Cleavage assays of procaspase 7 variants by initiator caspases were performed as followed. Initiator caspases ($> 100 \text{ nM}$) were preactivated at 37°C for 1 hr in high salt buffer prior to performing the assays to maximize caspase activity [27]. For gel analyses, a range of concentrations of activated initiator caspase was incubated at 37°C with a fixed amount of procaspase 7 (see figure legend for other assay conditions). After a fixed period of time, reactions were stopped by precipitating proteins with trichloroacetic acid (TCA)/acetone, resuspended in SDS/PAGE loading buffer, and resolved on a gradient gel. Estimation of k_{cat}/K_m was obtained using the relationship $p = (1 - e^{k_{cat}[E]/K_m t})$ [19] in which p is the fraction of procaspase 7 that is cleaved at time t (s), and $[E]$ is the initiator caspase concentration. The point at which 50% of the zymogen is processed ($p = 0.5$) was used to estimate k_{cat}/K_m . For pre-steady state activation assays, a fixed concentration of activated initiator caspase was incubated with various concentrations of procaspase 7 and the amidolytic activity on AcDEVD-pNA chromogenic substrate was measured continuously using a NanoQuant Infinite M200 or Infinite M1000 platereader (Tecan). Results were analyzed by non-linear regression using GraphPad Prism 5 software as previously described [8, 10, 27] to determine rates of activation. Importantly, site 2 mutations did not alter kinetic parameters the active enzyme significantly (Supplementary Table 1), which allowed us to easily compare these mutants. However, mutation of site 1 to LETD/LEHD caused the active enzyme to behave erratically precluding their thorough analysis by pre-steady state analyses.

SDS/PAGE and immunoblotting

Poly-acrylamide gels were performed using gradient (8–18%, w/v) acrylamide gels in the 2-amino-2-methylpropan-1,3-diol/glycine buffer system [28]. Gels were either stained using GelCode Blue (Pierce Chemical Co., Rockford, IL, U.S.A.) or transferred to Immobilon-P membrane (Millipore, Billerica, MA, U.S.A.) using 10 mM Caps, pH 11, and 10% (v/v) methanol for 45 min at 0.4 A and at 4°C. Immunoblots were carried out using standard

protocols with anti-His antibody (0.1 µg/mL; 34660; QIAGEN, Mississauga, ON, Canada), and revealed with WestPico SuperSignal (Pierce Chemical Co.).

RESULTS

Processing of caspase 7 activation site mutants

The cleavage site and its location within a substrate are two essential factors directing proteolysis efficacy [29]. At a given location in a substrate, the cleavage site sequence becomes critical. Based on results from cleavage of short peptidic substrates [26], it appeared that caspase 7's primary activation site sequence (site 1, IQAD₁₉₈↓S, caspase 7 residue numbering; Figure 1A) and the secondary site (site 2, NDTD₂₀₆↓A) within the IDC are suboptimal recognition motifs for initiator caspases. To better understand the basis of this apparent suboptimal sequence usage, we generated a series of caspase 7 activation site variants by mutating individual motifs to the preferred sequence recognized by initiator caspase 9 (LEHD motif) or caspase 8 (LETD motif) as determined using short peptidic substrates [26]. We first analyzed the ability of these mutants to auto-activate and characterized their proteolytic activity. Recombinant caspases were produced in bacteria as His-tagged proteins and purified by immobilized metal affinity chromatography. To prevent cleavage of the alternate site while studying a particular site (*i.e.* site 1 *vs.* site 2), the critical aspartate residues were mutated to an alanine *i.e.* IQAD→IQAA (site 1) or NDTD→NDTA (site 2). Throughout the study, cleavage site mutants are named with the sequence of both sites as site 1/site 2 pairs; thus, the WT (wild-type) enzyme would be termed caspase 7 IQAD/NDTD (P1 cleavage residues are underlined). Figure 1B shows that *E. coli* expression of site 1 mutants (caspase 7 LEHD/NDTA or caspase 7 LETD/NDTA) resulted in auto-processed enzymes displaying the typical large and small subunits. We noticed that a standard 8 hr-expression was insufficient to produce fully processed caspase 7 LEHD/NDTA (Figure 1B) when compared to WT caspase 7 suggesting that either the site is inadequate for caspase 7 self-activation or that the resulting enzyme is less active. To test the latter we measured the kinetic parameters of the processed enzyme. Interestingly, both caspase 7 site 1 mutants did not exhibit typical Michaelis-Menten behavior (not shown). Therefore, we resorted to pseudo-first order assays ($v/[S] \ll K_m$) to estimate activity. In such conditions, $k_{cat}/K_m \approx v/[S]/[E]$ and the specific activity is directly related to the substrate hydrolysis v and inversely related to substrate and enzyme concentrations. Using this method, we obtained specific activities that are 17 to 64 times less active than caspase 7 IQAD/NDTA (Table 1). These weak activities likely explain why we were unable to active site-titrate these enzymes.

In a second series of mutants, we modified activation site 2 to the motifs preferred by caspase 8 or caspase 9 respectively. Contrary to site 1 mutants, site 2 mutants (caspase 7 IQAA/LEHD and caspase 7 IQAA/LETD) did not auto-activate in bacteria (Figure 1B). To verify whether modifications to site 2 affected proteolytic activity, we determined the kinetic parameters of both mutants following their processing with caspase 8 and found k_{cat} and K_m values to be similar to WT caspase 7 (Table 1). Both mutants retained typical Michaelis-Menten behavior after processing with caspase 8 as compared to WT site 2 caspase 7.

Caspase 7 primary activation site is close to optimal for activation *trans* by initiator caspases

Having characterized caspase 7 cleavage site mutants, we addressed the ability of full-length and truncated initiator caspases to activate them. An important prerequisite to the use of a truncated caspase 9 is to demonstrate that initiator caspase recruitment domains do not influence substrate preference. Therefore, we tested full-length and CARD-caspase 9 against a combinatorial peptidic substrate library covering the P4-P2 cleavage site residues (Supplementary Figure 1) and found that both enzymes have the same substrate preference. Furthermore, similar experiments performed using full-length and truncated caspase-8 gave similar results (C. Pop and G.S. Salvesen, unpublished results). We first tested the capacity of full-length caspase 9 and a truncated version (lacking the caspase recruitment domain, CARD) to activate procaspase 7 (Figure 2A). In our assays, serial dilutions of activators were incubated with a fixed concentration of procaspase 7 IQAD/NDTA or LEHD/NDTA. Cleaved fragments are then resolved on SDS-PAGE. Figure 2A (top left) shows that processing of site 1 is not improved when the optimized LEHD motif is used compared to the IQAD sequence, which corresponds to an activation at WT site 1 [11]. This is also true for the activation by CARD-caspase 9, albeit the activation of all procaspases is better than with the full-length activator. Indeed, the lowest concentration used (35 nM) resulted in full cleavage of the procaspase 7. The discrepancy between both caspase 9 activators remains a mystery at this moment. However, by varying the incubation time of the assays, we estimated that procaspase 7 mutants LEHD/NDTA was activated by CARD-caspase 9 with a rate that is twice as fast than that of the WT site 1 (data not shown). Similarly, procaspase 7 LETD/NDTA is activated more efficiently (~2 folds) by caspase 8 as demonstrated by the fact that less activator was required to cleave 50% of zymogen (119 nM vs. 53 nM; Supplementary). These results demonstrate that activation site 1 is close to optimal for activation by the initiators.

Site 2 can be improved for activation by initiator caspases

We also performed on-gel activation assays with procaspase 7 site 2 mutants. Compared to procaspase 7 IQAA/NDTD (*i.e.* WT site 2), procaspase 7 IQAA/LEHD has a ~5-fold better activation rate by caspase 9, and ~4-fold increase using CARD-caspase 9 as an activator (Figure 2A). Because our gel-based assays are performed with a procaspase 7 concentration that may exceed the K_m value, it is possible that activation assays were not performed in pseudo-first order condition, which would greatly underestimate the calculated k_{cat}/K_m . Therefore, we performed pre-steady state assays to better characterize the activation at site 2 since we observed an important improvement in activation when this site is mutate. In these assays, serial dilutions of procaspase 7 are incubated with a fix amount of activator. The ensuing activation is then monitored using a caspase 7 substrate (Figure 2B, top panels). Using an equation describing the rate of activation of a protein in a coupled reaction, we determined activation rates that we plotted against procaspase 7 (Figure 2B, bottom panels) and calculated K_m , k_{cat} and k_{cat}/K_m using non-linear regression (Table 2). For all three activator caspases tested, we observed between 10–36-fold improvement in specific activity (k_{cat}/K_m) when site 2 is modified to the preferred recognition motif of either caspase 9 or caspase 8. Interestingly and irrespectively of the initiator caspase studied, we obtained activation rates at the mutated site 2 that are similar to the rates obtained for WT site 1.

Another finding is that for both forms of caspase 9 we tested, K_m values for WT site 1 (IQAD) are assumed to be high because we were unable to ascribe a Michaelis-Menten behavior to the activation of procaspase 7 IQAD/NDTA. Finally, a striking observation was that very low k_{cat} were obtained. The reason for that is unknown, but our k_{cat} values are consistent with previously published k_{cat}/K_m [4] ($\sim 10^4 \text{ M}^{-1}\text{s}^{-1}$) and K_m [30] ($\sim 100 \text{ nM}$). Taken together, we conclude that site 2 can be improved for activation.

Both activation sites can be improved in complete apoptotic paradigms

To further demonstrate that activation sites are not optimal, we used apoptotic models. First, we employed a cell-free system that recapitulates the intrinsic apoptotic pathway led by caspase 9 that we trigger with cytochrome *c* and dATP [24]. Extracts were prepared from cells expressing the flag-tagged catalytic mutant of the cleavage site mutants (named 'reporter'). By doing so, each reporter is incorporated into the apoptotic framework as a bystander substrate of initiator, thus reporting activation. Importantly, the reporter has no observable effect as indicated by the rate of AcDEVD-pNA hydrolysis (caspase 3/7 activity) in cell extract compared to that observed with extract from empty plasmid-transfected cells (Figure 3A, graph inset). The generation of cleaved flag-tagged reporter carrying WT sites (Figure 3A, middle panel) appears within 10 min of *cyt*c/dATP addition followed at 30 min by the cleavage of the death substrate hsp90 co-chaperone p23 [31]. Similar profiles were obtained with the reporter with IQAD/NDTA or LEHD/NDTA sites (Figure 3, left panels) demonstrating that in accordance with our activation kinetics (Table 2), site 1 is relatively optimal for caspase 9-mediated activation. On the contrary, no activation was observed at WT site 2 (IQAA/NDTD), which is what we observed in assays with recombinant proteins (Figure 2). Interestingly, its replacement with the LEHD motif allows some limited processing (Figure 3A, right panels). In all cases, p23 processing was not altered suggesting again that ectopic expression of caspase 7 activation reporter does not interfere with the apoptotic machinery.

Employing a different apoptotic model that uses the death ligand Trail to activate the extrinsic pathway (caspase 8/10), we showed that both cleavage sites can be improved by using the LETD motif (Figure 3B). Indeed, 293Ad cells transfected with a plasmid encoding the reporter with LETD/NDTA or IQAA/LETD sites showed better processing than with the respective WT site. Similarly to caspase 9, these results also mirrored our *in vitro* activation assays. Taken together, these results demonstrate that caspase 7 activation sites can be improved by substitution them with the preferred motif of the respective initiator caspase.

Self-activation of caspase-7 and cleavage site location

Caspase 7 does not self-activate at the endogenous concentration whereas the high concentration of enzyme achieved during *E. coli* expression allows self-activation. To better understand the mechanism by which caspase 7 autoactivates, we first inserted an optimal caspase 7 cleavage sequence (DEVD) at site 1. Like WT caspase 7, the introduction of a DEVD motif (caspase 7 DEVD/NDTD or caspase 7 DEVD/NDTA) resulted in a fully processed enzyme (Figure 4A). Unexpectedly, tracking of caspase 7 DEVD/NDTA processing during its expression revealed that the zymogen form could easily be produced, and that most of the processing occurs after 2 hours of expression, like WT caspase 7

(Figure 4B). This is likely due to the enzyme's weak intrinsic activity (Table 1) as similar to that observed for the LETD/LEHD site 1 mutants. Indeed, caspase 7 DEVD/NDTA did not display typical Michaelis-Menten behavior suggesting that the IQAD→DEV D substitution altered some fundamental properties of the enzyme similarly to site 1 mutation to LETD/LEHD. Further, by deconstructing the DEV D motif (IQAD→DQAD/DEAD), we realized that the P4 residue Ile₁₉₅ is critical in supporting robust enzyme activity whereas mutation of P3 Gln₁₉₆ alone (IQAD→IEAD) had a minimal impact on the activity (Table 1). We next asked why Ile₁₉₅ is so important as suggested by our data and recent work by Witkowski and Hardy [32]. One reason for the loss of activity of site 1 mutants with altered Ile₁₉₆ may reside in the intricate contacts between this residue of one catalytic unit of the dimer with the other seen in the crystal structure of inhibitor-bound caspase 7 [33]. In the crystal structure of inhibitor-bound caspase 7, Ile₁₉₅ is in close proximity to Lys₂₁₂' and Ile₂₁₃' of the other catalytic unit of the dimer (often called L2-L2' loop bundle). To further test this observation, these residues were mutated, and enzyme activity was assessed. As expected, disruptive mutation of Lys₂₁₂ to Met/Glu or Ile₂₁₃ to Ala/Glu significantly affected the activity of the caspase (Table 1). In support to the perturbation of caspase activity by non-conservative mutations, three of these mutants required longer expression times or *trans* activation by caspase 8 to obtain processed enzymes. Conversely, conservative amino acid substitutions (Lys₂₁₂→Arg or Ile₂₁₃→Met/Leu) preserved caspase 7 activity and proper activation time (Table 1).

We showed that despite the improvement of site 2 by introducing preferred initiator caspase recognition motifs, caspase 7's site 1 was superior for activation suggesting, as proposed by Timmer and colleagues [29], that location of the site within the IDC is also important. To address this, we first transferred the site 1 motif (in addition to the P1' serine) to site 2 generating caspase 7 IQAA/IQAD-S. Interestingly, this mutant was expressed only as a zymogen (Figure 4C). Moreover, transplanting site 2 motif (NDTD) to site 1 failed to produce a caspase able to auto-activate, even after 24 hrs (data not shown). This demonstrates that location of the activation site within the IDC is important. We then replaced site 2 by the preferred caspase 7 motif DEV D, thus generating caspase 7 IQAA/DEV D. Surprisingly, we obtained this protein in a fully processed form (Figure 1A), demonstrating that it is possible to cleave site 2 first. Nevertheless, this mutant displayed a ten-fold decrease in specific activity compared to WT caspase 7 (Table 1). Despite the reduced activity of caspase 7 IQAA/DEV D, this caspase activated so effectively that even a 25 min. expression immediately followed by immunoblotting failed to show the zymogen form of the enzyme (Figure 4D). Altogether, these results show that site 1 is better positioned than site 2 for activation in *trans*, but that the propensity for processing of site 2 can be improved significantly.

The interdomain connector length is a crucial determinant

The longer length of the IDC is postulated to be a crucial determinant in the ability of initiator caspases to display activity without cleavage [17]. Executioner caspases 3 and 7 have a shorter IDC and, contrary to the initiators, which do not require cleavage for activity [5, 6, 20], proteolysis is the driving force of executioners activation. Therefore, not only is the primary structure of caspase 7's IDC key to the activation process but also its length may

be important. To address this issue, we extended the IDC from 25 residues to 31 (the length of caspase 8's IDC) with the hope of creating an active executioner without cleavage of the IDC. To do so, and guided by the studies described above, we inserted 3 Gly-Ser pairs N-terminal of site 2 of the double cleavage site mutant to generate caspase 7 IQAA/NDTA+6 (Figure 5A). Figure 5B shows that this mutant did not self-process during bacterial expression contrary to the cleavable site 1 version of this construct (IQAD/NDTA+6). We also extended the IDC by adding 7 Gly-Ser pairs but were unsuccessful in obtaining an active enzyme (Table 1, data not shown). In a second strategy, we constructed two mutants by replacing the IDC of caspase 7 with that of caspase 8: a) DGIQADSGPINDTDANPRYKIPV → KGIPVETDSEEQPYLEMDLSSPQTRYIPD (linker casp-8a) to preserve Asp₁₉₂ [10], or b) QADSGPINDTDANPRYKIPV → PVETDSEEQPYLEMDLSSPQTRYIPD (linker casp-8b) to also preserve the critical Ile₁₉₅ of caspase 7 (dotted lines in Figure 5A). However, these mutants did not undergo auto-activation nor did they exhibit any proteolytic activity (Figure 5B and data not shown), but processing by caspase 8 did activate caspase 7/linker casp-8b (data not shown). A corollary to our hypothesis is that the length of the initiator caspase 8 IDC is optimal in order to support caspase 8 activity without cleavage of the linker. Therefore, as a validation of our strategy we performed the reverse approach with caspase 8, and attempted to create a caspase 8 requiring proteolysis for activity. To do so, two (Glu³⁷⁷-Gln, caspase 8 numbering system) or four residues (Glu³⁷⁷-Gln-Pro-Tyr) were deleted from caspase 8's IDC, and we compared the activity of these deletion mutants possessing a cleavable (caspase 8 VETD/LEMA) or uncleavable (caspase 8 VETA/LEMA) IDC (Figure 5A). Removal of 4, but not of 2, residues resulted in a caspase 8 requiring cleavage for activity (Figure 5B, Table 1). Indeed, caspase 8 VETA/LEMA-4 was inactive in high salt buffer whereas caspase 8 VETD/VETA-4 was cleaved (Figure 5B) and fully active compared to WT caspase 8 in the same conditions (Table 1). These results demonstrate that the length of the IDC is critical in the activation process of caspases.

DISCUSSION

All caspases have an interdomain connector that is poorly conserved amongst family members, yet it is always cleaved during activation. Cleavage of executioner caspases is the driving force for their activation [8–10] whereas cleavage of initiator caspase IDC is insufficient for activation [4–7, 34]. The IDC of human apoptotic caspases has two cleavage sites that are processed during apoptosis or *E. coli* expression. In this study, we addressed the structure-relationship of the two activation sites of executioner caspase 7.

Here, we show that the sequences of both activation sites of caspase 7 are suboptimal for activation by initiators *in vitro* and *in cellulo*. Changing site 1 to the preferred motif recognized by caspase 8 resulted in a higher activation rate whereas activation by caspase 9 seemed optimal. Interestingly, most site 1 mutants we examined have poor kinetic efficacies demonstrating that it also supports enzyme activity once cleaved. Indeed, crystal structures of many cleaved caspases show that residues upstream to site 1 are implicated in contacts with the neighboring catalytic unit within a dimer to form the so-called L2-L2' loop bundle (Supplementary A). Many studies [10, 32] have confirmed that Asp₁₉₂ and Ile₁₉₅ are critical for caspase 7 activity, explaining why many site 1 mutants have reduced kinetic

performance. In the crystal structures, these residues contact amino acids C-terminal to the IDC. We have tested two of these residues, Lys₂₁₂ and Ile₂₁₃, and found that only conservative mutations at these positions supported robust activity. Both residues are conserved in the closely related homolog caspase 3, whereas only the hydrophobic character of position 213 is preserved in other caspases. We speculate that the lack of conservation may explain why swapping the IDC between caspases failed to produce activity because the IDC must also complement features of the active site to stabilize it. Suboptimal cleavage sites in protease zymogen activation has been reported before such as for plasminogen [35]. In this particular context, structural requirements over-ride sequence preference, and so caspase 7 activation demonstrates an interesting analogy to the requirements of plasmin activation. Less than optimal activation processes pave the way for rigorous regulation. Indeed, coupled with the difference of reactivity towards its endogenous inhibitor XIAP [12], a controlled cleavage at two different sites can enhance regulation of the whole system.

Our data obtained with site 1 and site 2 mutants suggest that the location has considerable impact on the efficacy of activation. We had previously demonstrated that caspase 7 cannot directly undergo auto-activation at site 2 [18]. Unexpectedly, inserting the best motif recognized by caspase 7 (DEVD) at site 2 allowed caspase auto-activation directly at this site. However, we could not obtain a caspase able to self-activate using the site 1 motif (IQAD) despite the fact that we demonstrated that it was close to optimal for self-activation. Therefore, it seems that only an optimal motif could be used at site 2, but these data demonstrate that it is the positioning within the IDC that is critical. Neither the available crystal structures of the active form nor the ones of the zymogen form of caspase 7 reveal well-defined electron density for residues surrounding site 2 (listed in ref. [17], and refs. [32, 36, 37]). However, these structures show that residues close to or part of site 1 (Asp₁₉₂ to Gln₁₉₆) stabilize the active form by immobilizing the catalytic cysteine.

Recently, the NMR structure of monomeric procaspase 8 was published [38]. It shows the IDC adopting several different conformations, and potentially making several contacts with the core of the protein. Because of its short length, caspase 7's IDC cannot adopt these configurations. Instead, the available structures of procaspase 7 demonstrate that the IDC must travel from one end to the other end of the molecule as an extended loop with contacts made only by residues C-terminal to site 2. Therefore, we can propose that site 2 is not as accessible as site 1 because of these contacts, which restrain IDC movement. However, mutation of site 2 to DEVD would disturb these interactions enough to render that mutant highly susceptible to self-activation. Indeed, procaspase 7 structures show that residue P2-Thr₂₀₅ of site 2 contacts Lys₂₁₂' of the other molecule of the dimer, which is likely disrupted in the DEVD mutant (Supplementary B) [39, 40]. Furthermore, Ile₂₁₃' and the side chain of Lys₂₁₂' occupy a hydrophobic pocket, with the ϵ -amino group of the lysine participating in hydrogen bonds with a nearby water molecule. Because cleavage of the IDC results in an important repositioning of Lys₂₁₂ and Ile₂₁₃, we can assume that insertion of the IDC into the central cavity of the dimer is reversible allowing rapid exchange with the other IDC, thus rendering site 2 of both catalytic units less available for cleavage.

Finally, we also evaluated the impact of IDC length on the activity of caspase 7. Several reports have demonstrated that the zymogen of initiator caspases displays activity without

cleavage [5, 6, 20], which is not the case for executioners. The length of the IDC is one of the critical features that is hypothesized to allow activity without cleavage [17]. Consequently, our rationale was to elongate caspase 7's IDC hoping to produce an active caspase 7 without proteolysis. However, neither the insertion of a highly flexible sequence of up to 14 residues nor the replacement with the IDC of an initiator caspase succeeded in removing this requirement. A corollary to the IDC length hypothesis is that the initiator IDCs must permit activity without proteolysis. In fact, shortening the IDC by four residues was sufficient to produce a caspase 8 that also requires cleavage for its activation. This result reinforces the idea that IDC length is essential for the higher zymogenicity of initiator caspases compared to executioners, and that for executioners, it serves also to stabilize the active form. In their study, Keller and colleagues also concluded that caspase 8 IDC length, and the various determinants it contains, were critical in providing activity to the zymogen [38]. However, they could not establish how the length controls the auto-cleavage process of caspase 8 because IDC truncation mutants were expressed as separate subunits then refolded to yield the catalytically active protease. Recent studies on an uncleaved but active caspase 3 mutant [41] (shorter IDC than caspase 7) and the crystal structure of procaspase 6 [42] (longer IDC than caspase 7 and caspase 8) draw a more complex picture of how the IDC controls the zymogen state and the activity of caspases. It is likely that a combination of several molecular determinants rules this process on an individual basis and that general rules may not apply even amongst executioner caspases.

Caspase activation has been extensively studied, but many of the underpinning molecular determinants of activation remain unknown. Our results highlight the importance of the primary structure of caspase 7 IDC for its activation and activity. It also demonstrate, using an real-life proteolytic event, the need to also study molecular determinants other than the immediate cleavage site and substrate binding site to fully understand how proteases reach high level of efficacy and specificity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

afc	7-amino-4-(trifluoromethyl)coumarin
CARD	caspase recruitment domain
CrmA	cytokine response modifier protein

cyt c	cytochrome <i>c</i>
DED	death effector domain
DTT	dithiothreitol
IDC	interdomain connector
pNA	<i>p</i> -nitroanilide
TCA	trichloroacetic acid
Trail	tumor necrosis factor-related apoptosis-inducing ligand
WT	wild-type
XIAP	X-linked inhibitor of apoptosis protein
Z-VAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Figure 1. Expression of caspase 7 cleavage site mutants

(A) Schematic representation of caspase 7 and its IDC. The N-terminal peptide (N), the large and small subunits, and the IDC (sequence shown) are displayed. The star represents the catalytic cysteine. Cleavages of the IDC occur C-terminal to the motifs in boldface. (B) SDS/PAGE analysis of caspase 7 cleavage site mutants. It is noteworthy that all proteins lost the N-terminal peptide due to the activity of the enzyme despite the fact that some are purified as zymogen form. The underlined residues identify the processed cleavage sites.

Figure 2. Activation of procaspase 7 by initiator caspase 9 can be improved at site 2
(A) SDS/PAGE analysis of activation kinetics of various caspase 7 mutants with full-length caspase 9 (left panels) or truncated (CARD) caspase 9 (right panels). Assay conditions were as followed: left, 400 nM of the indicated procaspase 7, full-length caspase 9 (1/2 serial dilution from 500 nM down to 8 nM), 2 hr. at 37°C; right, 200 nM of the indicated procaspase 7, CARD-caspase 9 (2/3 serial dilution from 400 nM down to 35 nM), 12 min. at 37°C. TCA/acetone-precipitated proteins were resolved on SDS/PAGE and stained. A sample without initiator caspase (procasp-7) or with the maximum concentration of the initiator (casp-9 or CARD-casp-9) used was included as controls. The full-length zymogen (empty arrowhead), the form lacking the N-terminal peptide (-N), and the cleaved large subunit (Is) are indicated. It is noted that most procaspase 7 preparations lack the zymogen with the N-peptide because it is removed extremely rapidly. Arrows indicate the midpoint at

which 50% of the procaspase 7 is cleaved. Apparent k_{cat}/K_m was obtained as described in **Experimental**. It is noted that most values are underestimated because procaspase 7 concentrations are above the estimated K_m (see Table 2). Molecular weight markers are 97.4 (full-length caspase 9 panels only), 66.2, 45 (*), 31, 21.5, and 14.4 kDa. **(B)** Activation kinetics by full-length caspase 9 (left), truncated caspase 9 (middle), and truncated caspase 8 (right) of procaspase 7 site 2 mutants. Concentration range (2/3 serial dilution from 150 nM down to 3.9 nM) of the indicated site 2 mutants was incubated with the indicated initiator caspases in the presence of 400 μ M of the chromogenic substrate Ac-DEVD-pNA. The top graphs depict the amidolytic activity monitored over time at 405 nm for the 67 nM procaspase 7 samples. Values presented are background-corrected for the activity of the initiator caspase. Rates of procaspase 7 activation were estimated as described in **Experimental** and plotted against the procaspase 7 concentrations in the bottom graphs. Solid lines were obtained by non-linear regression of data using the Michaelis-Menten equation $v = V_{max,app}[S]/(K_{m,app} + [S])$ whereas interrupted lines are linear regressions and represent the particular case in which $[S] \ll K_m$ and apparent k_{cat}/K_m is obtained from the slope over activator concentration. Kinetic parameters are reported in Table 2. Note the scale differences of y-axes. In bottom graphs, dotted lines indicate the 67 nM procaspase 7 concentration values extracted from the top panels data. Activator concentrations are: full-length caspase 9, 100 nM; truncated caspase 9, 25 nM; truncated caspase 8, 25 nM.

Figure 3. Activation of procaspase 7 can be improved in a complete apoptotic system

A) Site 1 is optimal for caspase 9 activation but site 2 can be ameliorated. Immunoblot analyses of *cytc*/dATP-activated extract from 293Ad cells expressing the various procaspase 7 cleavage site mutants. After the indicated period of time, samples were boiled in gel loading buffer and analyzed using anti-flag, p23 (caspase substrate), and hsp90 (loading control). Because procaspase 7 has a C-terminal flag tag, the anti-flag antibody detects the full-length zymogen (empty arrowhead), the form lacking the N-terminal peptide (-N), and the cleaved small subunit (ss). The inset shows similar assays analyzed for caspase activation using the chromogenic substrate AcDEVD-pNA. It is noted that no difference is observed between *cytc*/dATP-activated samples in which procaspase 7 cleavage site mutants are individually expressed. The asterisk denotes the group of traces obtained in the absence of *cytc*/dATP for each corresponding extract. The underlined residues identify the processed cleavage sites. B) Both sites can be improved for activation by caspase 8. 293Ad cells transfected to express the indicated procaspase 7 activation site mutant were challenged

without (first lane) or with increasing doses of recombinant Trail ligand (0.5, 1, 2, 5, 10, 20, 50, or 100 ng/mL). Processing of flag-tagged procaspase 7 was monitored by immunoblotting with an anti-flag antibody at 12 hrs following Trail addition. The underlined residues identify the processed cleavage sites.

Figure 4. Auto-activation of caspase 7 mutants

(A) SDS/PAGE analysis of purified caspase 7 cleavage site mutants. zym., zymogen. (B) Self-activation at site 1. The indicated caspase 7 were expressed in 50 mL cultures for the indicated period of time, purified, and analyzed by SDS/PAGE. Arrowheads indicate the transition point from the zymogen to the active form; this transition is absent for caspase 7 IQAA/NDTD that cannot self-activate [18]. (C) Swapping activation sites sequences. The indicated caspase 7s were expressed for 8 hrs, purified, and analyzed by SDS/PAGE. (D) Self-activation at site 2. Lysates of *E. coli* expressing the indicated caspase 7s for 30 min. or 8 hrs were analyzed by immunoblotting using an anti-His tag antibody. It is noted that only processed caspase is detected for caspase 7 IQAA/DEVD. WT and caspase 7 IQAA/NDTD are presented as controls for fully processed and zymogen form respectively. The underlined residues identify the processed cleavage sites.

Figure 5. Changing the length of the IDC

(A) Schematic representation of caspase 8 and its IDC. DEDs, the large and small subunits, and the IDC (sequence shown) are displayed. The star identifies the catalytic cysteine. Cleavage of the IDC occurs C-terminal to the motifs in bold at two sites (Asp³⁷⁴, or Asp³⁸⁴, caspase 8 numbering system). The 6-residue insertion (in caspase 7) and 2/4 amino acid deletions in caspase 8 are indicated under the sequence. The dotted lines show which parts of the IDC were swapped between caspase 8 and caspase 7. (B) SDS/PAGE analysis of caspase 7 insertion and caspase 8 deletion mutants after 8 or 6 hrs of expression respectively. Caspase 7/linker casp-8b is shown. The underlined residues identify the processed cleavage sites.

Table 1

Kinetic parameters of caspases 7 and 8 cleavage site mutants *

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Caspase 7 (AcDEVD-Afc)			
WT	12.8 ± 0.9	2.7 ± 0.2	$2.1 \pm 0.3 \times 10^5$
IQAD/NDTA	22.6 ± 3.6	2.3 ± 0.1	$1.0 \pm 0.2 \times 10^5$
DEVD/NDTA	<i>n.d.</i>	<i>n.d.</i>	1.6×10^4
LETD/NDTA	<i>n.d.</i>	<i>n.d.</i>	1566
LEHD/NDTA	<i>n.d.</i>	<i>n.d.</i>	5773
DQAD/NDTA	<i>n.d.</i>	<i>n.d.</i>	1690
DEAD/NDTA	<i>n.d.</i>	<i>n.d.</i>	288
IEAD/NDTA	24.5 ± 0.6	3.4 ± 0.1	$1.4 \pm 0.1 \times 10^5$
IQAA/DEVD	58.8 ± 5.1	1.3 ± 0.1	$2.1 \pm 0.3 \times 10^4$
Caspase 8-activated caspase 7 (AcDEVD-Afc)			
IQAA/NDTD	13.8 ± 0.7	4.4 ± 0.1	$3.1 \pm 0.2 \times 10^5$
IQAA/LETD	13.3 ± 1.1	3.6 ± 0.1	$2.7 \pm 0.3 \times 10^5$
IQAA/LEHD	14.6 ± 0.8	3.2 ± 0.1	$2.2 \pm 0.2 \times 10^5$
Caspase 7 (AcDEVD-Afc)			
K212M [†]	<i>n.d.</i>	<i>n.d.</i>	2185
K212E	<i>n.d.</i>	<i>n.d.</i>	< 100
K212R	59.2 ± 4.2	3.6 ± 0.1	$6.2 \pm 0.7 \times 10^4$
I213A	<i>n.d.</i>	<i>n.d.</i>	3183
I213M	56.5 ± 5.4	2.6 ± 0.1	$4.7 \pm 0.6 \times 10^4$
I213E	<i>n.d.</i>	<i>n.d.</i>	< 100
I213L	78.2 ± 6.5	3.8 ± 0.1	$4.7 \pm 0.5 \times 10^4$
Caspase 7 insertion mutants (AcDEVD-Afc)			
IQAD/NDTA+6	36.6 ± 2.5	2.9 ± 0.1	$7.4 \pm 0.6 \times 10^4$
IQAA/NDTA+6		inactive	
IQAD/NDTA+14	27.7 ± 2.1	6.0 ± 0.1	$2.2 \pm 0.2 \times 10^5$
IQAA/NDTA+14		inactive	
Caspase 8 mutants (AcLETD-Afc)			
WT	1.3 ± 0.1	2.0 ± 0.2	$1.0 \pm 0.0 \times 10^6$
VETD/LEMA	0.3 ± 0.0	0.5 ± 0.0	$1.9 \pm 0.2 \times 10^6$
VETA/LEMA	21.3 ± 1.1	0.3 ± 0.0	$1.5 \pm 0.1 \times 10^4$
VETD/LEMA-2	8.6 ± 1.4	5.8 ± 0.21	$6.7 \pm 1.3 \times 10^6$
VETA/LEMA-2	43.7 ± 4.1	0.09 ± 0.01	2022 ± 388
VETD/LEMA-4	5.9 ± 0.5	3.4 ± 0.1	$5.8 \pm 0.6 \times 10^5$
VETA/LEMA-4		inactive	

* Kinetic parameters are averages $\pm$ standard deviation from 2–5 independent determinations. *n.d.*, not determined because the enzyme did not display Michaelis-Menten behavior at the substrate concentration range we used (see text). For these, k_{cat}/K_m were determined using pseudo-first order kinetics ($[S] \ll K_m$).

[†]This caspase 7 mutant was activated using excess caspase 8 during purification.

Table 2

Activation kinetics of procaspase 7 cleavage site 2 mutants *

Activator	Procaspase 7	$K_{m,app}$ (nM)	$k_{cat,app}$ (s^{-1})	$k_{cat,app}/K_{m,app}$ ($\times 10^4 M^{-1}s^{-1}$)
Caspase 9	IQAD/NDTA		<i>n.d.</i>	0.34
	IQAA/NDTD		<i>n.d.</i>	< 0.01
	IQAA/LEHD	180 $\pm$ 10	0.0007 $\pm$ 0.0001	0.36
CARD-caspase 9	IQAD/NDTA		<i>n.d.</i>	29.5
	IQAA/NDTD	76.0 $\pm$ 8.2	0.0020 $\pm$ 0.0001	2.6
	IQAA/LEHD	20.0 $\pm$ 14.5	0.005 $\pm$ 0.002	25
DEDS-caspase 8	IQAD/NDTA	38.9 $\pm$ 6.7	0.0019 $\pm$ 0.0002	4.9
	IQAA/NDTD		<i>n.d.</i>	< 0.3
	IQAA/LETD	17.5 $\pm$ 7.3	0.0012 $\pm$ 0.0004	7.3

* Values $\pm$ S.D. of 2–3 independent datasets of 4–10 samples are presented. *n.d.*, not determined because $[S] \ll K_m$. The parameters k_{cat} and K_m could not be determined independently because $K_m \gg [S]$.