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 IDC (interdomain connector) 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 caspase 8 or 9, we found an up to 36-fold improvement in activation. Moreover, cleavage at site 1 is preferred to site 2 because of its location within the IDC, since swapping sites does not lead to a more efficient activation. We also demonstrate the important role of Ile1395 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 necessity of 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.

Key words: activation mechanism, apoptosis, caspase 7, interdomain connector, protease

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 neighbouring cells. Apoptosis is driven by a family of ubiquitously expressed cytosolic cysteine peptidases called caspases. Although the primary role of caspases is to cause cellular demise [1], they are necessary also for some non-lethal processes [2,3].

Apoptotic caspases are divided into two groups: namely initiator (caspases 8, 9 and 10) and executioner (caspases 3, 6 and 7) caspases. 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 IDC (interdomain connector) 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 large subunit is also key in the interaction of caspases 3, 7 and 9 with the endogenous inhibitor XIAP (X-linked inhibitor of apoptosis protein) [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 caspases. Our work is facilitated by the availability of recombinant caspase 7...
of several X-ray structures of the active and the zymogenic 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 referred to as 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 those 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 pathways 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 tested the hypothesis that the IDC’s length constrains the caspase by requiring cleavage to become active. Our results demonstrate the importance of the primary structure of the IDC in the activity and activation of caspase 7.

EXPERIMENTAL

Reagents

All caspase substrates were obtained from Biomol and were calibrated using the molar absorption coefficient (ε) of free pNA (p-nitroanilide)/afc [7-amino-4-(trifluoromethyl)coumarin] after full hydrolysis using excess caspase 3. The irreversible caspase inhibitor Z-Val-Ala-DL-Asp-CH₂F [benzyloxycarbonyl-valyl-alanyl-DL-aspartyl-fluoromethane; also known as Z-VAD-FMK (‘benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone’)] used to active site-titrated caspases was purchased from MP Biomedicals. Other chemicals were from Sigma–Aldrich or Laboratoire MAT.

Plasmids, recombinant protein expression and purification

Site-directed mutagenesis was performed using overlapping PCR, and the ensuing cDNAs were sequenced to ensure their integrity. Full-length caspase 7 cDNA subcloned into pET-23b(+) (Novagen) [10] and ΔDEDs [mutant lacking DEDs (death effector domains)] caspase 8 cDNA in pET-15b (Novagen) [20] were used for all constructs. Full-length caspase 9 and ΔCARD [mutant lacking CARD (caspase recruitment domain)] caspase 9 were described previously [6]. Caspases, CrmA (cytokine response modifier protein) and p35 were produced in the BL21(DE3)pLysS (Novagen) Escherichia coli strain as described previously [19,21,22]. Pro-caspase 7 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. The remaining active caspase 8 was then neutralized with the poxvirus CrmA protein, which does not inhibit caspase 7 [22]. Protein concentrations of inactive caspases or of caspases that cannot be active site-titrated were determined using Coomassie blue 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 HEK (human embryonic kidney)-293Ad cells (MP Biomedicals) were propagated in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) fetal bovine serum (Wisent), 2 mM L-glutamine (Wisent) and antibiotics. For caspase 8-directed apoptosis, cells grown in 100 mm plates were transfected using FuGENETM 6 (Roche Diagnostics) and 6 μg of the indicated plasmid DNA then split into different 6-well plates after 24 h. After another 24 h, cells were left untreated or treated with the indicated concentration of recombinant human soluble Killer-TRAIL (Enzolife Sciences). Detergent extracts (mRIPA buffer [50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 1% (v/v) Nonidet P40, 0.5% deoxycholate and 0.1% SDS] with protease inhibitors) were prepared and analysed by the method described in [18] after 12 h of treatment. For caspase 9-directed caspase activation, cells grown in 150 mm plates were transfected using FuGENETM 6 (Roche) and 9 μg of the indicated plasmid DNA then split into three 150 mm plates after 24 h. After another 24 h, a hypotonic extract was prepared as described in [24] in the presence of 30 μg/ml RNase A, and programmed to activate caspases using 1 μM horse cytochrome c (cytochrome c) and 1 mM dATP (both from Sigma–Aldrich) for the indicated period of time. Reactions were stopped with SDS gel loading buffer. In both cases, samples were analysed by immunoblotting as previously described [25] using M2 (0.5 μg/ml; F1804; Sigma–Aldrich), p23 (1:2000; MA3-414; Pierce, Rockland, IL, U.S.A.) or anti-hsp90 (heat-shock protein 90) (0.05 μg/ml; 610419; BD Biosciences) antibody.

Biochemical characterization

All caspases were active site-titrated as previously described using Z-Val-Ala-DL-Asp-CH₂F or p35 (a pan-caspase inhibitor from baculovirus) as the titrant and Ac-DEVD-afc (A-caspase inhibitor, N-acetyl-Asp-Glu-Val-Asp-afc) (for caspase 7), Ac-IETD-afc (A-caspase inhibitor, N-acetyl-Ile-Glu-Thr-Asp-afc) (caspase 8) or Ac-LEHD-afc (A-caspase inhibitor, N-acetyl-Leu-Glu-His-Asp-afc) (caspase 9) as the 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 [10 mM Pipes, pH 7.2, 100 mM NaCl, 10% (w/v) sucrose, 0.1% (w/v) Chaps, 1 mM EDTA and 10 mM DTT (dithiothreitool)] for executioner caspases 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] << K_m) when K_m values were outside the
workable substrate range ($K_m > 200 \mu M$). In all cases, initiator caspases at concentrations above 100 nM were pre-incubated for 1 h in high salt buffer at 37 °C to promote dimerization and maximal activity of the peptidases. Fluorescence was acquired using an M1000 Infinite platteread (Tecan) or an FLX800 (BioTek Instruments). Results were analysed with GraphPad Prism 5 software.

**Caspase cleavage assay by initiator caspases**

Cleavage assays of pro-caspase 7 variants by initiator caspases were performed as follows. Initiator caspases (>100 nM) were preactivated at 37 °C for 1 h in high salt buffer prior to performing the assays for maximizing caspase activity [27]. For gel analyses, a range of concentrations of activated initiator caspase were incubated at 37 °C with a fixed amount of pro-caspase 7 (see the Figure legends for other assay conditions). After a fixed period of time, reactions were stopped by sedimenting proteins with trichloroacetic acid/acetone, resuspended in SDS/PAGE loading buffer and resolved on a gradient gel. Estimation of $k_{cat}/K_m$ was obtained using the relationship $p = \left(1 - e^{-k_{cat}/K_m} \cdot t\right)$ [19] in which $p$ is the fraction of pro-caspase 7 that is cleaved at time $t$ (in units of seconds) and [E] is the initiator caspase concentration. The point at which 50% of thezymogen 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 pro-caspase 7 and the amidolytic activity on Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) chromogenic substrate was measured continuously using a NanoQuant Infinite M200 or Infinite M1000 platteread (Tecan). Results were analysed by non-linear regression using GraphPad Prism 5 software as previously described [8,10,27] to determine the rates of activation. Importantly, site 2 mutations did not alter kinetic parameters of the active enzyme significantly (see Supplementary Table S1 at www.biosci.org/bsr/031/bsr0310283add.htm), 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**

PAGE was 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) or proteins were transferred to an Immobilon-P membrane (Millipore) using 10 mM Caps (pH 11) and 10% (v/v) methanol for 45 min at 0.4 A and at 4 °C. Immunoblotting was carried out using standard protocols with anti-His antibody (0.1 µg/ml; 34660; Qiagen) and were revealed with WestPico SuperSignal (Pierce).

**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 the cleavage of short peptidic substrates [26], it appeared that caspase 7’s primary activation site sequence (site 1, IQAD↓↓↓↓S, caspase 7 residue numbering; Figure 1A) and the secondary site (site 2, NDTD↓↓↓↓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 analysed 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 versus site 2), the critical aspartate residues were mutated to an alanine residue, 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 1(B) 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 h expression was insufficient to produce fully processed caspase 7 LEHD/NDTA (Figure 1B) when compared with 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 behaviour (results not shown). Therefore we resorted to pseudo-first-order assays ([S]≪$K_m$) to estimate activity. In such conditions, $k_{cat}/K_m = \nu/[S][E]$ and the specific activity is directly related to the substrate hydrolysis $\nu$ and inversely related to the substrate and enzyme concentrations. Using this method, we obtained specific activities that are 17–64 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. Unlike 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 the $k_{cat}$ and $K_m$ values to be similar to WT caspase 7 (Table 1). Both mutants retained typical Michaelis–Menten behaviour after processing with caspase 8 as compared with WT site 2 caspase 7.

**Caspase 7 primary activation site is close to optimal for activation in 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 for the
use of a truncated caspase 9 is to demonstrate that initiator CARD do not influence substrate preference. Therefore we tested full-length and ΔCARD caspase 9 against a combinatorial peptide substrate library covering the P4–P2 cleavage site residues (see Supplementary Experimental section and Figure S1 at http://www.biosci.org/bsr/031/bsr0310283add.htm) and found that both enzymes have the same substrate preference. Furthermore, similar experiments performed using full-length and truncated caspase 8 gave similar results [29a]. We first tested the capacity of full-length caspase 9 and a truncated version (lacking the CARD) to activate pro-caspase 7 (Figure 2A). In our assays, serial dilutions of activators were incubated with a fixed concentration of pro-caspase 7 IQAD/NDTA or LEHD/NDTA. Cleaved fragments are then resolved on SDS/PAGE. Figure 2(A) (top left) shows that processing of site 1 is not improved when the optimized LEHD motif is used compared with the IQAD sequence, which corresponds to an activation at WT site 1 [11]. This is also true for the activation by ΔCARD caspase 9, albeit the activation of all pro-caspases is better than with the full-length activator. Indeed, the lowest concentration used (35 nM) resulted in full cleavage of the pro-caspase 7. The discrepancy between the two caspase 9 activators remains a mystery at present. However, by varying the incubation time of the assays, we estimated that pro-caspase 7 mutant LEHD/NDTA was activated by ΔCARD caspase 9 with a rate that is twice as high as that of the WT site 1 (results not shown). Similarly, pro-caspase 7 LETD/NDTA is activated more efficiently (~2-fold) by caspase 8 as demonstrated by the fact that less activator was required for cleaving 50% of the zymogen (119 nM compared with 53 nM; Supplementary Figure S2 at http://www.biosci.org/bsr/031/bsr0310283add.htm). 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 pro-caspase 7 site 2 mutants. Compared with pro-caspase 7 IQAA/NDTD (i.e. WT site 2), pro-caspase 7 IQAA/LEHD has a ~5-fold better rate of caspase 9-mediated activation and ~4-fold increase using ΔCARD caspase 9 as an activator (Figure 2A). Because our gel-based assays are performed with a pro-caspase 7 concentration that may exceed the $K_m$ value, it is possible that activation assays were not performed in pseudo-first-order conditions, 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 because we observed an important improvement in activation when this site is mutated. In these assays, serial dilutions of pro-caspase 7 are incubated with a fixed 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 pro-caspase 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 a 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 irrespective 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 behaviour to the activation of pro-caspase 7 IQAD/NDTA. Finally, a striking observation was that very low $k_{cat}$ values were obtained. The reason for that is unknown, but our $k_{cat}$ values are consistent with previously published $k_{cat}/K_m$ [4] ($\sim 10^4 M^{-1} \cdot s^{-1}$) and $K_m$ values [30] ($\sim 100$ 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 cyt $c$ 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 the initiator, thus reporting activation. Importantly, the reporter has no observable effect as indicated by the rate of Ac-DEVD-pNA hydrolysis (caspase 3/7 activity) in the cell extract compared to that observed with the 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 agreement with our activation kinetics (Table 2), site 1 is relatively optimal for caspase 9-mediated activation. In contrast, 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 (tumour-necrosis-factor-related apoptosis-inducing ligand) to activate the extrinsic pathway (caspase 8/10), we showed that both cleavage sites can be improved by using the
Figure 2  Activation of pro-caspase 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 (ΔCARD) caspase 9 (right panels). Assay conditions were as follows: left, 400 nM of the indicated pro-caspase 7, full-length caspase 9 (1/2 serial dilution from 500 nM down to 8 nM), 2 h at 37°C; right, 200 nM of the indicated pro-caspase 7, ΔCARD caspase 9 (2/3 serial dilution from 400 nM down to 35 nM), 12 min at 37°C. Trichloroacetic acid/acetone-precipitated proteins were resolved on SDS/PAGE and stained. A sample without initiator caspase (pro-casp-7) or with maximum concentration of the initiator (casp-9 or ΔCARD-casp-9) used was included as control. The full-length zymogen (empty arrowhead), the form lacking the N-terminal peptide (−N) and the cleaved large subunit (ls) are indicated. Note that most pro-caspase 7 preparations lack the N-peptide because it is removed extremely rapidly. Arrows indicate the midpoint at which 50% of the pro-caspase 7 is cleaved. Apparent \( k_{\text{cat}}/K_m \) was obtained as described in the Experimental section. Note that most values are underestimated because pro-caspase 7 concentrations are above the estimated \( K_m \) (see Table 2). Molecular mass 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 pro-caspase 7 site 2 mutants. The 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 μM of the chromogenic substrate Ac-DEVD-pNA. The top graphs depict the amidolytic activity monitored over time at 405 nm for the 67 nM pro-caspase 7 samples. Values presented are background-corrected for the activity of the initiator caspase. Rates of pro-caspase 7 activation were estimated as described in the Experimental section and plotted against the pro-caspase 7 concentrations in the bottom graphs. Solid lines were obtained by non-linear regression of data using the Michaelis–Menten equation \( v = \frac{V_{\text{max,app}}}{[S]/(K_{\text{m,app}} + [S])} \), whereas broken lines are linear regressions and represent the particular case in which \([S]\ll K_m\) and apparent \(k_{\text{cat}}/K_m\) 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 pro-caspase 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.
LETD motif (Figure 3B). Indeed, HEK-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 substituting 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 auto-activates, 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/NDTA 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 h of expression, like WT caspase 7 (Figure 4B). This is likely due to the enzyme’s weak intrinsic activity (Table 1) similar to that observed for the LETD/LEHD site 1 mutants. Indeed, caspase 7 DEVD/NDTA did not display typical Michaelis–Menten behaviour, suggesting that the IQAD → DEVD substitution altered some fundamental properties of the enzyme similarly to site 1 mutation to LETD/LEHD. Further, by deconstructing the DEVD 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 results 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 the 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 a methionine/glutamine residue or Ile213 to an alanine/glutamine residue significantly affected the activity of the caspase (Table 1). In support of the perturbation of caspase activity by non-conservative mutations, three of these mutants required longer expression times or transactivation by caspase 8 to obtain processed enzymes. Conversely, conservative amino acid substitutions (Lys212 → arginine residue or Ile213 → methionine/leucine residue) 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 et al. [29], that location of the site within the IDC is also important. To address this, we first transfected 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 h (results 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 DEVD, thus generating caspase 7 IQAA/DEVD-S. 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 10-fold decrease in specific activity compared to WT caspase 7 (Table 1). Despite the reduced activity of caspase 7 IQAA/DEVD-S, this caspase was 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 IDC 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, unlike 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 three glycine–serine pairs N-terminal of site 2 of the double cleavage site mutant to generate caspase 7 IQAA/NDTA+6 (Figure 5A). Figure 5(B) shows that this mutant did not self-process during bacterial expression unlike the cleavable site 1 version of this construct (IQAD/NDTA+6). We also extended the IDC by adding seven glycine-serine pairs but were unsuccessful in obtaining an active enzyme (Table 1, and results not shown). In a second strategy, we constructed two mutants by replacing the IDC of caspase 7 with that of caspase 8: (a)

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**Figure 3** Activation of pro-caspase 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 cyt c/dATP-activated extract from HEK-293Ad cells expressing the various pro-caspase 7 cleavage site mutants. After the indicated period of time, samples were boiled in gel loading buffer and analysed using anti-FLAG, p23 (caspase substrate) and hsp90 (loading control). Because pro-caspase 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 analysed for caspase activation using the chromogenic substrate Ac-DEVD-pNA. Note that no difference is observed between cyt c/dATP-activated samples in which pro-caspase 7 cleavage site mutants are individually expressed. The asterisk denotes the group of traces obtained in the absence of cyt c/dATP for each corresponding extract. The underlined residues identify the processed cleavage sites. (B) Both sites can be improved for activation by caspase 8. HEK-293Ad cells transfected to express the indicated pro-caspase 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 pro-caspase 7 was monitored by immunoblotting with an anti-FLAG antibody at 12 h after TRAIL addition. The underlined residues identify the processed cleavage sites.
Mechanisms of caspase 7 activation

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 analysed by SDS/PAGE. Arrowheads indicate the transition point from the zymogen to the active form; this transition is absent from caspase 7 IQAA/NDTD that cannot self-activate [18]. (C) Swapping activation site sequences. The indicated caspase 7s were expressed for 8 h, purified and analysed by SDS/PAGE. (D) Self-activation at site 2. Lysates of E. coli expressing the indicated caspase 7s for 30 min or 8 h were analysed by immunoblotting using an anti-His tag antibody. Note that only processed caspase is detected for caspase 7 IQAA/DEVD. WT and caspase 7 IQAA/NDTD are presented as controls for the fully processed and zymogen forms respectively. The underlined residues identify the processed cleavage sites.

DGIQADSGPINDTDANPRYKIPV→KGIVETDSEEQPYLEMDLSSPQTRYIPD (linker casp-8a) to preserve Asp192 [10] or (b) QADSGPINDTDANPRYKIPV→PVETDSEEQPYLEMDLSSPQTRYIPD (linker casp-8b) to also preserve the critical Ile195 of caspase 7 (broken lines in Figure 5A). However, these mutants did not undergo auto-activation nor did they exhibit any proteolytic activity (Figure 5B and results not shown), but processing by caspase 8 did activate caspase 7/linker casp-8b (results not shown). A corollary to our hypothesis is that the length of the initiator caspase 8 IDC is optimal 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 with 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 IDC that is poorly conserved among 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 the present 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 of site 1 are implicated in contacts with the neighbouring catalytic unit within a dimer to form the so-called L2–L2 loop bundle (Supplementary Figure S3A at http://www.biosci.org/bsr/031/bsr0310283add.htm). Many studies [10,32] have confirmed that Asp192 and Ile213 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 homologue 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 proteasezymogen activation have 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 in reactivity towards its endogenous inhibitor XIAP [12], a controlled cleavage at two different sites can enhance the regulation of the whole system.

Our results 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 results 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 [17,32,36,37]). 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 pro-caspase 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 pro-caspase 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, pro-caspase 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
Figure S3B) [39,40]. Furthermore, Ile213 and the side chain of Lys212 occupy a hydrophobic pocket, with the ε-amino group of the lysine residue 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 thezymogen 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 are 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 with executioners, and that for executioners, it serves also to stabilize the active form. In their study, Keller et al. [38] also concluded that caspase 8 IDC length, and the various determinants it contains, were critical in providing activity to the zymogen. 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 and 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 pro-caspase 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 among 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. They also demonstrate, using a real-life proteolytic event, the need to also study molecular determinants other than the immediate cleavage site and the substrate binding site to fully understand how proteases reach high levels of efficacy and specificity.

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AUTHOR CONTRIBUTION
Dave Boucher designed, performed and interpreted the experiments, with the following exceptions: Véronique Blais did all of the in cellulo experiments and Marcin Drag made the substrate library and performed assaying screens with the library. Jean-Bernard Denault contributed to the conceptual and experimental design, and in writing the manuscript.
D. Boucher and others


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SUPPLEMENTARY ONLINE DATA

Molecular determinants involved in activation of caspase 7

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ΔCARD-caspase 9

Figure S1  Full-length and truncated (ΔCARD) caspase 9 have similar substrate preferences

Truncated (ΔCARD; top) or full-length (bottom) caspase-9 was assayed with a positional scanning substrate library (made in house) with P1 fixed as aspartic acid residue. The ordinate is the rate of hydrolysis reported as a percentage of the maximal rate observed. The abscissa provides the positionally defined L-residue. Two independent sets of data are presented per histogram.

SUPPLEMENTARY EXPERIMENTAL

Combinatorial library

The combinatorial library for substrate specificity screening of caspases was synthesized using the methodology described previously [1] and using semi-automatic FlexChem synthesizer. The ACC (7-amino-4-carbamoylmethylcoumarin) fluorophore was used for the synthesis, because it is the most suitable one for solid phase synthesis of PS-SCL (positional scanning substrate combinatorial library) [2,3]. Three sublibraries were generated: P2 sublibrary with the sequence Ac-X-Fix-Asp-ACC, P3 sublibrary with the sequence Ac-X-Fix-Asp-ACC and P4 sublibrary with the sequence Ac-Fix-X-Asp-ACC, where X represents an equimolar mixture of 19 natural amino acids (all amino acids omitting cysteine and methionine residues due to oxidation problems; norleucine was used to mimic methionine), ‘Fix’ represents a defined amino acid and Asp represents the fixed aspartic acid residue at P1 position. The N-termini of the tetrapeptides were capped with acetate (Ac). All ACC substrates were dissolved as stock solutions at a concentration of 50 mM in DMSO and stored frozen. Caspase substrate specificity assay conditions were as

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In vitro activation of pro-caspase 7 by initiator caspase 8 can be improved at both sites
SDS/PAGE analysis of activation kinetics of various caspase 7 mutants by caspase 8. The indicated pro-caspase 7 (200 nM) were incubated with a concentration range (2/3 serial dilution from 400 nM down to 35 nM) of caspase 8 for 8 min at 37°C. Trichloroacetic acid/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-8) was included as control. Arrows on the right indicate the position of the pro-caspase 7; arrowheads indicate the midpoint at which 50% of the pro-caspase 7 is cleaved. Apparent $k_{cat}/K_m$ was obtained as described in the Experimental section. Note that most values are underestimated because pro-caspase 7 concentrations are above the estimated $K_m$ (see Table 2 in the main text). Molecular mass markers are 200, 116, 97.4, 66.2, 45, 31 (marked), 21.5, 14.4 and 6.5 kDa.

Table S1 Kinetic parameters of caspase 7 cleavage site mutants on Ac-DEVD-pNA in activation assay buffer (high salt)
n.d., not determined because the enzyme did not display classical Michaelis–Menten behaviour at the substrate concentration range used.

<table>
<thead>
<tr>
<th>Caspase</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (x10⁴ M⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>51.2</td>
<td>1.52</td>
<td>3.0</td>
</tr>
<tr>
<td>IQAD/NDTA</td>
<td>65.0</td>
<td>1.37</td>
<td>2.1</td>
</tr>
<tr>
<td>LETD/NDTA</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEHD/NDTA</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase 8-activated caspase 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IQAA/NDTD</td>
<td>32.8</td>
<td>0.62</td>
<td>1.9</td>
</tr>
<tr>
<td>IQAA/LETD</td>
<td>39.7</td>
<td>1.02</td>
<td>2.6</td>
</tr>
<tr>
<td>IQAA/LEHD</td>
<td>27.5</td>
<td>0.90</td>
<td>3.3</td>
</tr>
</tbody>
</table>

follows: 100 μl reaction volume, temperature 37°C, caspase buffer, less than 1.5% DMSO, 100 or 250 μM (assuming approx. 277 nM or 693 nM per single substrate) and total final substrate mixture concentration and enzyme concentration was 0.5–5 μM. Release of ACC fluorophore was monitored continuously with excitation at 355 nm and emission at 460 nm with an assay time of 60 min. Analysis of the results was based on total RFU (relative fluorescence unit) for every sub-library, setting the highest value to 100% and adjusting the other results accordingly.
Figure S3 Contacts made by caspase 7’s IDC

Representation of the environment of Lys^{212} and Ile^{213} in the active (A) and zymogen (B) forms of caspase 7. The whole structures are presented as cartoon models as a dimer in which the large (blue) and small (cyan) subunits are colour-coded. Insets show residues surrounding Lys^{212} and Ile^{213} (mesh). Amino acids from the right catalytic unit are labelled with prime positions. Visible residues from site 1 and site 2 are shown, and labelled according to the position with respect to the scissile bond of the cleavage site. Water molecules are presented as green spheres. In the active form, the inhibitor Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp aldehyde) (orange) is bound to the active site. It is noteworthy that no residue is visible beyond Gln^{196} of site 1. In the inactive conformation, the yellow loops depict parts of the IDC for which there was no electron density. The active and zymogen forms were generated using PDB structures 1F1J [4] and 1GQF [5] respectively.
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