Title: Characterization of caspase-7 interaction with RNA

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Abstract

Apoptosis is a regulated form of cell death essential to the removal of unwanted cells. At its core, a family of cysteine peptidases named caspases cleaves key proteins allowing cell death to occur. To do so, each caspase catalytic pocket recognizes preferred amino acid sequences resulting in proteolysis, but some also use exosites to select and cleave important proteins efficaciously. Such exosites have been found in a few caspases, notably caspase-7 that has a lysine patch (K38KKK) that binds RNA, which acts as a bridge to RNA-binding proteins favouring proximity between the peptidase and its substrates resulting in swifter cleavage. Although caspase-7 interaction with RNA has been identified, in-depth characterization of this interaction is lacking. In this study, using in vitro cleavage assays, we determine that RNA concentration and length affect the cleavage of RNA-binding proteins. Additionally, using binding assays and RNA sequencing, we found that caspase-7 binds RNA molecules regardless of their type, sequence, or structure. Moreover, we demonstrate that the N-terminal peptide of caspase-7 reduces the affinity of the peptidase for RNA, which translates into slower cleavages of RNA-binding proteins. Finally, employing engineered heterodimers, we show that a caspase-7 dimer can use both exosites simultaneously to increase its affinity to RNA because a heterodimer with only one exosite has reduced affinity for RNA and cleavage efficacy. These findings shed light on a mechanism that furthers substrate recognition by caspases and provides potential insight into its regulation during apoptosis.
Introduction

Apoptosis is the most common form of cell death occurring in the human body; it is notably useful in removing obsolete, damaged, infected, or degenerating cells. As a regulated process, apoptosis can be triggered by either internal or external cues leading to the activation of the intrinsic or extrinsic apoptotic pathway, respectively. The intrinsic pathway leads to the permeabilization of the outer membrane of mitochondria, leaking of cytochrome c, and the subsequent dimerization and activation of caspase-9 via the apoptosome. Similarly, following the ligation of specific receptors of the TNFα family, the extrinsic pathway leads to the formation of a death-inducing signalling complex (DISC) that causes the dimerization and activation of caspase-8 and -10. Both pathways converge in the activation by cleavage of executioner caspases-3, -6, and -7 [1-3]. The executioner caspases are key components because their activation leads to full-blown apoptosis, and it is both the activation and inactivation of many cytosolic and nuclear proteins in multiple cellular pathways by caspase-mediated proteolysis that cause cell demise. Thus far, more than 2,000 substrates have been identified [4, 5], but only the cleavage of a few may be necessary for cell death to occur, while most proteins are likely bystander substrates. Consequently, to achieve apoptosis, caspases must interact selectively and cleave important substrates such as the inhibitor of caspase-activated DNase (ICAD) [6, 7] and the poly(ADP-ribose) polymerase 1 (PARP-1) [8, 9].

A caspase recognizes its substrates via the binding of specific amino acid sequences by the substrate-binding pocket next to the catalytic site. All caspases prefer cleaving peptide bonds after an aspartic acid (P1 residue), but while executioner caspases-3 and -7 prefer an aspartic acid in the P4 position (4 amino acids upstream of the scissile bond; DxxD motifs), caspase-6 favours small hydrophobic amino acids such as a valine at the P4 position (VxxD motifs) [10, 11]. Even though caspase-3 and -7 have the same primary structure preference, their substrate repertoire and kinetics differ [12]. This dichotomy can be explained, at least partly, using exosites, which are secondary binding sites separate from the catalytic site that enhance the cleavage of specific substrates by increasing their affinity. To date, caspase exosites have been described for human caspase-1 [13, 14], caspase-4 [13], caspase-6 [15, 16], caspase-7 [17], and caspase-8 [18]. For caspase-1 and -4, hydrophobic amino acids in a β-sheet interact with a hydrophobic pocket on the substrate gasdermin D, promoting cleavage [13, 14]. Caspase-6’s exosite is in its N-terminal domain and is comprised of a tri-arginine motif, R42RR, that interacts directly with protein substrates such as lamin C and DVL3 [15, 16]. In caspase-8, residues 414-427 interact with residues near the KL loop of the baculoviral inhibitor p35 to enhance interaction and protease inhibition [18]. In caspase-7, the intrinsically disordered N-terminal domain contains at least two exosites. First, amino acids 36 to 45 interact directly with the HSP90 co-chaperone p23 [19], while for PARP-1 and other RNA-binding proteins (RNA-BPs), a well-defined patch of four lysine residues (K38KKK) directly interact with RNA and promotes the cleavage of protein substrates due to the mutual binding to RNA [17, 20]. The K38KKK motif is modular, meaning that it can interact by itself without the catalytic domain of caspase-7, and its interaction with RNA relies solely on charges because substitution of lysine residues for arginine residues maintains the exosite function; the introduction of negatively charged residues hampers its interaction with RNA.
Although uncovered, this caspase-7/RNA interaction is poorly understood and differs from other caspases’ exosites, which interact directly with proteins. In this study, we performed biochemical experiments to understand better how caspase-7 interacts with RNA and RNA-binding substrates using the prototypical substrate PARP-1. We uncovered that caspase-7 binds RNA non-specifically and requires optimal RNA concentration and length for optimal proteolysis of RNA-BPs. We determined that the caspase-7/RNA affinity is in the low micromolar range and demonstrated that caspase-7’s N-terminal peptide (N-pep) reduces exosite-binding to RNA, providing a means to regulate that exosite. Finally, using engineered caspase-7 heterodimers, we showed that both exosites of a caspase-7 dimer contribute to the affinity for RNA and faster proteolysis of PARP-1.

Results

The concentration and length, but not the type of RNA, are key to optimal PARP-1 cleavage

To characterize caspase-7 exosite interaction with RNA and its RNA-binding substrates, we performed cleavage assays with PARP-1 as a prototypical RNA-binding substrate; assays were performed as previously described with FLAG-tagged PARP-1-containing cell extracts [20]. We employed immunoblots densitometry on unsaturated bands for the full-length substrate to estimate cleavage rates. Moreover, we have previously shown that in the experimental conditions used, there is no caspase activation [20] occurring in the extracts, and it has also been demonstrated that caspase-7 does not activate other caspases [21]. Consequently, the cleavage of substrates seen in assays results from the caspase added to cell extracts.

Because RNA brings together the protease and its substrates, we first determined the optimal RNA concentration. We initially depleted extracts from RNA with RNase and, following RNase inhibition, reintroduced purified total RNA at different concentrations (Figure 1A). We have also calculated the cleavage rate $k$ (see Material and Methods) for the different RNA concentrations and plotted them (Figure 1B). As we had shown before [20], depletion of RNA reduced the cleavage efficacy of PARP-1 by caspase-7 by 76-fold (mean $k$ values $2.28 \times 10^6$ M$^{-1}$·s$^{-1}$ for untreated versus $2.98 \times 10^5$ M$^{-1}$·s$^{-1}$ for RNase-treated extracts). Reintroduction of RNA enhanced the cleavage efficacy up to a concentration between 10 and 30 ng/µl (mean $k$ values of $1.95 \times 10^6$ M$^{-1}$·s$^{-1}$ and $2.03 \times 10^6$ M$^{-1}$·s$^{-1}$, respectively); this maximal cleavage rate is like the rate obtained with untreated extracts. Above 30 ng/µl, additional RNA reduced cleavage efficacy, suggesting that too much RNA somehow hampers the interaction between caspase-7 and PARP-1. Of note, we were unable to find an RNA concentration resulting in a better cleavage rate than that of the diluted cell extracts we used.

Next, we evaluated the optimal length of RNA molecules promoting the interaction between caspase-7 and PARP-1. We repeated the previous experiments using 30 ng/µl of RNA of different lengths obtained by in vitro transcription. Because the RNA concentration is fixed, there is the same number of RNA-binding sites, but they are not distributed equally as longer RNA molecules have more binding sites per molecule. In
Figure 2A, treatment with RNase reduced the cleavage of PARP-1. Upon adding 100-nt RNA molecules, the cleavage efficacy was enhanced and was further enhanced with 200-nt RNA molecules. The addition of 300-nt or 1000-nt RNA molecules did not further improve the cleavage of PARP-1. RNA molecules of 400, 600, and 800-nt were also tested but gave the same results as the 200-nt (data not shown). Thus, we assume that a minimal RNA length of 100-200-nt is optimal to bridge caspase-7 and PARP-1, and that a length of up to 1000-nt does not lead to a significant decrease in interaction efficacy, at least at the concentration of RNA used.

To support our previous speculation that caspase-7 binds any type of RNAs [20], we compared by RNA sequencing the total RNA (input group) and RNA retained by a caspase-7-affinity column (bound group) from three pooled biological replicates (Figure 5B; see Material and Methods). Before sequencing, the abundant ribosomal RNA (rRNA) was removed from the samples because the initial analysis of RNA integrity showed that the peaks for the 18S and 28S rRNAs from both the input and the caspase-7-bound fractions had similar intensities, meaning that caspase-7 binds to rRNA equally. Analyses showed that RNAs sequenced in both groups were associated with a total of 19,086 genes. Of those, 1209 (6.3%) were found only in the input and 591 (3.1%) in the caspase-7-bound group. Most RNA sequences, associated with 17,286 genes (90.6%), were found in both groups suggesting that caspase-7 largely binds RNA in an unspecific manner. The RNA sequences found in both groups were further analyzed to compare the sequences associated with a specific gene in the bound versus the input group; Figure 3A presents the log₂ (bound/input) ratios of the 17,286 genes. Most genes clustered near a log₂ ratio of 0 (no difference), with 84.4% of genes within a 2-fold RNA difference and 95.6% within a 4-fold difference. In Figure 3B, the same analyses as in Figure 3A were done without sequences with a low number of reads, i.e., less than five transcripts per million (TPM; see Material and Methods). The resulting RNA sequences were associated with 10,945 genes, of which 48 (0.4%) were found in the input group only and 57 (0.5%) in the bound group only. RNAs associated with a total of 10,840 genes (99.0%) and were found in both groups showing that most RNAs shown in Figure 3A that were either in the input or bound group only were RNAs with a low read count. Consequently, most genes clustered near a log₂ ratio of 0; 96.5% of genes are within 2-fold RNA difference in the bound and input groups, and 99.9% are within 4-fold. In Figure 3C, the percentage of each RNA type found in the input and the caspase-7-bound groups are shown; those results include both the overlapping sequences and the individually bound by each group. All RNA types were represented in similar proportions in both groups. Because ribosomal RNA (rRNA) was depleted before sequencing, we noticed that the most abundant RNA type in both groups is protein-coding (mRNA), 98.1% and 98.2% of sequences in the input and bound groups, respectively. Other RNA types found are long non-coding (0.02% input versus 0.02% bound), microRNAs (miRNA; 0.22% input versus 0.22% bound), transcribed pseudogenes (0.13% input versus 0.12% bound), remaining rRNAs (0.09% input versus 0.08% bound), small nucleolar RNAs (snoRNAs; 0.71% input versus 0.66% bound), and small nuclear RNAs (snRNAs; 0.71% input versus 0.68% bound). These results show that caspase-7 binds every type of RNA with seemingly no preference. Taken together, these results show that there is an optimal concentration as well as a minimal length of
RNA resulting in optimal interaction and cleavage of PARP-1, and that any type of RNA can be used to bridge caspase-7 and its RNA-binding substrates.

The N-terminal peptide of caspase-7 reduces RNA-binding and cleavage of RNA-binding proteins

During apoptosis, caspase-7’s N-terminal peptide (N-pep) is removed by caspase-3 [22], and our previous work suggests that the N-pep may be a means to regulate the exosite [17]. Because we have uncovered that RNA was mediating the effect of the exosite, we assessed if the N-pep modifies caspase-7 affinity for RNA, thus, affecting the cleavage of RNA-BPs in general. We compared the cleavage by caspase-7 bearing the D23A substitution, which prevents the removal of the N-pep during bacterial expression [22], to that by WT (N-pep removed) and the exosite-less mutant KEEK [17, 20]. As shown in Figure 4A, compared with WT caspase-7, D23A caspase-7 has a ~12-fold lower cleavage rate of PARP-1, but remains better than the KEEK mutant, which is ~128-fold slower. Furthermore, the addition of RNase resulted in reduced cleavage of PARP-1 by both WT and D23A caspase-7, while the KEEK mutant was not affected. Importantly, WT, KEEK, and D23A caspase-7 have similar kinetic parameters on a small peptidic substrate (Table 1), demonstrating that the catalytic site of caspase-7 is not affected by the presence or absence of the N-pep. To ensure that the N-pep was acting negatively on the exosite and not as a peptide that negatively interfered with all protein substrates, we analyzed the cleavage of two additional RNA-BPs, RNA-binding motif protein 26 (RBM26) and U2-associated protein SR140 (SR140). As with PARP-1, D23A caspase-7 has a reduced cleavage of both RNA-BPs (Figure 3B, C) by ~6-fold compared to WT caspase-7 while remaining affected negatively by the RNase treatment. We also analyzed the cleavage of a non-RNA-BP, the HSP90 co-chaperone p23, and found that its cleavage was not affected by the presence of the N-pep or RNase treatment.

The reduction of RNA-BP cleavage by caspase-7’s N-pep suggests a decrease in affinity for RNA. To verify this, we determined the RNA-binding capability of D23A caspase-7 compared to WT and KEEK caspase-7. First, we set up caspase-7-affinity columns consisting of immobilized metal affinity chromatography resins (IMAC) saturated with 6xHis-tagged caspase-7 proteins (Figure 5A). RNA (8 µg) was applied to the column, washed three times, and eluted using a high-salt solution; Figure 5B shows the RNA quantification of every fraction of every column. As seen in the elution fractions, only WT caspase-7 retained a substantial amount of RNA (average of 4.0 µg) while the KEEK and D23A mutant retained minimal amounts (< 0.5 µg), and resin alone retained no RNA (< 0.1 µg). Conversely, less RNA was found in the flowthrough and washing fractions of WT caspase-7 than the others. Figure 5C shows a sample of each elution fraction separated by non-denaturing agarose gel electrophoresis; RNA was only visible in the elution fraction of WT caspase-7.

We estimated the binding affinity of WT caspase-7 to RNA by fluorescence anisotropy using a 30-mer uracil RNA oligonucleotide bearing a 3 prime 6-carboxyfluorescein (FAM) and compared it to that of the D23A and KEEK caspase-7 mutants, and caspase-3. We incubated this oligonucleotide at a concentration of 1 nM with varying
concentrations of caspase to calculate the anisotropy resulting from the binding of the fluorescent RNA (see Material and Methods). As shown in Figure 5D, the binding of caspase-7 to the probe shows a sigmoidal binding curve spanning ~2 order of magnitude between the lowest and highest anisotropy value with a calculated $K_d$ of 3.2 µM (95% confidence interval: 1.2 to 8.1 µM; see Discussion). D23A caspase-7 affinity is 4-fold lower than WT caspase-7 with a calculated $K_d$ of 12.3 µM (95% CI: 8.1 to 20.0 µM). The D23A mutant reached a higher anisotropy maximum because it has more amino acids creating a larger complex with the RNA probe. The data for the KEEK caspase-7 mutant and WT caspase-3 did not allow us to calculate the $K_d$ precisely since their binding curves are incomplete (right shift). Because their complex with RNA should be roughly the same size as the one for WT caspase-7, we fixed the maximum anisotropy value to the value obtained for WT caspase-7 and estimated a $K_d$ of 63 µM (95% CI: 8 to 128 µM) for KEEK caspase-7 and 48 µM (95% CI: 16 to 67 µM) for WT caspase-3. The anisotropy experiments show that the N-pep of caspase-7 reduced the affinity for RNA and that the four-lysine exosite is the main contributor to RNA-binding; caspase-3 has minimal affinity for RNA. Altogether, these results show that caspase-7’s N-pep reduces the affinity of caspase-7 for RNA, resulting in a reduced cleavage rate of RNA-BPs.

A caspase-7 dimer uses its two exosites for optimal RNA-binding and PARP-1 cleavage

Caspase-7 is an obligate dimer made of two active sites and two available exosites, suggesting that either one or two exosites may contribute to RNA-binding and substrate cleavage. The exosite of caspase-7 resides in the N-terminal domain, which consists of a 62-amino acid stretch with no defined structure [23-25], and its length allows it to reach the vicinity of the active site of the same caspase molecule (in cis) or the other (in trans) within a dimer. To understand how the exosite works in conjunction with the active site, we generated caspase-7 heterodimers (Figure 6A) using a bicistronic expression vector and sequential tag purifications (see Material and Methods). Each molecule of a dimer has a Strep-tag with an active catalytic site (noted A$^+$) or a His-tag with an inactive catalytic site (A$^-$), and either a functional (E$^+$) or non-functional exosite (E). First, we generated a heterodimer with two active sites to ensure that the addition of the Strep-tag does not affect the kinetics of the enzyme (Table 1); indeed, that heterodimer has similar kinetic parameters as WT caspase-7 (His/His). Heterodimers with only one active site were generated (Figure 6B), and their characterization shows that they have similar kinetic parameters as WT caspase-7 (Table 1), except for the heterodimer with one exosite in trans that has a 32% reduction in $k_{cat}/K_M$ compared to that of WT caspase-7, which should not lead to a major difference in PARP-1 cleavage. We compared all heterodimers for their efficacy at cleaving PARP-1 (Figure 6C). Cleavage by WT caspase-7 was twice as fast as by the heterodimer bearing one active site and two exosites, even if twice as much of the heterodimer was used in this experiment, suggesting that once bound, an additional active site on the same dimer slightly enhance the chance of interacting with the substrate and cleaving it. Removal of both exosites (E$^+$:E$^-$) resulted in a ~32-fold decrease in cleavage rate compared to the heterodimer with two exosites (E$^+$:E$^+$). Interestingly, the heterodimer with one
The exosite in cis (E$^+$A$^-$:E$^-$$^$A$^+$) is 8-fold less efficacious than the one with two exosites (E$^+$A$^-$:E$^-$$^$A$^+$), while the one with the exosite in trans (E$^+$A$^-$:E$^-$$^$A$^+$) is 32-fold slower. These results suggest that a caspase-7 dimer requires both exosites for optimal cleavage, but it can use one, albeit with less efficacy. To explain the difference in cleavage seen between two exosites and one exosite per dimer or the difference between the cis and trans heterodimers, we determined their binding affinity for RNA using fluorescence anisotropy (Figure 6D). Binding curves for the two caspase-7 dimers with a single exosite (E$^+$A$^-$:E$^-$$^$A$^+$ and E$^+$A$^-$:E$^-$$^$A$^+$) sit between the binding curves for the two exosites and exosite-less dimers. For both heterodimers with a single exosite, data were insufficient to calculate affinity values without limiting the maximal anisotropy value. The estimated $K_d$ for the heterodimer with one exosite in cis is 18.6 µM (95% CI: 10.4 to 25.9 µM). In contrast, the estimated $K_d$ for the trans dimer is 9.8 µM (95% CI: 5.0 to 14.1 µM), which is ~6 and ~3-fold lower in affinity for RNA than WT caspase-7, respectively. Of note, even though the trans dimer (E$^+$A$^-$:E$^-$$^$A$^+$) has a better RNA-binding affinity than the cis dimer (E$^+$A$^-$:E$^-$$^$A$^+$), it is the latter that has the faster PARP-1 cleavage rate (Figure 6C). From these results, we conclude that both exosites of a caspase-7 dimer can bind the same RNA molecule (see Discussion), which would explain why the dimer with two exosites has a higher affinity for RNA and a faster PARP-1 cleavage rate.

Discussion

We have previously uncovered that caspase-7 employs a four-lysine patch to bind RNA, bringing caspase-7 closer to RNA-BPs, allowing more efficacious proteolysis of these substrates. To date, only a handful of proteases are known to bind RNA in humans [20, 26, 27], which makes it all too necessary to understand these interactions better.

First, we characterized the interaction between RNA and caspase-7 by modifying the concentrations of RNA. The addition of total RNA enhances the cleavage rate up to a concentration of 10-30 ng/µl, at which the highest cleavage rate is obtained; then, the cleavage rate decreases as the RNA concentration is increased further. PARP-1 also binds RNA, including every type of RNA we identified as bound to caspase-7, and PARP-1 binds them with affinities ranging from 10 to 900 nM [28-30]. Because we determined that the affinity of caspase-7 for a short RNA molecule is in the low micromolar range, the limiting factor is likely caspase-7’s affinity for RNA. Consequently, the increase in the cleavage rate is governed by the binding of caspase-7 to RNA until the RNA molecules become too numerous, and caspase-7 binds RNA molecules without bound PARP-1 resulting in lower cleavage rates. The optimal concentration of RNA we determined was specific to PARP-1 in our assay conditions and most likely differed for other RNA-BPs and cellular contexts. Nonetheless, we assume that the general principle of an optimal RNA concentration for the cleavage of a specific RNA-BP applies. Indeed, Thomas et al. [26] showed that the cleavage of the RNA-BP hnRNP C1 by the protease granzyme B, which also binds RNA, has an optimal RNA concentration that leads to efficient cleavage. As with caspase-7 and PARP-1, 31 and 125 ng of total RNA resulted in optimal cleavage of hnRNP C1 by granzyme B, while lower and higher RNA quantity resulted in suboptimal proteolysis. In a cellular context where caspase-7 may bind many types of RNAs, our results suggest that proteins that
also interact with different or abundant RNAs, such as PARP-1, are more likely to ‘meet’ caspase-7 and get cleaved than proteins binding to a limited subset of RNA molecules.

We also studied the effect of RNA length on the cleavage rate and found that a 200-nt or longer RNA molecule was optimal for PARP-1 proteolysis. PARP-1 can bind RNA as small as 19-nt-long [29], and we showed that caspase-7 can bind a 30-mer oligonucleotide, so we conclude that the difference observed between the 100 and the 200-nt RNA is not due to the inability of either protein to bind short RNAs. Because RNA can adopt multiple conformations, which could affect the binding of caspase-7 and PARP-1, we can surmised that the 100 and 200-nt RNA have more similar structures than longer RNA molecules, which is supported by the RNA structure predictor RNAfold [31]. Nevertheless, despite having different conformation, the 200, 300, and 1000-nt RNAs showed no difference in enhancing PARP-1 cleavage, meaning that RNA structure is probably unimportant. Therefore, we assume that a minimal RNA length is required for optimal RNA-BP cleavage because it would allow caspase-7 to bind the same RNA molecule as its substrate in an optimal conformation. Conversely, shorter RNA molecules could lead to a suboptimal conformation which would impact the cleavage rate.

In our previous study [20], we speculated that RNA-binding was nonspecific in large part because of the simplicity of the exosite motif. An RNA sequencing experiment was done to confirm that caspase-7 bound non-specifically to RNA and validated this hypothesis. Indeed, we found a high overlap of sequences in the input and caspase-7-bound groups and similar numbers of sequences associated with each gene show that caspase-7 does not bind RNA in a sequence or structure-specific manner. It is even more salient after removing sequences with a low count from the analysis. Furthermore, there was no specific type of RNA preferred by caspase-7. Of note, transfer RNAs were not correctly identified in our study because we used a method unsuitable for this type of RNA [32]. In agreement with our results, Castello et al. [33] have suggested that RNA-BPs with poly-lysine patches similar to caspase-7 make electrostatic interactions with the phosphate backbone of RNA mimicking basic tails of DNA-BPs and would, therefore, interact non-specifically with RNA. Additionally, in our previous work [20], we showed that caspase-7 cleaves many proteins binding different types of RNA faster in the presence of total RNA, suggesting that caspase-7 binds different RNAs to promote the cleavage of different types of RNA-BPs. The binding of caspase-7 to RNA and the ensuing cleavage of RNA-BPs likely lead to the inactivation of many RNA-related pathways.

We demonstrated that the presence of the N-pep reduces the cleavage of three RNA-BPs by caspase-7, while not affecting that of a non-RNA-BP, but not to the same extent as the loss of the exosite or the absence of RNA. Using caspase affinity columns and fluorescence anisotropy, we showed that caspase-7 with its N-pep has a reduced affinity for RNA, which explains why it has reduced cleavage of RNA-BPs. In line with our results, Denault and Salvesen [21] have shown that caspase-7 lacking its N-pep is more lethal in cellulo than a caspase-7 that cannot be removed, suggesting that this peptide restricts caspase-7 in some way. They also showed that caspase-7 without its N-pep cleaves PARP-1 faster than WT caspase-7, although enhanced cleavage of a non-RNA-BP was also observed. The N-pep of caspase-7 is charged negatively (net
charge of -9; MADDQGCIEEQGVEDSANEDSV), which likely interacts with the positively charged exosite and, thus, compete with RNA for binding; it might also repulse the negatively charged backbone of RNA. Either hypothesis explains the reduced affinity for RNA of the D23A caspase-7 mutant compared to WT. The N-pep of the other executioner caspases has also been found to act as a silencer of these enzymes [34, 35]. Caspase-6 also has an exosite composed in part of three arginine residues [15, 16], R<sup>32</sup>RR, which resembles that of caspase-7, but has not been shown to bind nucleic acids. However, we do not think it would share the same mechanism of N-pep inhibition because it has only a net charge of -2 (MSSASGLRRGHPAGGEENMTETD). Thus, the N-pep of caspase-7 represents an example of exosite regulation. Because the exosite is comprised of four lysine residues and that these residues are known targets of many post-translational modifications that may affect charges, there are potentially many ways to regulate that exosite. A somewhat similar mechanism exists in caspase-4, in which cleavage at Asp289 is essential for the exosite function and cleavage of gasdermin D [13]. After cleavage, the N-terminus of the caspase small subunit interacts with a hydrophobic patch of gasdermin D, which is impossible before activation.

Using engineered caspase heterodimers, we demonstrated that a dimer uses either one or preferentially two exosites to bind RNA. Indeed, a caspase-7 dimer with two exosites has a better affinity for RNA than a dimer with only one exosite, in cis or trans configuration. In circumstances where the RNA concentration is low compared to that of caspase-7 (e.g., anisotropy experiments with a small oligonucleotide), it is more likely that both exosites of a dimer bind the same RNA molecule than at higher RNA concentrations (e.g., PARP-1 cleavage assays). Nevertheless, the higher affinity of the WT caspase-7 for RNA than a one exosite-heterodimer points to binding to the same molecule, at least in the fluorescence anisotropy experiments. Better RNA affinity should translate into faster cleavage of RNA-BPs, which our results show. When combining cleavage results of PARP-1 from all relevant cleavage assays, we conclude that a WT caspase-7 dimer is the most efficacious at cleaving PARP-1 followed by the caspase with the N-pep still attached (D23A), a dimer with one exosite (in cis) and finally by the caspase-7 mutant without a functioning exosite (KEEK); in binding assays, we observe the same order of affinity. Only the heterodimer with one exosite in trans showed diverging results between affinity and cleavage. Even though both heterodimers with one exosite have similar affinities, it seems that they are not equal when it comes to cleaving PARP-1, the cis heterodimer being better. This might be explained by a structural positioning that does not favour the interaction between PARP-1 and the active site of caspase-7. Furthermore, having two active sites (WT caspase-7; homodimer) may confer a slight edge over the same concentration of active sites spread on more molecules (E<sup>+</sup>A<sup>+</sup>:E<sup>+</sup>A<sup>+</sup> heterodimer), suggesting an increase in probabilities of substrate recognition by an active site when in pair in a dimer. Of note, the binding curve of WT caspase-7 shows a plateau mid-way in the data that is reminiscent of a two-site binding curve with differential affinities or identical sites with negative cooperativity. Since WT caspase-7 is a homodimer with two identical binding sites, we do not expect a different affinity. Also, because the binding curve of WT caspase-7 is shifted to the left compared with the heterodimers bearing one exosite, our results suggest positive cooperativity and not negative. Furthermore, when the data is
analyzed using a function for binding with cooperativity, a Hill coefficient of ~1 is obtained, suggesting no cooperativity; an analysis with a two-site specific binding equation did not lead to conclusive results. Consequently, we believe that both exosites of a caspase-7 dimer bind the same RNA molecule in some conditions, thus, enhancing affinity and cleavage rates of RNA-BPs as we have shown in this study. On another note, because our assays were performed in a controlled in vitro environment, it is difficult to predict if our results would translate fully to cellular context in which RNA concentration is highly variable between cells and constantly changing. We have shown that in cellulo the exosite of caspase-7 promotes the cleavage of PARP-1 [17], and Denault and Salvesen [21] offered hints that the N-pep removal might also benefit the exosite in cellulo. We also must consider that some RNAs are part of complex structures limiting the access to those RNAs (such as rRNA in ribosomes) and that there is competition with other RNA-BPs of which some have a better affinity for RNA than caspase-7. Additionally, it has been shown that polyadenylated RNAs, mostly mRNAs and a few non-coding RNAs, are degraded during apoptosis when mitochondrial outer membrane permeabilization occurs [36, 37]. With mRNA representing between 1 and 5% of the total RNA, their degradation would not affect by much the total concentration of RNA but could affect the cleavage of mRNA-BPs by caspase-7.

Because caspase-7 uses its two exosites either simultaneously or individually, it would be worthwhile to show whether caspase-7 uses mechanisms like the ones employed by other nucleic acid-BPs with dual binding sites, such as DNA sliding and intersegment transfer. DNA sliding is the one-dimensional diffusion of a protein along the sugar-phosphate backbone of DNA [38], while an intersegment transfer is the transfer of the DNA-BP on a distant part of the same molecule or to another molecule via the detachment of one of the binding sites and its reattachment somewhere else [39]. The DNA-BPs using those mechanisms usually have one intrinsically disordered region with a net positive charge that binds non-specifically to DNA [40], characteristics shared by caspase-7 with RNA [19]. Moreover, it was shown that those mechanisms are more frequent when the charges are moderate and clustered near the middle or towards the N-terminus [40], which once again matches the clustered caspases-7 exosite. In conclusion, our study provides much-needed information on a protease with RNA-binding capability and identifies the N-pep of caspase-7 as a negative regulator of RNA-binding.

Materials and methods

DNA constructs and plasmids

Human caspase-7 (GenBank: NM_001227.5) and caspase-3 (NM_004346.4) cDNAs subcloned in the pET-23b(+) vector (Novagen) to obtain caspase expression plasmid with a C-terminal 6xHis tag were used. Human caspase-8 (NM_033355.3) lacking death effector domains (ΔDEDs; a.a. 217 to 479; no tag) subcloned in the pET-15b(+) was also used, as well as full-length human PARP-1 (GenBank: NM_001618.4) with an N-terminal FLAG-tag in the pCMV-Tag2B vector. To generate caspase-7 heterodimers, Strep (WSHPQFEK)-tagged caspase-7 was inserted in pET-23b(+) vectors already

Materials and methods

DNA constructs and plasmids

Human caspase-7 (GenBank: NM_001227.5) and caspase-3 (NM_004346.4) cDNAs subcloned in the pET-23b(+) vector (Novagen) to obtain caspase expression plasmid with a C-terminal 6xHis tag were used. Human caspase-8 (NM_033355.3) lacking death effector domains (ΔDEDs; a.a. 217 to 479; no tag) subcloned in the pET-15b(+) was also used, as well as full-length human PARP-1 (GenBank: NM_001618.4) with an N-terminal FLAG-tag in the pCMV-Tag2B vector. To generate caspase-7 heterodimers, Strep (WSHPQFEK)-tagged caspase-7 was inserted in pET-23b(+) vectors already
containing a His-tagged caspase-7. Strep-tagged caspase-7 was obtained by PCR by amplifying caspase-7 cDNA from a pET-23b(+) vector using the T7 promoter and an oligonucleotide containing a C-terminal Strep-Tag II followed by a stop codon and a Xba I restriction site (CGTCTAGATTTTTTCGAACTGCGGTGGGCTCCAGGATCCCTTGACTGAAGTAGAGGAGGTTC). Because the pET-23b(+) vector contains a Xba I restriction site before the ribosome binding site (RBS) and the coding sequence, restriction digest with Xba I generated the following DNA fragments (Xba I-spacer-RBS-START-Casp7-Strep-Tag II-STOP-Xba I). Those DNA fragments were inserted into pET-23b(+) vector upstream of a His-tagged caspase-7, thus generating a bicistronic vector. The caspase-7 proteins lacked the N-pep (a.a. 1-23) to hasten heterodimer self-activation. All other DNA constructs were obtained by overlapping PCR and subcloning, and all were sequenced to ensure their integrity.

Recombinant caspases
Caspases used in this study were expressed, purified, and characterized as previously published [41], and adapted for heterodimers. The bicistronic pET-23b(+) vectors were transformed in Escherichia coli BL21(DE3)pLysS (Novagen), and bacteria were grown in 2xYT medium with 50 µg/ml ampicillin and 25 µg/ml chloramphenicol at 37ºC. The expression was carried out at 18ºC for 16 h with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Bio Basic). Bacteria were harvested by centrifugation, resuspended in 50 mM Tris, pH 8.0, plus 100 mM NaCl (buffer A), and kept frozen at -80ºC before purification. For purification, bacteria were thawed, sonicated, and lysates were clarified by centrifugation and filtration to remove debris. Then, proteins were purified using immobilized metal affinity chromatography columns (IMAC) to isolate His-containing dimers. Once bound to the resin, a bacterial lysate containing untagged ΔDEDs caspase-8 and 2 mM β-mercaptoethanol was incubated to enhance the generation of the active cleaved form of the heterodimer, and resin was washed extensively with 50 mM Tris, pH 8.0, plus 500 mM NaCl. Heterodimers were eluted with an imidazole gradient (0-200 mM in buffer A), and fractions were collected. Fractions containing caspase-7 were pooled and further purified on a Strep-Tactin Superflow column (IBA Lifesciences) following the manufacturer’s protocol (Strep-tag purification short protocol), extensive washing steps [100 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)] to remove any His-His homodimers leaving only His-Strep tag-containing heterodimers. All caspase preparations were active-site titrated with the irreversible caspase inhibitor N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk; Enzo Life Sciences), their kinetic parameters (k\text{cat}, K_M, and k_{cat}/K_M) were determined using acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluorocoumarine fluorogenic substrate (Ac-DEVD-Afc; Enzo Life Sciences), and all kinetic data were analyzed with GraphPad Prism 8.

Cell culture, transfection, and extracts
AD-293 cells (Agilent) in which we previously abolished caspase-7 expression using CRISPR/Cas9 gene-editing technique (hereafter AD-293_C7KO) [20] and HCT 116
(American Type Culture Collection) cell lines were used. Cells were grown in DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (all from Wisent). For transfection of PARP-1 into AD-293^{C7KO} cells, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s protocol. For either cell extracts or RNA purification, cells were harvested by centrifugation, washed in cold PBS, and frozen at -80°C until needed. To prepare protein extracts, cells were incubated on ice for 30 min. in lysis buffer [50 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.4, 100 mM NaCl, and 1% (v/v) Nonidet P-40 containing non-caspase proteases inhibitors [10 µM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64); 10 µM 3,4-dichloroisocoumarin; 1 mM 1,10-ortho-phenanthroline, and 10 µM leupeptin; Sigma-Aldrich], centrifuged at 18 000 x g for 15 min., and the supernatant was aliquoted and frozen at -80°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (bicinchoninic acid; Thermo Fisher Scientific). As we have shown previously [20], this protocol does not activate any endogenous caspase that could interfere with our experiments.

Cleavage assays
Two µg of AD-293^{C7KO} cell extracts containing human full-length FLAG-tagged PARP-1 were incubated with varying concentrations of caspase (0.2-400 nM) at 37°C for 30 min. in caspase buffer [10 mM 1,4-Piperazinediethanesulfonic acid (PIPES) pH 7.4, 100 mM NaCl, 0.1% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (w/v) sucrose, and 10 mM dithiothreitol (DTT)]; for other substrates, 10 µg of cell extracts were used. Caspases were pre-activated for 20 min. at 37°C in caspase buffer. Each cleavage assay series had a control sample without caspase to ensure that the substrate is not cleaved or degraded by any other protease than the added caspase. Cleavage reactions were stopped by the addition of gel loading buffer and boiling and analyzed by immunoblotting. Additional sample treatments were performed as indicated in figure legends and included RNase A (1 µg/ml, 30 min. at 25°C; Thermo Fisher Scientific), RNasin (40 units, 30 min. at 25°C; Promega Corporation), and the addition of RNA (0.1-500 ng/µl for whole-cell RNA and 30 ng/µl for in vitro transcribed RNA).

SDS-PAGE and immunoblotting
Protein samples were separated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in an 2-Amino-2-methyl-1,3-propanediol (ammediol; Sigma-Aldrich) buffer system [42]. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) in 10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11, and 10% (v/v) methanol [43]. Membranes were blocked in PBS-T-milk [0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk] and incubated overnight with primary antibodies in either PBS-T-milk or PBS-BSA [3% (w/v) BSA; Wisent] followed by secondary antibodies incubation for 2 h in PBS-T-milk. The antibodies used were FLAG M2 (1:10,000; Sigma-Aldrich; #F1804); HRP-conjugated anti-mouse (1:5,000; Cell Signaling Technologies; #7076); HRP-conjugated anti-rabbit (1:5,000; Cell Signaling Technologies; #7074); p23 (1:5,000; Thermo Fisher Scientific;
MA3-414); RBM26 (1:2,000; Abcam; ab74198); and SR140 (1:3,000; Santa Cruz Biotechnology; sc398718). The immunoblots were visualized using the Immobilon Crescendo (Millipore) HRP substrate and a VersaDoc 4000mp (BioRad) imaging system. The Quantity One software (BioRad) was used for densitometry analyses of unsaturated images.

**Hydrolysis rate determination**

The immunoblots from PARP-1 cleavage assays were quantified to obtain the proportion of cleaved PARP-1 (100% minus percentage of full-length PARP-1). Then, the hydrolysis rate was calculated using the pseudo-first-order rate equation \( p = 1 - e^{-kt} \) (\( p \), the proportion of cleaved PARP-1; \( k \), cleavage rate; \( E \), caspase concentration; \( t \), time in seconds). For a complete description of the method, refer to Martini et al. [19].

**RNA purification, in vitro transcription, and sequencing**

Total RNA from HCT 116 cells was purified using the RNeasy Kit (QIAGEN). To produce RNA molecules of a specific length, *E. coli* carA gene sequence (carbamoyl-phosphate synthase small chain; NC_000913.3, 29651-30799 bp) was amplified with a T7 promoter upstream and terminated at different length using PCR. These PCR fragments were purified using a QIAquick PCR purification kit (QIAGEN) and transcribed using the MEGAscript T7 transcription kit (Thermo Fisher Scientific). *In vitro* transcribed RNAs were purified using TRIzol (Invitrogen) reagent following the manufacturer’s protocol and analyzed by denaturing agarose gel electrophoresis [40 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0, 10 mM sodium acetate, 1 mM EDTA pH 8.0, 6.67% (v/v) formaldehyde, and 1.5% (w/v) agarose] to verify length and integrity. For RNA sequencing experiments, RNA was collected from three independent experiments, purified with RNeasy kit to remove PBS, salts, and RNasin before RNA sequencing, and analyzed using an Agilent RNA 6000 Nano kit to verify RNA integrity. All samples had high-quality RNA, RNA integrity numbers >9.7 on a scale of 1 (degraded RNA) to 10 (intact RNA). Ribosomal RNA was removed using the Ribo-Zero Gold rRNA Removal Kit (Illumina) before library creation (using NEBNext Ultra II Directional RNA Library Prep Kit, Illumina). Biological replicates were pooled to form two distinct groups (input and bound; see the following section) for sequencing. Following sequencing, the transcriptome was treated as follows: trimming of the adaptor sequences with Trimmomatic [44], reads alignment with Bowtie 2 [45], and quantification in transcripts per million (TPM) using RSEM [46]. The RNA sequencing, alignment, and quantification were done locally at The Université de Sherbrooke RNomics Platform.

**Caspase affinity columns**

Columns were made as previously reported [20] with minor modifications. Briefly, small IMAC columns were saturated with 6xHis-tagged caspases, RNA was added (8 µg in 200 µl PBS and 40 units of RNasin), washed three times with PBS, and eluted in 300 µl
PBS plus 1.25 M NaCl. All fractions were kept on ice, RNA was quantified using an Infinite NanoQuant M200 plate reader (Tecan) and analyzed by agarose gel electrophoresis.

**Fluorescence Anisotropy**

A 30-uracil RNA oligonucleotide with a 3' FAM (30rU-FAM; from Integrated DNA Technologies) was used at a final concentration of 1 nM in buffer [50 mM 2-Amino-2-(hydroxymethyl)-1,3-propanediol, pH 7.2, 100 mM NaCl and 10 mM DTT; same buffer for proteins] containing 1 U/µl of RNasin. The FAM-labeled oligonucleotide and a serial dilution of the different caspases were incubated in a total volume of 30 µl for 10 min. at room temperature in a black 384-well microplate to reach equilibrium. Fluorescence anisotropy measurements were made using an Infinite M1000 plate reader (TECAN). Before anisotropy calculation, the background fluorescence from proteins only (blank) was subtracted from raw fluorescence data, and the fluorescence data were corrected for the G-factor, which was determined following a protocol specific for the plate reader (TECAN). Each experiment was done in duplicates, read five consecutive times, and each experiment was repeated an additional three times (n=4). The binding affinities for each caspase with the fluorescent RNA was determined in GraphPad Prism 8 using the equation:

\[
a_{\text{obs}} = a_{\text{min}} + (a_{\text{max}} - a_{\text{min}}) \cdot \frac{(K_d + RNA + X) - \sqrt{(K_d + RNA + X)^2 - 4 \cdot X \cdot RNA}}{2 \cdot RNA}
\]

where \(a_{\text{obs/max/min}}\) is the observed/maximal/minimal anisotropy value, \(K_d\) is the dissociation constant, \(RNA\) and \(X\) are the molar concentrations of the FAM-labelled RNA and caspase, respectively [47]. To estimate the affinities of the different caspases with incomplete binding curves, \(a_{\text{max}}\) was restricted so that it could not be higher than the value obtained for WT caspase-7. Fluorescence anisotropy graphs show the average anisotropy value for each caspase concentration, the standard error, and the binding curve without restriction for the maximal anisotropy.

**Data Availability**

RNA sequencing data are available on the Figshare platform at https://doi.org/10.6084/m9.figshare.14612148.v1.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

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Contributions
A.D. and J.-B.D. designed research; A.D. performed research; A.D. contributed new reagents and analytic tools; A.D. and J.-B.D. analyzed data; and A.D. and J.-B.D. wrote the paper.

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Abbreviations
DNA-BPs, DNA-binding proteins; IMAC, immobilized metal affinity chromatography; mRNA, messenger RNA; N-pep, N-terminal peptide; PARP-1, poly(ADP-ribose) polymerase 1; RNA-BPs, RNA-binding proteins; rRNA, ribosomal RNA.

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Figure Legends

Figure 1. RNA concentration affects the cleavage efficacy by caspase-7.
(A) Two µg of extracts from 293<sup>C7KO</sup> cells expressing FLAG-tagged PARP-1 were left untreated (-), treated with RNase (+) alone, or sequentially treated with RNase, RNasin (RNase inhibitor), and added purified RNA (+0.1 - 500 ng/µl of total RNA). Then, extracts were incubated for 30 min. at 37°C with a two-fold serial dilution of WT caspase-7 (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.6, 0.8, 0.4, 0.2 nM; the concentration range and time are indicated in the black wedge). PARP-1 was detected using an anti-FLAG antibody by immunoblotting. Black arrows point to the sample with approximately 50% cleavage measured by densitometry. Molecular weight markers are identified on the left side of the first immunoblot and repeated. (B) PARP-1 cleavage rate k (see Material and Methods) was calculated from immunoblots from A and other replicates (n=3) and plotted. Individual values and the mean are presented. On the abscissa, the RNase treatment alone (0) and the untreated initial lysate are shown.

Figure 2. RNA length influences the optimal interaction between caspase-7 and PARP-1.
(A) Two µg of extracts from 293<sup>C7KO</sup> cells expressing FLAG-tagged PARP-1 were left untreated (-), treated with RNase (+), or sequentially treated with RNase, RNasin, and supplemented with 30 ng/µl of purified RNA of fixed length (+100 to 1000 nt; see Material and Methods). Then, extracts were incubated for 30 min. at 37°C with a two-fold serial dilution of WT caspase-7 starting at 12.5 nM (highest concentration). Black arrows indicate the sample showing ~50% cleavage; open arrow indicates that the sample with ~50% cleavage is out of range. (B) Denaturing agarose gel of the purified RNA used in A.

Figure 3. Caspase-7’s RNA-binding is nonspecific.
(A) Top Venn diagram of all the genes identified in the input (black), fraction bound by caspase-7 (light grey), or both (intersection) with the number of genes in each group. Bottom The histogram of the overlapping genes which, on the abscissa, compares the number of RNAs associated to a gene in the caspase-7 bound group versus the input in log<sub>2</sub> scale. Each column contains genes with a log<sub>2</sub> ratio ≥ than the x value to its left and < than the x value to its right. The number of genes in each column is indicated on the top. (B) Same as in A but omitting all genes with low reads (<5 TPM). (C) Pie charts presenting proportions of the different RNA types identified in the input and the caspase-7-bound fraction. The input RNA group includes RNAs associated with 18,495 different genes, while the RNAs identified in the caspase-7-bound group are associated with 17,877 genes.

Figure 4. Caspase-7’s N-pep interfere with the cleavage of RNA-binding proteins.
(A) Two µg of extracts from 293<sup>C7KO</sup> cells expressing FLAG-tagged PARP-1 were incubated for 30 min. at 37°C with a two-fold serial dilution of WT, D23A, or KEEK caspase-7 starting at 200 nM (highest concentration). Extracts were left untreated (-) or treated with RNase (+). (B-D) Same as A but using 10 µg of cell extracts and 400 nM of caspase as the highest concentration. Antibodies against RBM26 and SR140 (two
RNA-BPs), and p23 (non RNA-BP) were used. Closed and open arrowheads represent the full-length protein and the cleaved protein, respectively.

**Figure 5. Caspase-7’s N-pep reduces the affinity for RNA.**

(A) Caspase-7 proteins used to setup affinity columns were visualized by SDS-PAGE and Coomassie staining. The large (LS) and small (SS) subunits of the caspase are visible; D23A has a larger LS due to the N-pep; some zymogen remains. The symbols used in A are conserved throughout the figure. (B) IMAC resin was saturated with the indicated caspase-7 or nothing (beads). Eight µg of total RNA was applied to the columns, washed three times, and eluted with a high-salt buffer solution; RNA from each fraction was quantified by spectrophotometry. Values from three independent experiments and the means are presented. (C) Elution fractions from B were analyzed by non-denaturing agarose gel electrophoresis along with the input. (D) RNA-binding analyzed using fluorescence anisotropy. Different caspase concentrations (~100–20,000 nM) were incubated with 1 nM of a 30rU-FAM fluorescent RNA molecule in a 384-well plate. The anisotropy was calculated as described in Material and Methods. For each caspase concentration, the mean value and the standard errors are presented as well as the associated binding curves obtained by non-linear regression.

**Figure 6. A caspase-7 dimer with two exosites is better at binding RNA.**

(A) Schematic representation of the different caspase dimers used. The large and small catalytic subunits are shown with the catalytic site as a star (green, active; red, inactive C285A mutant), and the exosite is represented by a green (WT) or a red box (KEEK). The EA:EA nomenclature represents the exosite’s status (E⁺ or E⁻) and that of the active site (A⁺ or A⁻) of each heterodimer. (B) Coomassie gel stain of the heterodimers generated. The large (LS) and small (SS) subunits of the caspase are visible; some zymogen remains. (C) Two µg of extracts from 293C7KO cells expressing FLAG-tagged PARP-1 were incubated for 30 min. at 37°C with a two-fold serial dilution of the indicated dimers starting at 200 nM (highest concentration). Importantly, the caspase concentration used is based on the active site concentration, not dimer concentration. Therefore, there is twice the concentration of heterodimers (only one active site per dimer) than WT dimers. (D) RNA-binding was analyzed using fluorescence anisotropy as in Figure 5D. The data for WT (black) and KEEK (red) caspase-7 are from Figure 5D and serve as references.
Table 1. Kinetic parameters of the various caspases used in this study.

| Caspases            | $k_{cat}$ (s$^{-1}$) | $K_M$ (µM)   | $k_{cat}/K_M$ ($\times 10^5$·M$^{-1}$·s$^{-1}$) |
|---------------------|----------------------|--------------|---------------------------------|
| WT Caspase-7 $(E^+A^+:E^+A^+)$ | 5.7 ± 0.9            | 29.3 ± 2.3   | 1.9 ± 0.4                        |
| KEEK caspase-7      | 4.9 ± 0.6            | 31.7 ± 5.1   | 1.5 ± 0.4                        |
| D23A caspase-7      | 6.2 ± 0.7            | 33.1 ± 2.4   | 1.9 ± 0.4                        |
| Heterodimers       |                      |              |                                 |
| (Active/Active)     |                      |              |                                 |
| 2 exosites         | 6.0 ± 0.3            | 27.8 ± 0.7   | 2.2 ± 0.2                        |
| No exosite         | 5.8 ± 1.1            | 30.7 ± 6.2   | 1.9 ± 0.7                        |
| Heterodimers       |                      |              |                                 |
| (Active/Inactive)  |                      |              |                                 |
| 2 exosites $E^+A^+:E^+A^-$ | 7.7 ± 0.5            | 44.2 ± 3.2   | 1.7 ± 0.2                        |
| No exosite $E^-A^+:E^-A^-$ | 5.9 ± 0.1            | 34.8 ± 1.2   | 1.7 ± 0.1                        |
| 1 exosite $cis$    | 6.0 ± 0.6            | 37.8 ± 8.5   | 1.6 ± 0.5                        |
| 1 exosite $trans$  | 4.6 ± 0.6            | 36.8 ± 1.4   | 1.3 ± 0.2                        |

The mean value ± the standard error (n=3) of kinetic parameters of the caspases used is shown. Refer to the text for a description of each caspase.
