FRET Evidence that an Isoform of Caspase-7 Binds but Does Not Cleave its Substrate

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***Abstract*— Caspase-7 is one of the executioner proteases in cellular apoptosis. Its kinetics has been monitored using biosensors based on the principle of fluorescence resonance energy transfer (FRET). Here, a caspase-7 biosensor (named vDEVDc) using fluorescent proteins as the donor and acceptor of FRET was used to study the biochemical properties of caspase-7. An active isoform of caspase-7 with the 56 N-terminal residues truncated (named 57casp7) cleaved the vDEVDc biosensor at the recognition sequence, resulting in a FRET efficiency decrease of 61%. In contrast, another caspase-7 isoform with the 23 N-terminal residues truncated (named 24casp7) bound the vDEVDc biosensor without cleaving the substrate, resulting in a FRET increase of 15%. The kinetics of the two caspase-7 isoforms were studied by monitoring the FRET change of the vDEVDc biosensor over time, which showed an exponential substrate cleavage and binding curve for the 57casp7 and 24casp7 isoform, respectively. Lastly, we modeled caspase-7 binding to the vDEVDc biosensor and estimated a FRET emission ratio increase of 16.2% after binding to caspase-7, which agrees with the 15% experimental result. We showed that two isoforms of Caspase-7 with differently truncated prodomain exhibit different enzymatic properties, namely binding by the 24casp7 isoform and hydrolysis by 57casp7. We also demonstrated that our FRET biosensor (vDEVDc) can be used to detect not only the substrate cleavage event, but also the substrate binding event.**

I. Introduction

CASPASE are a family of cysteine proteases associated with cellular apoptosis with high substrate specificity [1]. In living cells, caspase-7 is expressed as a dimeric zymogen with a 23-residue N-terminal prodomain and remains as dimer after activation [2, 3]. During the activation process, initiator caspases, such as caspase-8, proteolyse after the Asp198 residue of pro-caspase-7 located between the large p20 and small p11 subunits [1, 3]. Also, the prodomain of caspase-7 is cleaved after the Asp23 residue. The crucial cleavage in caspase-7 activation is at Asp198 because the cleavage at Asp23 alone does not produce an active caspase-7 [4]. Thus, the caspase-7 isoform truncated after Asp23 (named 24casp7, Fig. 1A) is inactive. In contrast, other studies of pro-caspase-7 showed that when truncated after Pro56 (named 57casp7, Fig. 1A), the resulting mutant is capable of autoprocessing itself into the active form [1, 3, 5].

Protein based fluorescence resonance energy transfer (FRET) caspase biosensors have been created to measure the caspase kinetics both in vitro and in living cells [6]. FRET is the transfer of energy that occurs through resonance between two fluorophores (one is the donor and the other, acceptor) with a spectral overlap in the donor emission and acceptor excitation [7]. The FRET efficiency (E%) depends on the relative distance and orientation between the fluorophore pair [7]. Caspase biosensors using FRET are typically created by sandwiching a caspase recognition substrate with a cyan fluorescent protein (CFP) donor and yellow fluorescent protein (YFP) acceptor [8-14]. Before proteolysing the substrate, the donor and acceptor are in close proximity restricted by the short bridging substrate, which gives rise to high FRET efficiency when excited by donor excitation. Once caspase cleaves the substrate, the fluorescent proteins are separated and FRET drops. By measuring the change in the FRET emission ratio (defined as the emission intensity of the acceptor divided by the donor), we can estimate the percentage of cleaved biosensors over time, which provides information on the caspase kinetics. In this paper, we show that the 57casp7 isoform cleaves our designed FRET caspase-7 biosensor, while the 24casp7 isoform binds but does not cleave.

II. Results and Discussion

A. Biosensor characterization

Our caspase-7 biosensor (named vDEVDc) was cleaved by the 57casp7 isoform (Fig. 1D), while a control biosensor (named v4Gc) was not (Fig. 1C). The vDEVDc biosensor consisted of a 24 amino acid linker containing the DEVD recognition sequence sandwiched by Venus [15] and ECFP (Fig. 1B). In contrast, the v4Gc control biosensor did not contain the recognition sequence (Fig. 1B). Prior to treatment with the active 57casp7 isoform, the emission spectra of both the vDEVDc and v4Gc biosensor had a strong peak emission of Venus (528 nm) due to FRET from ECFP to Venus (Fig. 1C, D). As the v4Gc biosensor had a shorter linker, it displayed a larger FRET as shown by a larger Venus emission peak relative to ECFP (475 nm) peak in the emission spectra (Fig. 1C). After treatment of the vDEVDc biosensor with the active 57casp7 isoform, the peak emission of Venus decreased and ECFP increased due to an expected loss of FRET after cleavage of the biosensor (Fig. 1D). In contrast, the v4Gc biosensor was not cleaved by the 57casp7 biosensor (Fig. 1C). Therefore, these results

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showed that the vDEVDc biosensor was cleaved specifically by the 57casp7 isoform.

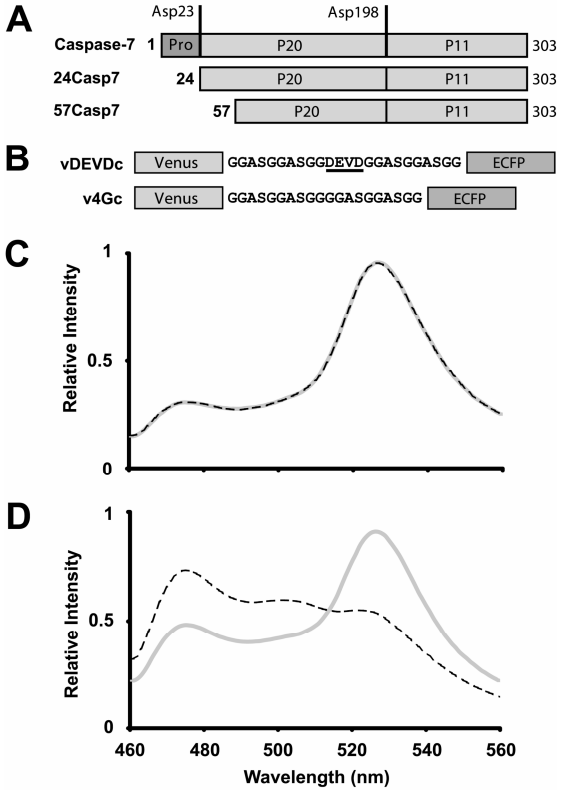

Fig. 1. Schematic diagrams and characterization of the 57casp7 isoform. A, Schematic diagram of the caspase-7 isoforms. B, Schematic diagram of the biosensors. The DEVD recognition sequence is underlined. The emission spectra before (solid gray) and after 30 minutes (dotted black) treatment with the 57casp7 isoform on C, the v4Gc control biosensor and D, the vDEVDc biosensor. All protein concentrations were 10 ± 1 μM.

B. Characterization and kinetics of the Caspase-7 isoforms

The 24casp7 isoform had no protease activity in vitro, but recognized and bound to the substrate of the vDEVDc biosensor (Fig. 2). A spectrum was recorded immediately after mixing the 24casp7 isoform with both the vDEVDc and v4Gc biosensors. After incubating for an hour at room temperature, the spectrum of this solution was recorded again. Comparing the two spectrums from before and after the incubation period, we observed that the FRET emission ratio of the vDEVDc biosensor increased by 15% (Fig. 2D). This increase of FRET indicated a conformational change in the biosensor that does not involved proteolytic cleavage. In order to isolate the cause of the increase of FRET to the 24casp7 isoform, two control experiments were performed. In the first experiment, both the v4Gc and vDEVDc biosensor were incubated alone for one hour at room temperature. The emission spectrums did not change during this period (Fig. 2A, C). This shows that any changes to the spectrum over time were not caused by intrinsic properties of the v4Gc and vDEVDc biosensors. In the second experiment, the 24casp7 isoform was mixed with the v4Gc control biosensor and incubated for an hour. The spectrums from before and after this incubation period were also identical (Fig. 2B). This result verified that the 24casp7 isoform does not interact with the v4Gc biosensor, whose structure is identical to the vDEVDc biosensor without the DEVD recognition sequence. Therefore, the vDEVDc biosensor specifically interacted with the 24casp7 isoform in the DEVD recognition sequence of the vDEVDc biosensor. Since this interaction was not cleavage of the DEVD recognition sequence, 24casp7 isoform instead must have bound the DEVD sequence.

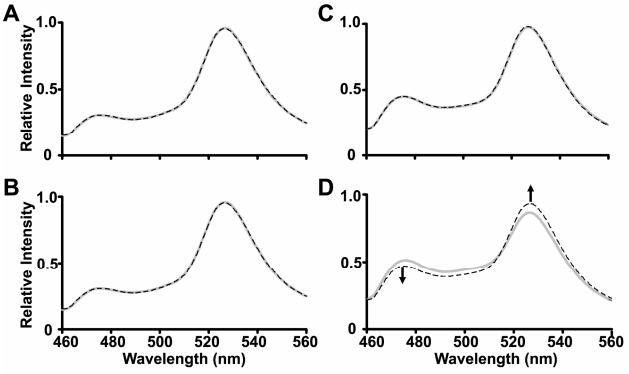

Fig. 2. Characterization of the 24casp7 isoform. The spectra was measured before (solid grey) and after (dotted black) an hour incubation at room temperature for A, the v4Gc biosensor, B, the 24casp7 isoform + v4Gc biosensor, C, the vDEVDc biosensor and D, 24casp7 + the vDEVDc biosensor. All protein concentrations were 10 ± 1 μM.

In time-lapsed experiments, the 24casp7 and 57casp7 isoforms exponentially bound and cleaved the vDEVDc biosensor, respectively (Fig. 3). Similar controls to the above experiments were used to obtain the time-lapsed spectrums using an excitation wavelength of 440 nm every minute over a period of 20 to 30 minutes. In these time-lapsed experiments, the solution was repeatedly sampled leading to an irreversible photobleaching of the biosensors. For example, in the control experiment of the vDEVDc biosensor alone, the FRET emission ratio decreased 2.5% by photobleaching (Fig. 3A). Similar photobleaching was observed in experiments with the v4Gc biosensor (data not shown). As this effect was omnipresent in all time-lapsed spectrums, the measured FRET emission ratios were the results of both photobleaching and activity of the caspase-7 isoforms. Further experiments with the 57casp7 isoform + the vDEVDc biosensor, as expected, showed a 67% exponential decrease of the FRET emission ratio (Fig. 3C), which overshadowed the decrease of FRET caused by photobleaching. In contrast, experiments with the 24casp7 isoform + the vDEVDc biosensor showed an 8% exponential increase of FRET (Fig. 3B). The FRET emission ratio decrease from photobleaching was evident during the first 4 minutes. Thus, these results indicated that the binding between the 24casp7 isoform and the vDEVDc biosensors occurred exponentially.

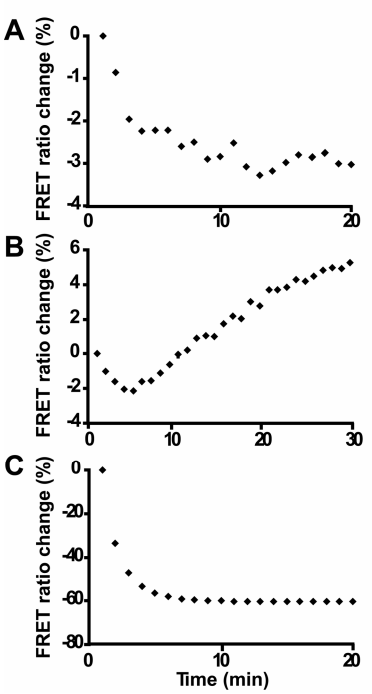

Fig. 3. Time-lapsed experiments of A, the vDEVDc biosensor, B, the 24casp7 isoform + vDEVDc biosensor and C, the 57casp7 isoform + vDEVDc biosensor.

C. Computational modeling of the 24casp7 isoform binding to the vDEVDc biosensor

Computational models of the vDEVDc biosensor before and after binding to caspase-7 estimated a FRET emission ratio of ~16.2%, which is consistent with our experiment results of 15% (Table I) (Fig. 4 and 5). The binding of caspase-7 to its inhibitor was used to model the binding of the vDEVDc biosensor to the active site of the 24casp7 isoform [16]. Both the caspase-7 free and bound cases had relatively low median FRET efficiency (E%) values at approximately 3%, however, at least 15% of the models in the caspase-7 bound case had a greater propensity for models with an E% larger than 20% which explained the corresponding shifts in their mean E%. For the caspase-7 free case (Fig. 4A), the average E% was 7.8% with corresponding distance and orientation factors values of 74.8 ± 10 Å and 0.470, respectively (Table 1) (Fig. 5). Note that the distance factor displayed a Gaussian-like distribution whereas the orientation factor distribution was more scattered. One commonly used constant for κ2 is 0.475, assuming the donor-acceptor orientations do not change during the lifetime of the excited state [17]. Our simulation results for orientation factor were consistent with that assumption since the orientation of the donor-acceptor pair has a large rotational freedom around the flexible linker in the caspase-7 free case. For the caspase-7 bound case, however, based on the dimer conformation of caspase-7, the E% could originate from the intra- or inter-molecular FRET effect (Fig. 4B). The simulated E%, distance factor, and orientation factor averages for the intra- and inter-molecular FRET effects were (15.3%, 71.7 ± 17 Å, 0.594) and (3.4%, 98.9 ± 17 Å, 0.559), respectively (Table 1) (Fig. 5). Since the intramolecular FRET was almost 5-fold larger compared to the intermolecular effect due to the smaller distance factor, the intramolecular effect was the dominant factor and thus, its FRET efficiency is representative of the overall caspase-7 bound FRET efficiency. Note also that both κ2 values deviated from the constant 0.475 in the caspase-7 free case. This was expected because the donor-acceptor pair has reduced rotational freedom due to dimer conformation of the caspase-7 and the vDEVDc biosensor complex. An increase of X% in FRET efficiency means an X% energy loss from the donor and an X% energy gain to the acceptor. The change in FRET emission ratio (the division of acceptor emission by donor emission after donor excitation), R, can then be estimated by the following equation:

$$R_{new} \approx R_{old} \times \frac{(1+X\%)}{(1-X\%)}$$

Since the increase in FRET efficiency from caspase-7 free to bound is 7.5%, there is an approximately 16.2% increase in FRET emission ratio after caspase-7 binding.

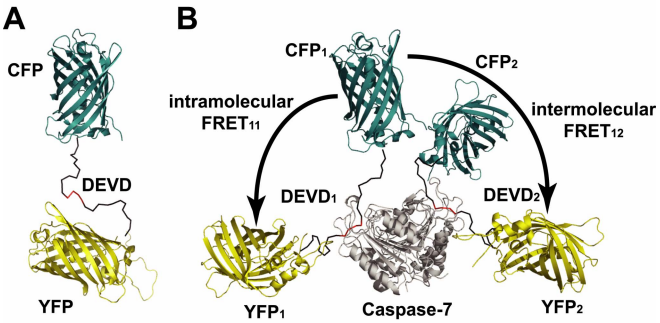

Fig. 4. Structural models of A, the unbound vDEVDc biosensor, B, the vDEVDc biosensors bound to dimeric caspase-7.

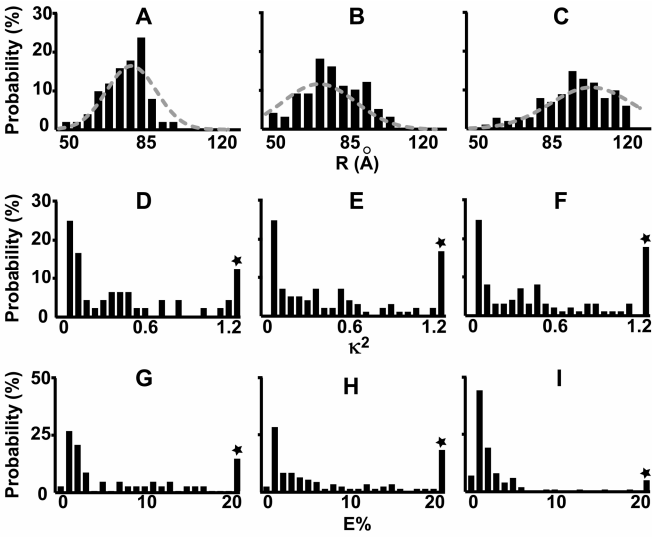

Fig. 5. Statistics of the modeling results. A, B, C, the distance distribution (R) between the CFP and YFP chromophores for the caspase-7 free models, caspase-7 bound models (intramolecular FRET) and caspase-7 bound models (intermolecular FRET), respectively. D, E, F, the FRET orientation factor (κ2) for the caspase-7 free models, caspase-7 bound models (intramolecular FRET) and caspase-7 bound models (intermolecular FRET), respectively. G, H, I, the overall FRET efficiency (E%) for the caspase-7 free models, caspase-7 bound models (intramolecular FRET) and caspase-7 bound models (intermolecular FRET), respectively.

TABLE I
MODELING STATISTICAL RESULTS

	R (Å)	κ2	E%
Caspase-7 free	74 ± 10	0.47	7.74%
Caspase-7 bound (Intra-molecular)	71 ± 17	0.59	15.31%
Caspase-7 bound (Inter-molecular)	98 ± 17	0.56	3.38%

III. CONCLUSION

We designed the vDEVDc biosensor for monitoring caspase-7 activity and discovered that it responds differently to two isoforms of caspase-7 – 24casp7 and 57casp7. The 57casp7 isoform exhibited the normal proteolytic activity and caused the vDEVDc biosensor to lose FRET. The 24casp7 isoform, on the other hand, caused the FRET emission ratio of vDEVDc to increase by 15%. We isolated the cause of this FRET increase to the putative binding of the 24casp7 isoform to the DEVD substrate in our vDEVDc biosensor. Furthermore, our experiments showed that upon incubating with the 24casp7 isoform, the FRET of the vDEVDc biosensor increases exponentially over time, which resembles the kinetics of a typical binding reaction. To further test the binding hypothesis, we simulated the FRET change of the vDEVDc biosensor from before and after binding to caspase-7 yielding a 16.2% increase, which agreed with the 15% FRET emission ratio increase observed experimentally. Hence, we conclude that 24casp7 isoform lacks its native proteolytic activity but is capable of recognizing and binding to its substrate. Last, this work expands the application of caspase FRET biosensors to a different aspect of caspase activity beyond proteolysis.

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