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 (Fig. 1D) while a control biosensor (Fig. 1C) was not cleaved (Fig. 1D). In contrast, the v4Gc control biosensor did not contain the recognition sequence sandwiched by Venus [15] and ECFP (Fig. 1B). Prior to treatment with the active 57casp7 isoform, the emission spectra of both the vDEVDc and v4Gc biosensor showed 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...
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 involve 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.

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.
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 $\kappa^2$ 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 $\kappa^2$ 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:

$$R_{new} = R_{old} \times \frac{(1 + X\%) \times \left(1 - X\% \ln R_{old}ight)}{(1 - X\%) \times \left(1 + X\% \ln R_{old}\right)}$$

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.
### 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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### References


### Table I: Modeling Statistical Results

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<th>R (Å)</th>
<th>k2</th>
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<tr>
<td>Caspase-7 free</td>
<td>74 ± 10</td>
<td>0.47</td>
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<tr>
<td>Caspase-7 bound (Intra-molecular)</td>
<td>71 ± 17</td>
<td>0.59</td>
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<tr>
<td>Caspase-7 bound (Inter-molecular)</td>
<td>98 ± 17</td>
<td>0.56</td>
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