Simultaneous Real-Time Detection of Initiator- and Effector-Caspase Activation by Double Fluorescence Resonance Energy Transfer Analysis

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Abstract. Fluorescence resonance energy transfer (FRET) with green fluorescent protein (GFP) variants has become widely used for biochemical research. In order to expand the choice of fluorescent range in FRET analysis, we designed various color versions of the FRET-based probes for caspase activity, in which the substrate sequence of the caspase was sandwiched by donor and acceptor fluorescent proteins, and studied the potential of these color versions as fluorescent indicators. Six color versions were constructed by a combination of cyan fluorescent protein (CFP), GFP, yellow fluorescent protein (YFP), and DsRed. Real-time monitoring in single cells revealed that all probes could detect caspase activation during tumor necrosis factor (TNF)-α-induced cell death as a fluorescent change. GFP-DsRed and YFP-DsRed were as sensitive as CFP-YFP, and CFP-DsRed also showed a large fluorescent change. By using two probes, CFP-DsRed and YFP-DsRed, we carried out simultaneous multi-FRET analysis and revealed that the initiator- and effector-caspases were activated almost simultaneously in TNF-α-induced cell death. These findings may give experimental bases for the development of novel techniques to analyze multi-events simultaneously in single cells by using FRET probes in combination.

Keywords: fluorescence resonance energy transfer, green fluorescent protein, tumor necrosis factor-α, cell death, caspase

Introduction

Many probes for various physiological reactions have been developed with green fluorescent protein (GFP) variants by using a similar strategy as that used with cameleon, the Ca²⁺-sensing fusion protein developed by Miyawaki et al. (1 – 9). The cameleon consists of cyan fluorescent protein (CFP), calmodulin, M13 peptide, and yellow fluorescent protein (YFP). This fusion protein senses Ca²⁺ as the change of fluorescence resonance energy transfer (FRET) efficiency between CFP and YFP. Calmodulin binds M13 in the presence of Ca²⁺, which causes conformational change in cameleon, resulting in a change in the distance between and relative orientation of CFP and YFP. This change alters the FRET efficiency from CFP to YFP; therefore, Ca²⁺ can be monitored as the fluorescent change (1).

CFP and YFP are the most frequently used pair for analysis by FRET. This pair is suitable for FRET analysis because the spectral overlap between the emission of the donor protein (CFP) and the excitation of the acceptor protein (YFP) is sufficient for energy transfer, and their ranges of fluorescence are far apart enough to be separated by measuring devices such as fluorescent microscopy (10). However, there are limitations for the CFP-YFP pair. It is impossible, for example, to use the CFP-YFP FRET probe for simultaneous measurement with other probes that are made of GFP variants or have

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fluorescein structure. If more choice of FRET probes is available from wider fluorescence ranges, it would allow us to analyze multi-events simultaneously occurring in living cells.

In this paper, we developed caspase-sensors of various colors by using cyan, green, yellow, and red fluorescent proteins and assessed their ability to detect the caspase activation in living single cells. Based on the findings obtained, we tried to perform multi-event FRET analysis and clarify the temporal relationships between biochemical reactions during cell death.

**Materials and Methods**

**Plasmid construction**

Plasmid encoding CY-sensor, YFP-peptide-CFP, was generated as previously reported (11). The sequence encoding 11 amino acids at the C-terminus of YFP was eliminated in this construct. The C-terminal truncated forms of the CFP (or GFP) gene were generated by PCR with primers containing the NheI site or BspEI site and pECFP-C1 (or pEGFP-C1; Clontech, Palo Alto, CA, USA) as a template, and the restricted fragment was inserted into the NheI/BspEI sites of the CY-sensor to generate a plasmid carrying truncated CFP (or GFP) at the N-terminus. DsRed was generated from pDsRed2-C1 (Clontech) by PCR, at the AgeI/NotI sites, and the restricted fragment was inserted into the AgeI/NotI sites of the CY-sensor to generate a plasmid carrying truncated CFP (or GFP) at the C-terminus. CG-, CR-, GR-, and YR-sensors were generated with a combination of these elements. The AgeI/BsrGI fragment from pEGFP-C1 was inserted into the AgeI/BsrGI sites of the CY-sensor to generate the 11-amino acids at the C-terminus of YFP was obtained from the whole cell region of normal-shaped spherical cells, respectively.

**Cell culture and transfection**

HeLa cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 10% fetal calf serum (Invitrogen Corp., Carlsbad, CA, USA). Plasmid encoding the sensor protein was transfected into HeLa cells using Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After 12 – 24 h incubation with the transfection reagent, the cells were washed with PBS and cultivated on dishes suitable for assay in medium containing 500 μg/ml of G418 for an additional 1 – 3 days until the assay was performed.

**Western blotting**

Cells cultured in a plastic dish were washed with PBS and lysed with 1 × SDS loading buffer. The samples dissolved in 1 × SDS loading buffer were incubated at 95°C for 2 min, and then they were loaded onto SDS-polyacrylamide gels (10%). Proteins were separated at 20 mA and then blotted to PVDF membranes in Tris-glycine transfer buffer at 100 V for 2 h. The membrane was incubated with block ace (Dainippon Pharmaceutical, Osaka) for 1 h, anti-GFP peptide antibody (Clontech, diluted with 0.1 × block ace to 1:1,000) for 2 h, and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Chemicon International Inc., Temecula, CA, USA; diluted with 0.1 × block ace to 1:10,000) for 1 h. The membrane was washed with TBS-T 3 times for 5 min after the incubation with the antibody. All of these incubations were performed at room temperature. The membrane was developed with the ECL chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

**Measurement of fluorescent spectra of the sensors in HeLa cells**

Spectral imaging was performed with LSM510META (Carl Zeiss, Jena, Germany) (12). Cells expressing one of the sensors were observed by excitation light at 458 nm (Ar laser), emitted fluorescence was separated by a grating, and the separated fluorescence were detected by 24 photomultiplier tubes (PMT) that were set to detect fluorescence at 468 – 714 nm. Each PMT detected fluorescence in the 10.7-nm wavelength range. So, the fluorescent spectrum at 468 – 714 nm was obtained with 10.7-nm resolution. Cell death was induced by incubation with tumor necrosis factor (TNF)-α (100 ng/ml) and cycloheximide (CHX, 10 μg/ml) for 6 h. Fluorescent spectra of living and dead cells were obtained from the whole cell region of normal-shaped and spherical cells, respectively.

**Real-time imaging with FRET sensors**

Transfected cells were cultured on a cover glass (25-mm diameter, 0.15 – 0.18-mm thickness) for 1 – 3 days. Cells were treated with TNF-α/CHX and then incubated under the usual culture condition for 1 – 2 h before analysis. Analyses were carried out by confocal laser-scanning fluorescent microscopy using a Carl Zeiss LSM510 system. During the observation, the media were buffered with 10 mM hepes buffer (pH 7.4), and the cells were maintained at 35 – 37°C. DIC images and grayscale images for fluorescence channels were obtained every 2 min unless otherwise described. Excitation lights for the FRET probe (458 nm for the CG-, CY-, GY-, and CR-sensors; 488 nm for the GR- and YR-sensors) were provided by an Ar laser with a 458 or 488 dichroic mirror. Images of the FRET probe were obtained separately for both donor and acceptor.
fluorescence using a dichroic mirror and band-pass emission filters as shown in Table 1. Images were processed and quantified using MetaFluor software as follows: The average pixel intensity of the fluorescence of the whole cell region was determined for each channel. The ratio value was calculated as the average pixel value of the fluorescent ratio, (fluorescent intensity for the acceptor channel) / (fluorescent intensity for the donor channel), in the whole cell region. As cells changed their morphology during the observation, the whole cell region was determined separately in each image.

Results

Construction and characterization of FRET probes

We developed plasmids expressing caspase sensors as shown in Fig. 1a. A 12-amino-acid peptide derived from poly(ADP-ribose)polymerase (PARP) that is a well-known substrate of effector caspases was sandwiched by two different fluorescent proteins (an example of CFP-YFP is shown in Fig. 1a). The peptide sequence contains a caspase recognition site in the middle, and this fusion protein was cleaved mainly by caspase-3 (11). CFP-GFP, CFP-YFP, GFP-YFP, GFP-DsRed, GFP-DsRed, and YFP-DsRed were used as the donor-acceptor pairs. We named these fusion proteins CG-, CY-, GY-, CR-, GR-, and YR-sensor, respectively (Table 1). These fusion proteins show FRET in their intact form, whereas in the presence of active caspase, the peptide sequence is cleaved, CFP and YFP are far apart, and the fusion proteins do not show FRET any longer. The fluorescent ratio of acceptor/donor reflects the amount of FRET, so we used the reduction of this value as an index of caspase activation.

HeLa cells expressing one of these fusion proteins

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Fusion protein</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>beam splitter</td>
</tr>
<tr>
<td>CG</td>
<td>GFP-peptide-CFP</td>
<td>458</td>
<td>515</td>
</tr>
<tr>
<td>CY</td>
<td>YFP-peptide-CFP</td>
<td>458</td>
<td>515</td>
</tr>
<tr>
<td>GR</td>
<td>GFP-peptide-DsRed</td>
<td>488</td>
<td>545</td>
</tr>
<tr>
<td>CR</td>
<td>CFP-peptide-DsRed</td>
<td>488</td>
<td>545</td>
</tr>
</tbody>
</table>

* N-terminal CFP, GFP, and YFP were in a truncated form in which 11 amino acids at the C-terminus were eliminated, and His10 was present at the C-terminus of CG, CY, and GY. Excitation light was obtained by Ar laser and a 458 or 488 dichroic mirror. Emitted fluorescence was separated by a 515 or 545 dichroic mirror, and the fluorescence of the donor and that of the acceptor were obtained through band pass emission filters.

**Table 1.** Measurement conditions for real-time analysis by LSM510

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Fusion protein</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>GFP-peptide-CFP</td>
<td>458</td>
<td>515</td>
</tr>
<tr>
<td>CY</td>
<td>YFP-peptide-CFP</td>
<td>458</td>
<td>515</td>
</tr>
<tr>
<td>CR</td>
<td>CFP-peptide-DsRed</td>
<td>488</td>
<td>545</td>
</tr>
<tr>
<td>GR</td>
<td>GFP-peptide-DsRed</td>
<td>488</td>
<td>545</td>
</tr>
<tr>
<td>YR</td>
<td>YFP-peptide-DsRed</td>
<td>488</td>
<td>545</td>
</tr>
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Fig. 1. Small peptide sandwiched by two different fluorescent proteins can be a caspase-sensor, a: Fusion protein that consists of a PARP-derived 12-amino-acid peptide sandwiched by CFP and YFP exhibits FRET in its intact form. In the presence of active caspases, the peptide is cleaved, and the fusion protein does not exhibit FRET. Caspase activation can be detected by measuring the fluorescence of CFP and YFP. b: Six caspase-sensors expressed in HeLa cells were cleaved by cell death stimuli. HeLa cells expressing one of the sensors were incubated in the presence or absence of TNF-α/CHX for 6 h. The arrow and arrowhead indicate the full length and cleaved fragments of the sensors.
were treated with TNF-α/CHX. After 6-h exposure, the sensor proteins in cells were extracted and analyzed by western blottting. All 6 fusion proteins were detected in their intact forms in non-treated HeLa cells (arrow in Fig. 1b), and small fragments were detected in cells treated with TNF-α/CHX (arrowhead in Fig. 1b), indicating that the fusion proteins were cleaved by cell death stimuli, as expected. The antibody used in this analysis reacts with CFP, GFP, and YFP, but not with DsRed. Therefore, CG-, CY-, and GY-sensor showed two cleaved fragments corresponding to the N- and C-terminal C/G/YFP, whereas CR-, GR-, and YR-sensor showed only one cleaved fragment corresponding to the N-terminal C/G/YFP.

Figure 2 shows the fluorescent spectra of the probes in living or dead cells. Comparing the fluorescence of living and dead cells, all sensors showed an increase of donor fluorescence and/or a reduction of acceptor fluorescence in response to cell death stimuli. This change results in a reduction of fluorescent ratio of acceptor/donor that is an index of FRET. These sensors were designed to show a reduction of FRET with caspase activation, so these results suggest that all 6 fusion proteins work as expected and can detect caspase activation as fluorescent change in living cells.

For simultaneous application of two or more fluorescent probes, minimum spectral overlap between probes is one of the important conditions. The spectra in Fig. 2 give us a clue to determine a suitable combination of probes for multi-probe analysis. CG-, CY-, or GY-sensor has the least fluorescence in the red-fluorescence region (>600 nm), so it is possible to use this fluorescent region for another dye. We can use a red-fluorescent dye that has fluorescence in this region together with CG-, CY-, or GY-sensor simultaneously. On the other hand, YR-sensor has the least fluorescence in the blue-cyan region (<500 nm), so blue-cyan-fluorescent dye is applicable with this probe for the purpose of simultaneous fluorescence imaging. The color variations of FRET probe may be useful for multi-probe analysis.

Real-time detection of caspase activation in living cells

Next, we applied the sensor proteins to real-time measurement. HeLa cells expressing one of the sensor proteins were analyzed with a time resolution of 2 min by laser-scanning confocal fluorescent microscopy. Figure 3 shows typical images (a) and fluorescent changes (b) during cell death. HeLa cells expressing GR-sensor were treated with TNF-α/CHX. An increase of donor protein fluorescence (GFP), a reduction of acceptor protein fluorescence (DsRed), and a reduction of the fluorescent ratio of acceptor/donor (DsRed/GFP) were observed in each cell at a different time. Caspases began to work at the point when the fluorescent ratio began to decrease.

All sensors showed similar changes, meaning that all sensors were useful for real-time detection of the caspase activation in a living cell, although the apparent sensitivity was different between sensors. In order to compare the sensitivity of these sensors to detect the caspase activation, the amount of the fluorescent change was calculated. We defined the start point and the end...
point of the reduction of the fluorescent ratio as follows: the start point was the point after which the value decreased over four continuous points or more, the value decreased more than 10% in total, and the reduction of the value was not because of artificial noise such as focus shift; the end point followed the start point and was the point at which the value stopped decreasing. The sensitivity of the probe was calculated as $\Delta R = (R_{\text{end}} - R_{\text{start}})/R_{\text{start}}$, where $R_{\text{start}}$ and $R_{\text{end}}$ were the fluorescent ratio at the start point and the end point, respectively. Figure 4 shows $\Delta R$ for each probe. GR and YR, as well as CY, showed the highest $\Delta R$. They each showed a more than 50% change during cell death. CR showed a slightly lower $\Delta R$, but its change was still 44% on average. CG and GY were less sensitive, probably because the fluorescent spectra of the donor and the acceptor were so similar that our system could not effectively measure FRET between them. CY vs GR, CY vs YR, or GR vs YR were not significantly different, and any other comparisons were significantly different by the Games-Howell test ($P<0.05$).

**Simultaneous multi-event analysis using two FRET probes**

Finally, we tried to perform multi-FRET measurement. We constructed a YR-initiator caspase sensor and a CR-effector caspase sensor by changing the caspase substrate sequence in the sensor and applied them to real-time imaging analysis simultaneously in order to reveal the temporal relationships between the initiator caspase activation and the effector caspase activation in the same cell. The caspase substrate sequences were derived from procaspase-3 and PARP, respectively, and their sequences are shown in Fig. 5a. These sensors were cleaved mainly by caspase-8/9 and caspase-3, respectively (11).

Simultaneous measurement of these sensors was performed under the multi-track scanning mode, in which two sets of excitation-detection conditions were used alternatively. CFP fluorescence by excitation at 458 nm was measured in the first track, and YFP and DsRed fluorescence by excitation at 488 nm was mea-

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**Fig. 3.** Real-time imaging of caspase activation in living HeLa cells during cell death. HeLa cells expressing GR-sensor were treated with TNF-α/CHX, and fluorescent images were obtained every 2 min. a: DIC images (upper panels) and fluorescent ratios (Red/Green, lower panels) are shown in grayscale. The indicated time represents the time after the addition of TNF-α/CHX. Scale bar, 10 μm. b: The fluorescent ratio of cells were plotted. Cell 1, 2, or 3 corresponds to the cells shown in panel a. c: The mean pixel intensity in arbitrary fluorescent units (a.u.) for each channel was plotted. The fluorescence of cell No. 3 from panel a is shown. Open circle, GFP; open triangle, DsRed; closed diamond, ratio of Red/Green. Asterisks on the x-axis indicate the time points of the images in panel a.

**Fig. 4.** Comparison of the sensitivity of various caspase-sensors. The amount of change of the fluorescent ratio during cell death ($\Delta R$) was determined in each cell as described in the text. Bars represent means ± S.D. The number of cells used in each analysis is shown in parentheses.
sured in the second track. The time difference of scanning between tracks is about 3 – 8 s. Figures 5b and 5c show control studies with cells expressing only one of the probes. These control studies were conducted in the same conditions as Fig. 5d. Figures 5b and 5c indicate that the YR- and CR-sensor could detect initiator- and effector-caspase activation as an increase of YFP and CFP signal, respectively, and the contamination of the signal between the YFP and CFP channels was negligible. So, we used an increase of the YFP and CFP signal as index of the initiator- and the effector-caspase activation, respectively. The DsRed signal in

Fig. 5c was derived from direct excitation of DsRed in the CR-sensor by the excitation light at 488 nm and was increased when the cell shrank because fluorescent proteins were concentrated in the cell.

Figure 5d shows typical data of multi-probe analysis with the YR-initiator caspase sensor and CR-effector caspase sensor. In this cell, the fluorescence was dramatically changed at 150 – 160 min after TNF-α/CHX treatment. The YFP and CFP signal began to increase almost simultaneously, suggesting that initiator caspase and effector caspase were initially activated within a short time period. Figures 5e – 5g show three
other examples. We observed more than 30 cells in at least 3 independent experiments and found that all dying cells showed similar results.

Discussion

In this study, we developed various color versions of caspase-sensors with CFP, GFP, YFP, and DsRed and revealed that various combinations are applicable in FRET analysis. CY, CR, GR, and YR pairs are preferable FRET pairs that possess a high ability to detect the caspase activation.

The sensitivity shown in Fig. 4 represents the apparent FRET change that depends on the measuring system and was determined by three factors. 1) Intrinsic FRET efficiency: All 4 fluorescent proteins had different fluorescent characteristics; therefore, the levels of FRET efficiency in 6 probes differed from each other. 2) Excitation crosstalk: The acceptors were excited directly by the excitation light. 3) Emission crosstalk: The acceptor channel was contaminated with the donor signal, and vice versa, because the setting shown in Table 1 could not perfectly separate the signals from the donor and the acceptor. The differences in these factors cause the difference of sensitivity among the sensors. Factors 2) and 3) reduce the apparent FRET change in the measurement. In the case of the CG-sensor, for example, fluorescent spectrum of donor and acceptor are so similar that the intrinsic FRET efficiency may be high, but excitation and emission crosstalk may also be high, much higher than in other sensors (e.g., CY-sensor), resulting in the relatively low sensitivity of this probe in our measurement system. Crosstalk effects are undesirable for detection, but it is impossible to completely eliminate these effects in the current measurement system. Maybe we could obtain different results by using spectral imaging in which emission crosstalk is eliminated (12).

According to the characteristics of the fluorescence spectrum, the CY probe seems to be one of the best for FRET-detection. However, the probe is not suitable for imaging with confocal laser microscopy, because the normal argon ion laser, the most common one in confocal microscopes, is not suitable for the excitation of CFP. The blue laser is the most suitable for the excitation, but it is not common in confocal laser microscopes. In this paper, we had to use the argon ion laser emitting 458 nm and the special emission filters optimized for the confocal ratio-imagings of caspase activation using the CY probe (11). On the contrary, the GR probe and the YR probe can be efficiently excited at 488 nm emitted by the normal argon ion laser and imaged with a set of emission filters for fluorescein and a set for rhodamine, with which almost all of the confocal microscopes are equipped. In addition, the GR probe is useful for the detection of caspase activation in flow cytometry, because almost all of the normal flow cytometers are also usually equipped with the laser and the emission filters.

DsRed-containing “red”-sensors have several characteristics that are different from other “non red”-sensors. As previously reported (13), it takes longer for DsRed to mature and emit red fluorescence than it takes for GFPs, and DsRed fluorescence tends to decrease during real-time observation, which may cause a reduction of the apparent sensitivity. These characteristics must be considered when any analysis is performed with these sensors, but as shown in Figs. 4 and 5, red-sensors have a potential similar to that of the CY-sensor and are very useful for multi-color imaging.

It has been reported that DsRed is useful as a fusion tag and a partner for FRET (13, 14). Erickson et al. analyzed the potential of DsRed as a FRET partner with CFP and GFP (14). Mizuno et al. developed a Ca\(^{2+}\) sensing fusion protein using Sapphire and DsRed (13). And recently, Karasawa et al. used two novel fluorescent proteins, namely the cyan-emitted and orange-emitted fluorescent proteins from Acropora sp. and Fungia concinna, respectively, as a FRET pair, and measured caspase-3 activity in cells (15). These results combined with our results indicate that various fluorescent proteins including GFP derivatives, DsRed, and others are useful for FRET analysis. By choosing the appropriate two fluorescent proteins as the FRET pair, we can customize the fluorescent range of FRET-based imaging probes to fit the analysis, which would expand the flexibility of simultaneous multi-event analysis.

By using the CR and YR developed in this study, we were able to analyze two FRET probes simultaneously in the same cells. In several reports, the initiator caspase activity and the effector caspase activity were measured in living cells (8, 9, 11). In these reports, however, each activity was measured independently in different cells. To our knowledge, the present study is the first report that analyzes these activities in the same cell. The results directly reveal the temporal relationships between these caspase activities. It takes a long time for cells to start the initiator caspase activation after drug treatment, but it takes a relatively short time for cells to start the effector caspase activation after the initiator caspase activation. The caspase cascade is initiated at the last stage of cell death signaling, and it proceeds within a short time period.

Acknowledgments

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