

Real-time detection of caspase-2 activation in a single living HeLa cell during cisplatin-induced apoptosis

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Abstract. Caspase-2 is important for the mitochondrial apoptotic pathway, however, the mechanism by which caspase-2 executes apoptosis remains obscure. We carry out the first measurements of the dynamics of caspase-2 activation in a single living cell by a FRET (fluorescence resonance energy transfer) probe. Two FRET probes are constructed that each encoded a CRS (caspase-2 or caspase-3 recognition site) fused with a cyan fluorescent protein (CFP) and a red fluorescent protein (DsRed) (CFP-CRS-DsRed). Using these probes, we found that during cisplatin-induced apoptosis, caspase-2 activation occurred more slowly than did activation of caspase-3; additionally, caspase-2 activation was initiated much earlier than that of caspase-3. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2187013]

Keywords: caspase-2; fluorescence resonance energy transfer; cyan fluorescence protein; red fluorescence protein from *Discosoma*; cisplatin.

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1 Introduction

Apoptosis plays an important role in the normal development of multicellular organisms. Defects in apoptosis underpin both tumorigenesis and drug resistance, often causing chemotherapy to fail.¹ A full understanding of the key molecular events during apoptosis will be very useful for anticancer drug design, therapy,¹ and screens.²

Caspases mediate apoptosis via two major pathways:^{3,4} the mitochondrial pathway (intrinsic) and the death receptor pathway (extrinsic). Caspase-2, an initiator caspase, was first identified in the death receptor pathway;⁵ however, several reports have recently shown that this caspase is required for mitochondrial permeabilization and apoptosis^{6,7} to begin. This result⁸ indicates that caspase-2 is the initiator caspase in the mitochondrial pathway, not caspase-9 as previously reported, challenging the traditional view that the mitochondrion is the central regulator of apoptosis.

The activation of caspases is a central event in apoptosis.^{8,9} Therefore, the scientific community has a strong interest in measuring the dynamics of caspase activation. Molecular probes based on the fluorescence resonance energy transfer (FRET) technique have been widely applied in the last few years to detect the dynamics of caspase activation during apoptosis, successfully tracking the activities of caspase-3 (Refs. 10–13), caspase-8 (Refs. 14 and 15), and caspase-9 (Ref. 9); however, the dynamics of activation of the initiator caspase,

caspase-2, and the mechanism by which caspase-2 executes apoptosis, remain obscure.¹⁶

In this study, we designed and synthesized a FRET probe to visualize the activities of caspase-2 during cisplatin-induced apoptosis. We also constructed a FRET probe specifically sensitive to caspase-3 by inserting a tandem substrate site (DEVD) into the donor/acceptor pair, to compare activation of these two caspases. After experimenting with popular designs of FRET probes that use enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescence protein (EYFP) as the FRET donor and acceptor,^{13,15} respectively. We chose the ECFP/DsRed2 (*Discosoma* red fluorescent protein) pair instead of the ECFP/EYFP pair because the former possesses minimal “crosstalk”^{17,18} and is insensitive to change in proton (H⁺) concentration during apoptosis.⁹

FRET is a distance-dependent physical process in which energy is transferred from a fluorophore in an excited state to another fluorophore by means of intermolecular long-range dipole-dipole coupling rather than by radiation.^{19,20} Based on this method, the FRET probe is a powerful tool for detecting protease activities in living cells. The pentapeptide VDVA is the preferred substrate for caspase-2. In contrast to other known caspases, it requires tetrapeptide specificity for cleavage. This cleavage site is unique to caspase-2 and unsusceptible to cleavage by other caspases with closely similar peptide specificity.²¹ Consequently, we biosynthesized the CD2 by sandwiching the linker containing the pentapeptide VDVA between two fluorescent proteins (CFP/DsRed2).

Using this FRET probe, we imaged the dynamics of caspase-2 activation in a living cell for the first time. We found that during cisplatin-induced apoptosis, caspase-2 acti-

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vation occurred more slowly, but was initiated much earlier, than that of caspase-3. This result provides some useful information toward understanding the mechanism by which caspase-2 executes apoptosis.

2 Materials and Methods

2.1 Gene Construction

For this study we constructed two different recombinant FRET probes, CFP-VDVAD-DsRed2 and CFP-DEVD-DsRed2; they were each made by fusing two fluorescent proteins (ECFP and DsRed2) with a linker containing the caspase-2 cleavage sequence^{10,22} (VDVAD) and the caspase-3 cleavage sequence^{9,11,13} (DEVD), respectively. To construct CFP-VDVAD-DsRed2 (CD2) and CFP-DEVD-DsRed2 (CD3), the ECFP-EYFP concatemer from YC2.1 (a gift from A. Miyawaki) was subcloned into PMD18-T (CLONTECH) by *HindIII* and *EcoRI*. The enhanced mutants ECFP and EYFP are referred to using the more concise names CFP and YFP throughout. The *HindIII/SacI* fragment from the PMD18 was replaced with the *HindIII/SacI*-digested polymerase chain reaction (PCR) product. The primers for CD2 were as follows: forward, 5'-ATAAGCTTGGCGCCGCCACCATGGT-GAC-3', reverse, 5'-AGGAGCTCGGATCCATCTGCGA-CATCGACCTCTTCTGTCAGTTGGTCATGC-3'. The primers for CD3 were as follows: forward, 5'-ATAAGCTT-GCGGCCGCCACCATGGTGAC-3', reverse, 5'-AGGAG-CTCACCACCATCGACCTCATCTCTTCTGTCAGTTGG-TCATGC-3'. According to this protocol, the VDVAD and DEVD sequences were introduced into the N terminal of CFP, respectively. Then, DsRed2 was amplified from pDsRed2 (CLONTECH) using the following primers: forward, 5'-ACTAGAGCTCATGGCCTCCTCCGAGAACG-3', reverse, 5'-ACAGAAATCTACAGGAACAGGTGGTGG-3'. The amplified fragment was digested and ligated into CFP-VDVAD-YFP/PMD18-T using *SacI* and *EcoRI*, which replaced YFP with DsRed2. CFP-VDVAD-DsRed2 was also subcloned into pcDNA3 to generate pcDNA3-CFP-VDVAD-DsRed2, (CD2) which could express in the mammalian cells. PcDNA3-CFP-DEVD-DsRed2 (CD3) was generated by the same procedure.

2.2 Expression, Purification, and Cleavage of these Recombinant FRET Probes *in Vitro*

To identify the sensitivity and specificity of CD2 and CD3 to caspase activation, the recombinant FRET probes (CD2 and CD3) were purified for *in vitro* analysis. CFP-VDVAD-DsRed2 and CFP-DEVD-DsRed2 were, respectively, subcloned to a prokaryotic fusion protein expression vector pET-28b and expressed in BL21 (DE3) *Escherichia coli* bacteria; these bacteria were cultured in Luria-Bertani (LB) at 37 °C and expression induced by 1-mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 4 h at 30 °C when grown to $A_{600\text{ nm}}=1$. Cells were harvested by centrifugation at $5000 \times g$ for 10 min, washed in binding buffer (10-mM imidazole, 20-mM phosphate, 0.5-M NaCl), then resuspended in 20 ml lysis buffer, and, subsequently, were lysed by repeated cycles of freezing and thawing plus sonification. The recombinant protein was further purified by Ni-NTA resin (Qiagen).

Recombinant human active caspase-2 and caspase-3 were purchased from BioVision. One unit of recombinant human caspase was defined as the enzyme activity that cleaves 1 nmol of the individual caspase substrate (VDVAD-pNA for caspase-2, DEVD-pNA for caspase-3) per hour at 37 °C. Five micrograms of the purified recombinant protein were incubated with 1 unit of each caspase in 10 μ l of reaction buffer (50-mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol, and 10-mM dithiothreitol) at 37 °C for 1.5 h and then cleavage products of the FRET probes were analyzed by Western blot and spectroscopic measurements.

2.3 Cell Culture and Gene Transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with penicillin G sodium (100 units/ml), streptomycin sulfate (100 μ g/ml), and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Transfection of HeLa cells was performed using the GeneJammer® transfection reagent (Stratagene). Transfected cells were selected by exposure to Geneticin (800 μ g/ml, Sigma), and stable cell lines were cloned. The properties of CD2 and CD3 were analyzed using CD2 or CD3 expressing HeLa cells by Western blot and spectroscopic measurements.

2.4 Western Blotting

Purified fusion proteins were prepared for *in vitro* caspase assay as follows: the polyhistidine-tagged FRET sensor protein was expressed in bacteria and purified using a Ni-NTA affinity column. Then, the purified FRET probes CD2 or CD3 were incubated with caspase-2 or caspase-3 for 60 min at 37 °C. The samples were incubated for 10 min at 95 °C and then loaded for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Whole cell extracts were prepared as follows: HeLa cells that stably expressed CD2 or CD3 were collected at 800 rpm for 8 min and washed with phosphate-buffered saline (PBS), and then the cell pellet was resuspended in lysis buffer [62.5-mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 1-mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, and 5 μ g/ml aprotinin]. The cell lysates were immediately incubated for 10 min at 95 °C, the cell debris was pelleted, and the samples were loaded for SDS-PAGE.

Proteins were separated at 120 V for 1.5 h in SDS-PAGE (12%) and subsequently transferred onto a polyvinylidene difluoride membrane (PVDF, Amersham). Following blotting, the membranes were probed with anti-GFP antibody (1:3000) (Clontech) in triethanolamine-buffered saline (TBS) with 0.5% Tween 20. The immunoblots were then probed with horseradish-peroxidase-conjugated secondary goat anti-rabbit IgG (immunoglobulin G) antibody (1:5000) (Bio-Rad), and the bands were detected using the ECL Western-blotting analysis system (Amersham).

2.5 Spectroscopic Measurements

The purified recombinant proteins (CD2 and CD3) were transferred into a quartz cuvette (total volume 1.5 ml), and then incubated respectively with a 3-unit recombinant human active caspase-2 or caspase-3 at 37 °C in the sample holder of

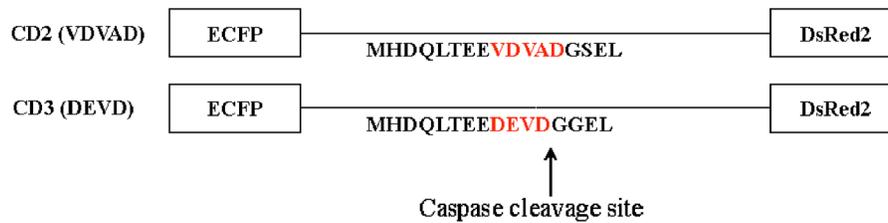


Fig. 1 Schematic representation of the biosynthetic FRET probes. The linker of CD2 is 17-amino-acid peptide MHDQLTTEEVDVADGSEL, which contains a caspase-2 recognition site sequence. It is different from CD3 (a 16-amino-acid peptide MHDQLTTEEDEVDGGEL with a linker containing a caspase-2 recognition site sequence). (The cleavage site is indicated by an underline.)

the spectrofluorometer (LS-50B, Perkin-Elmer, Norwalk, Connecticut). Fluorescence emission spectra from 450 to 600 nm were recorded with an excitation wavelength at 433 nm.

The CD2 or CD3 expressing HeLa cells were each divided into two groups. One of the groups was treated by cisplatin for 8 h and the other was not. The cells were harvested, respectively, washed twice with PBS, resuspended in PBS (total volume 1 ml), and then transferred into a quartz cuvette, which was placed inside a sample holder of the spectrofluorometer (Hitachi RF4500). The fluorescent emission spectrum was obtained by performing a spectrum scanning analysis with an excitation wavelength of 433 nm.

2.6 Imaging Analysis System for FRET Measurements

FRET measurements were performed as described previously^{17,22} on an inverted fluorescent microscope (Olympus IX70, Japan). FRET filter cubes for CFP/DsRed2 were as follows: CFP (425-445HQ, DM450, 460-510HQ, Olympus); DsRed (BP510-550, DM570, BA590, Olympus); FRET (436/20X, 455DCLP, 620/60M, Omega Optical).

Images were captured using a cooled CCD camera (Micro-max 5 MHz, Roper Scientific). Photographs were taken with Winview32 software (Roper Scientific). The ratio of FRET (R_{FRET}) was calculated by Matlab7.0 software as follows:²³

$$R_{\text{FRET}} = \frac{I_{\text{FRET}} - I_{\text{DsRed}} \times a - I_{\text{CFP}} \times b}{(I_{\text{DsRed}} \times I_{\text{CFP}})^{1/2}},$$

where I_{FRET} , I_{DsRed} , and I_{CFP} are intensities in each region of interest (ROI) under FRET, DsRed, and CFP filter sets; a is a norm of the percentage of DsRed bleed-through; and b is a norm of the percentage of CFP bleed-through under the FRET filter set. In our current imaging system, the values of a ranged from 2.25 to 8.30%, and the averaged value was 4.74% ($n=6$). The values of b ranged from 2.54 to 5.09% and the averaged value was 3.70% ($n=14$).

3 Results

3.1 Characterization of the FRET Probes (CD2 and CD3) for Activated Caspases

We generated two FRET probes by fusing a CFP and a DsRed2 protein with a specialized 17-amino-acid (CD2) or 16-amino-acid (CD3) linker. The linkers each contain the optimum caspase-2 cleavage sequence^{16,22} (VDVAD) and caspase-3 cleavage sequence^{9,11,13} (DEVD), respectively (Fig. 1). Before caspases were activated, FRET from CFP to

DsRed2 occurred because the two fluorescent proteins were covalently linked together. After caspases activation, cleavage of the linker peptide abolished the FRET (Fig. 1). To determine the sensitivity and specificity of CD2 and CD3 to activated caspases, we first examined these FRET probes for activated caspases *in vitro*. The recombinant FRET probes (CD2 and CD3) were expressed in BL21 (DE3) *E. coli* (Novagen), and purified by Ni-NTA resin (Qiagen). The changes of FRET efficiency in response to activated caspase cleavage were measured using a spectrofluorometer. The results are shown in Figs. 2(a) and 2(b). After adding 3 units of activated caspases (BioVision) into the solution of CD2 or CD3, we observed a significant decrease of emission at the 580-nm wavelength during each 10-min scanning interval. The emission spectra normalized at the donor emission peak, enabling the acceptor emission to stand for the FRET donor/acceptor ratio. The ratio value gradually decreased as time elapsed [Figs. 2(a) and 2(b)]. These results demonstrate that the CD2 and CD3 are sensitive to the activated caspase-2 and caspase-3, respectively. To further determine the caspase specificity of these probes, the CD2 or CD3 protein was incubated with either caspase-2 or caspase-3 for 1.5 h, and the products were analyzed by Western blot [Fig. 2(c)]. We observed that CD2 proteins were cleaved by caspase-2 after a 1.5-h incubation period [Fig. 2(c), lane 2], while CD3 proteins were not cleaved by caspase-2 under the same conditions [Fig. 2(c), lane 1]. Similarly, CD3 proteins can be cleaved by caspase-3 [Fig. 2(c), lane 4] but cannot be cleaved by caspase-2 [Fig. 2(c), lane 3]. These results indicate that the CD2 and CD3 are recognized uniquely by the activated caspase-2 and caspase-3, respectively.

3.2 Caspase Assay for CD2 and CD3 in Apoptotic HeLa Cell Extract

Cisplatin is one of the most effective and widely used anti-cancer drugs. Recent studies have indicated that caspase-2 (Ref. 6) and caspase-3 (Ref. 24) were activated in cisplatin-treated HeLa cells. To determine the sensitivity of CD2 and CD3 to caspases during apoptosis, the two FRET probes were each transfected into their respective HeLa cells. HeLa cells that stably expressed the recombinant FRET probes were then treated with cisplatin (10 $\mu\text{g}/\text{ml}$). Figures 3(a) and 3(b) display the emission spectra of cisplatin-treated and cisplatin-untreated HeLa cells, which were transfected with CD2 or CD3 and were excited at 433 nm. The cisplatin-untreated group peaked at 585 nm, but the cisplatin-treated groups did not peak at this level. To provide evidence that caspases

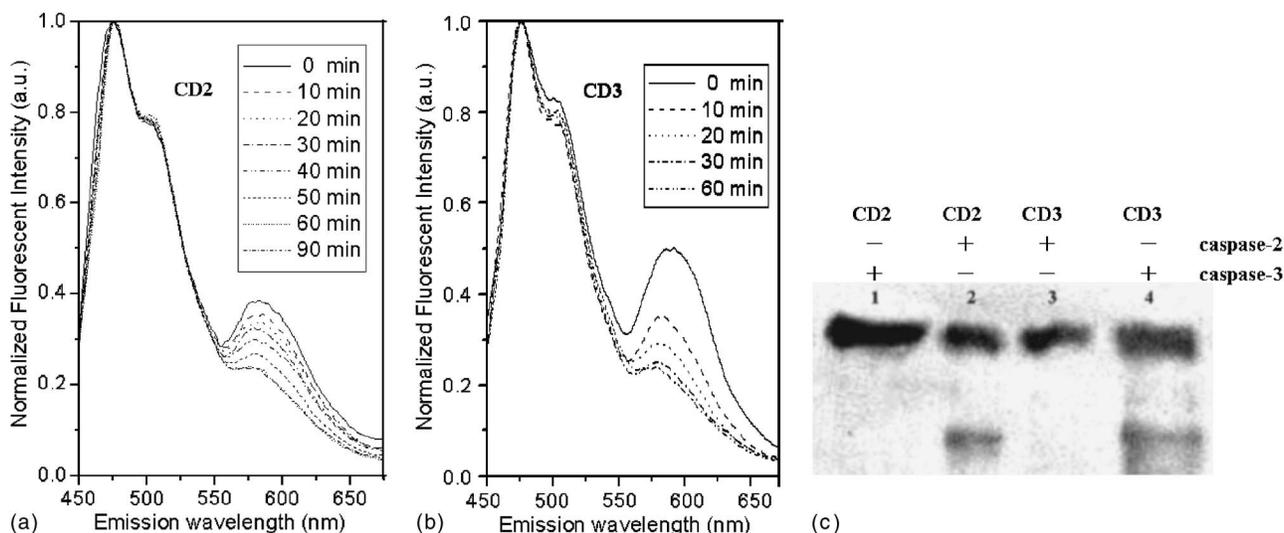


Fig. 2 Characterization of the FRET probes. (a) The emission spectra were measured using a spectrofluorometer (excitation wavelength =433 nm) while the recombinant CD2 protein was incubated with caspase-2 (final concentration=2 unit/ml) for 1.5 h. (b) The emission spectra were measured using a spectrofluorometer (excitation wavelength=433 nm) while the recombinant CD3 protein was incubated with caspase-3 (final concentration=2 unit/ml) for 1 h. (c) Western blot analysis of the recombinant CD2 and CD3 proteins exposed by activated caspases; the purified CD2 protein was incubated with caspase-3 (lane 1) and caspase-2 (lane 2) for 1.5 h, and the purified CD3 protein was incubated with caspase-2 (lane 3) and caspase-3 (lane 4) for 1.5 h (final concentration=2 unit/ml).

cleaved the FRET probe in living HeLa cells, we applied Western blot analysis of cellular extracts after 7 to 12 h of cisplatin treatment, showing that the CD3 was efficiently cleaved into two monomers, CFP and DsRed [Fig. 3(c), lane 2]. Similarly, the efficiency of cleavage could be detected in the cells transfected by CD2 [Fig. 3(d), lane 2]. In the control group of cisplatin-untreated HeLa cells, however, no cleavage was detected [Fig. 3(c), lane 1, and Fig. 3(d), lane 1].

3.3 FRET Imaging of the Dynamics of Caspase-2 and Caspase-3 Activation in Living HeLa Cells during Cisplatin-Induced Apoptosis

To monitor caspase-2 or caspase-3 activation in living cells, HeLa cells that stably expressed the CD2 or CD3 fusion proteins were kept in a 96-well plate for 24 h, and imaging analysis was started immediately after the cells were exposed

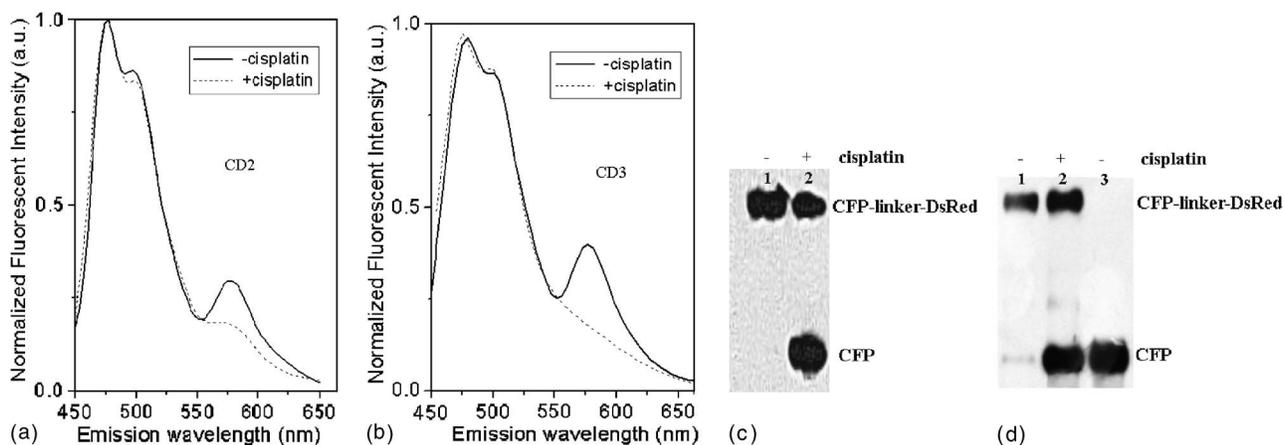


Fig. 3 Properties of CD2 and CD3 in cisplatin-induced apoptosis. (a) HeLa cells that stably expressed the CD2 fusion proteins were incubated with cisplatin (10 μ g/ml) for 7 to 12 h. The cell suspensions were then subjected to spectral analysis with excitation wavelength at 433 nm. (b) HeLa cells that stably expressed the CD3 fusion proteins were incubated with cisplatin (10 μ g/ml) for 7 to 12 h. The cell suspensions were then subjected to spectral analysis with excitation wavelength at 433 nm. (c) Cleavage assay of CD2 in lysates, prepared from cisplatin-treated HeLa cells which stably expressed CD2; its cleavage was then examined by Western blotting using an anti-GFP antibody. Lane 1, the lysates were prepared from cisplatin-treated HeLa cells. Lane 2, the lysates were prepared from cisplatin-untreated HeLa cells. Lane 3, the lysates were prepared from the CFP expressing HeLa cells. (d) Cleavage assay of CD3 in lysates, prepared from cisplatin-treated HeLa cells that stably expressed CD3; its cleavage was then examined by Western blotting using an anti-GFP antibody. Lane 1, the lysates were prepared from cisplatin-treated HeLa cells. Lane 2, the lysates were prepared from cisplatin-untreated HeLa cells.

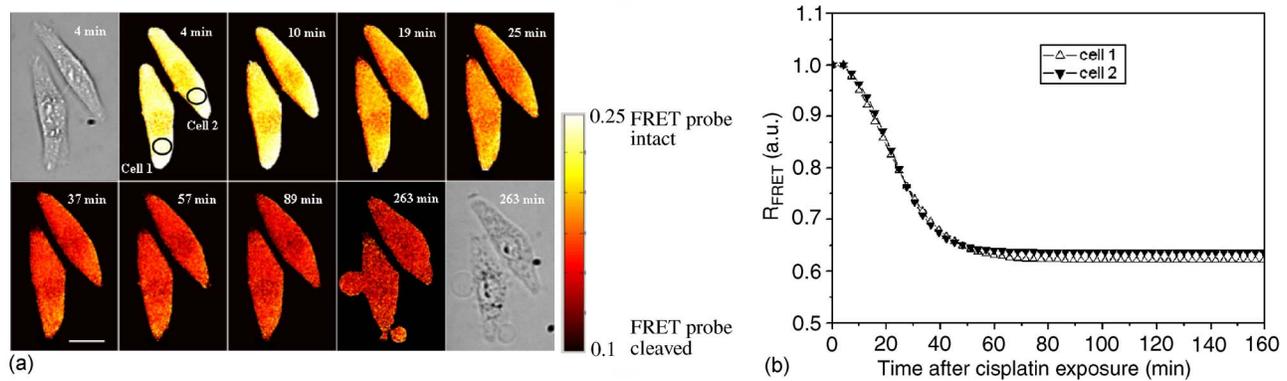


Fig. 4 Single-cell imaging FRET changes of CD2 in response to caspase activation during cisplatin-induced apoptosis in living HeLa cells. (a) Ratio of FRET images of the CD2 expressing HeLa cells. HeLa cells that stably expressed the CD2 fusion proteins were cultured in a 96-well plate for 24 h; imaging analysis was started immediately after exposure to cisplatin (30 $\mu\text{g}/\text{ml}$). Scale bar, 20 μm . (b) Dynamics of activation of caspase-2 in individual HeLa cells during cisplatin-induced apoptosis as examined in (a). The values of R_{FRET} are from the ROI, which is indicated by a circle.

to cisplatin (30 $\mu\text{g}/\text{ml}$). Using a three-filter imaging system, the time series of the ratio of FRET images of CD2 and CD3 expressing HeLa cells are shown in Figs. 4(a) and 5(a), respectively. The FRET efficiency of CD2 and CD3 was significantly decreased in response to the cleavage of the probe. The values of R_{FRET} were obtained from the ROI in the different cells and were plotted as a function of time [Figs. 4(b) and 5(b)]. From these figures, we can observe that the activation of caspase-2 and caspase-3 was completed before the cell changed morphologically (such as membrane blebbing and cell shrinkage). Furthermore, once the activation of caspase-3 initiated in the cytosol, the activation process completed rapidly in 10 min [Fig. 5(b)]. However, in comparison to the dynamics of caspase-3 activation, caspase-2 activation occurs very slowly but ends earlier [Fig. 4(b)].

4 Discussion

Caspase-2 appears to be necessary for the onset of apoptosis triggered by DNA damage.²¹ Cisplatin is an anticancer drug of DNA damage and is widely used in clinical therapy. Therefore, we selected cisplatin to induce apoptosis for this study of the activation of caspase-2. For comparison with the activa-

tion of caspase-2, we constructed another FRET probe specifically sensitive to caspase-3 by inserting a tandem substrate site (DEVD) into the donor/acceptor pair.

An *in vitro* caspase assay demonstrated that the CD2 and CD3 are sensitive specifically to the activated caspase-2 and caspase-3, respectively. However, we observed that the purified recombinant CD2 and CD3 were not cleaved completely by activated caspase-2 and caspase-3 *in vitro* (Fig. 2). This incomplete cleavage may be caused by the decrease in activity of recombinant human active caspases at 37 $^{\circ}\text{C}$, and the subsequent decrease in the progress of the reaction, because the caspases used were only stable for 1 yr at -70°C (Bio-Vision). The FRET efficiency is highly sensitive to the relative orientation and distance between the two fluorophores.²⁵ Spectral analysis showed that CD3 has higher ratio values of 580/475 nm than does CD2 both *in vitro* [Figs. 2(a) and 2(b)] and in suspensions of cells [Figs. 3(a) and 3(b)]. These higher ratios were due to the fact that the linker of CD3 is composed of 16 amino acids, shorter than that of CD2 (17 amino acids). The spectroscopic properties of the CD2 or CD3 stably expressing HeLa cells were similar [Figs. 3(a) and 3(b)]. In the control group, an emission peak of DsRed2 at 580 nm was observed when CD2 or CD3 was excited at 430 nm. How-

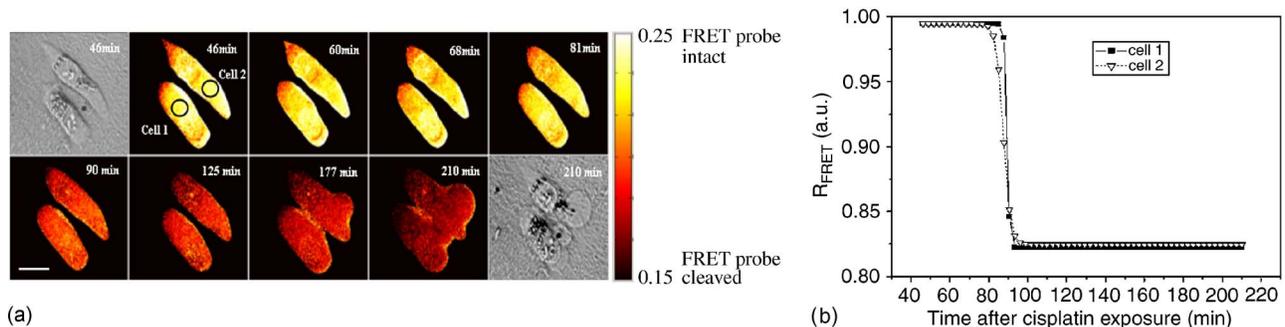


Fig. 5 Single-cell imaging FRET changes of CD3 in response to caspase activation during cisplatin-induced apoptosis in living HeLa cells. (a) Ratio of FRET images of the CD3 expressing cells; HeLa cells that stably expressed the CD3 fusion proteins were cultured in a 96-well plate for 24 h; imaging analysis was started 40 min after exposure to cisplatin (30 $\mu\text{g}/\text{ml}$). Scale bar, 20 μm . (b) Dynamics of activation of caspase-3 in individual HeLa cells during cisplatin-induced apoptosis as examined in (a). The values of R_{FRET} are from the ROI, which is indicated by a circle.

ever, this emission peak of DsRed2 at 580 nm disappeared in the cisplatin-treated group. These results demonstrate that the caspase-2 or caspase-3 activity could be indicated by the change in FRET efficiency of CD2 [Fig. 3(a)] or CD3 [Fig. 3(b)]. However, in the cisplatin-treated group, we observed that the cleaved efficiency of CD2 did not reach the efficiency level of CD3. The *in vitro* caspase assay demonstrated the same tendency. The results show that about 90% of CD3 protein was cleaved by caspase-3 after a 30-min incubation period [Fig. 2(b)], while only 30% of CD2 protein was cleaved under the same conditions [Fig. 2(a)]. This finding implies that the activation property of initiator caspase (caspase-2) differs from that of the effector caspase (caspase-3).

For further determination of the specific properties of caspase-2, CD2 was applied to detect caspase-2 activity in single living cells. To detect the changes of the FRET efficiency of CD2, a temporal order of ratio images was calculated by the formula for R_{FRET} , as shown in Fig. 4(a). The values of R_{FRET} from the ROI can be plotted as a time-dependent function [Fig. 4(b)]. Therefore, we can quantitatively analyze the dynamics of caspase-2 activation in a single living cell. These results show that the caspase-2 activation profile was significantly different from that of caspase-3. During cisplatin-induced apoptosis, the activation of caspase-2 happened gradually, taking at least 30 min ($n=11$), while the activation of caspase-3 rapidly reached its maximum within a short period because ratio changes were completed in less than 10 min [Figs. 5(a) and 5(b)]. We also observed that the activation of caspase-2 and caspase-3 adhered to a strictly temporal order. This result demonstrates that, in the mitochondrial apoptotic pathway, caspase-2 activation occurs upstream of caspase-3 in cisplatin-induced apoptosis.

The FRET probes constructed for this study are not only powerful tools for studying the caspase-2 mechanisms in apoptotic cells, they are also favorable for use in high-throughput drug screens due to their nontoxicity and insensitivity to changes in environment.

Acknowledgments

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References

1. R. W. Johnstone, S. W. Lowe, and A. A. Ruefli, "Apoptosis: a link between cancer genetics and chemotherapy," *Cell* **108**, 153–164 (2002).
2. J. Neefjes and N. P. Dantuma, "Fluorescent probes for proteolysis: tools for drug discovery," *Nat. Rev. Drug Discovery* **3**, 58–69 (2004).
3. S. W. Fesik and Y. Shi, "Controlling the caspases," *Science* **294**, 1477–1478 (2001).
4. S. Kumar and D. L. Vaux, "A Cinderella caspase takes center stage," *Science* **297**, 1290–1291 (2002).
5. L. Wang, M. Miura, L. Bergeron, H. Zhu, and J. Yuan Ich-1, "An ICE/ced-3-related gene, encodes both positive and negative regula-

6. P. Lassus, X. Opitz-Araya, and Y. Lazebnik, "Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization," *Science* **297**, 1352–1354 (2002).
7. Y. Guo, S. M. Srinivasula, A. Druihe, T. Fernandes-Alnemri, and E. S. Alnemri, "Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria," *J. Biol. Chem.* **277**(16), 13430–13437 (2002).
8. N. A. Thornberry and Y. Lazebnik, "Caspases: enemies within," *Science* **281**, 1312–1316 (1998).
9. K. Takemoto, T. Nagai, A. Miyawaki, and M. Miura, "Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects," *J. Cell Biol.* **160**, 235–243 (2003).
10. X. Xu, A. L. Gerard, B. C. Huang, D. C. Anderson, D. G. Payan, and Y. Luo, "Detection of programmed cell death using fluorescence energy transfer," *Nucleic Acids Res.* **26**, 2034–2035 (1998).
11. L. Tyas, V. A. Brophy, A. Pope, A. J. Rivett, and J. M. Tavare, "Rapid caspase-3 activation during apoptosis revealed using fluorescence resonance energy transfer," *EMBO Rep.* **1**, 266–270 (2000).
12. K. Q. Luo, V. C. Yu, Y. Pu, and D. C. Chang, "Application of the fluorescence resonance energy transfer method for studying the dynamics of caspase-3 activation during UV-induced apoptosis in living HeLa cells," *Biochem. Biophys. Res. Commun.* **283**, 1054–1060 (2001).
13. M. Rehm, H. Dussmann, R. U. Janicke, J. M. Tavare, D. Kogel, and J. H. Prehn, "Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process," *J. Biol. Chem.* **277**, 24506–24514 (2002).
14. R. Onuki, A. Nagasaki, H. Kawasaki, T. Baba, T. Q. Uyeda, and K. Taira, "Confirmation by FRET in individual living cells of the absence of significant amyloid beta-mediated caspase 8 activation," *Proc. Natl. Acad. Sci. U.S.A.* **99**, 14716–14721 (2002).
15. K. Q. Luo, V. C. Yu, Y. Pu, and D. C. Chang, "Measuring dynamics of caspase-8 activation in a single living HeLa cell during TNF-induced apoptosis," *Biochem. Biophys. Res. Commun.* **304**, 217–222 (2003).
16. J. D. Robertson, M. Enoksson, M. Suomela, B. Zhivotovsky, and S. Orrenius, "Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity," *EMBO Rep.* **5**, 643–648 (2002).
17. M. G. Erickson, D. L. Moon, and D. T. Yue, "DsRed as a potential FRET partner with CFP and GFP," *Biophys. J.* **85**, 599–611 (2003).
18. H. Kawai, T. Suzuki, T. Kobayashi, H. Sakurai, H. Ohata, K. Honda, K. Momose, I. Namekata, H. Tanaka, K. Shigenobu, R. Nakamura, T. Hayakawa, and T. Kawanishi, "Simultaneous real-time detection of initiator—and effector—caspase activation by double fluorescence resonance energy transfer analysis," *J. Pharmacol. Sci.* **97**(3), 361–368 (2005).
19. E. A. Jares-Erijman and T. M. Jovin, "FRET imaging," *Nat. Biotechnol.* **21**, 1387–1394 (2003).
20. T. Chen, S. H. Zeng, Q. Luo, Z. H. Zhang, and W. Zhou, "High-order photobleaching of green fluorescent protein inside live cells in two-photon excitation microscopy," *Biochem. Biophys. Res. Commun.* **291**, 1272–1275 (2002).
21. B. Zhivotovsky and S. Orrenius, "Caspase-2 function in response to DNA damage," *Biochem. Biophys. Res. Commun.* **331**, 859–867 (2005).
22. B. B. Wolf and D. R. Green, "Suicidal tendencies: apoptotic cell death by caspase family proteinases," *J. Biol. Chem.* **274**, 20049–20052 (1999).
23. Z. Xia and Y. Liu, "Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes," *Biophys. J.* **81**, 2395–2402 (2001).
24. M. Horky, G. Wurzer, V. Kotala, M. Anton, B. Vojtesek, J. Vacha, and J. Wesierska-Gadek, "Segregation of nucleolar components coincides with caspase-3 activation in cisplatin-treated HeLa cells," *J. Cell. Sci.* **114**, 663–670 (2000).
25. K. Takemoto, T. Nagai, and A. Miyawaki, "A high-throughput method for development of FRET-based indicators for proteolysis," *Biochem. Biophys. Res. Commun.* **319**, 72–77 (2004).