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Detection of programmed cell death using fluorescence energy transfer

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ABSTRACT

Fluorescence energy transfer (FRET) can be generated when green fluorescent protein (GFP) and blue fluorescent protein (BFP) are covalently linked together by a short peptide. Cleavage of this linkage by protease completely eliminates FRET effect. Caspase-3 (CPP32) is an important cellular protease activated during programmed cell death. An 18 amino acid peptide containing CPP32 recognition sequence, DEVD, was used to link GFP and BFP together. CPP32 activation can be monitored by FRET assay during the apoptosis process.

Green fluorescent protein (GFP) has been used as a highly sensitive reporter to monitor gene expression and transfer in cells (1). When two differently colored mutants of GFP, such as EGFP (enhanced green fluorescence protein) and EBFP (enhanced blue fluorescence protein), are covalently linked together within a few manometers distance, fluorescence resonance energy transfer (FRET) can be detected (2,3). Such transfer is characterized by a reduction of fluorescence intensity of the donor fluorophore (EBFP) and re-emission of fluorescence at acceptor fluorophore (EGFP) wavelengths. Disruption of the covalent linkage between two fluorophores, such as EGFP and EBFP, effectively eliminate the FRET effect. This disruption can be caused by specific protease cleavage of a peptide linking EGFP to EBFP.

Activation of intracellular proteases such as caspase-3 (CPP32) is an important event in programmed cell death. It has been demonstrated that tumor necrosis factor (TNF), Fas ligand and chemotherapeutic drugs, are able to induce apoptosis by activating CPP32 (4–7). Substrates of CPP32 share a consensus recognition and cleavage amino acid sequence: DEVD (4). Detection of CPP32 activation in live cells will greatly facilitate monitoring of the apoptosis process in live cells. To design a convenient assay of CPP32 protease activation, we constructed a hybrid protein EGFP–EBFP, linked together by an 18 amino acid region containing a consensus DEVD sequence (Fig. 1A). The BFP mutant (EBFP) has a maximum excitation wavelength at 380 nm and emits maximally at 440 nm. The GFP mutant (EGFP) has a maximum excitation wavelength at 488 nm and maximum emission at 511 nm. GSGS sequences were used to flank the DEVD sequence to make this region soluble and accessible to cleavage by CPP32.

The EGFP–EBFP hybrid protein expression vector (pGDB) was constructed by inserting EGFP, DEVD linker, and EBFP into the pCI-neo vector (Promega). The protein kinase Rip has previously been shown to be involved in TNF-induced apoptosis pathway (8,9). Over-expression of Rip alone induces apoptosis. The human Rip gene was cloned by PCR amplification from a cDNA library and inserted into the pCR3.1 (Invitrogen) expression vector to construct pCMV-Rip. To detect Rip induced apoptosis, pGDB was transiently co-transfected into 293 cells with pCMV-Rip. After transfection (24–36 h), cells co-transfected by pCMV-Rip showed characteristic apoptotic morphology, being round and condensed (Fig. 1C). Adherent and non-adherent cells were then harvested for both western analysis of CPP32 protease cleavage of the hybrid protein EGFP–EBFP and for monitoring of CPP32 activation by FACS analysis of the specific FRET effect.

In cells co-transfected with both pGDB and pCMV-Rip, the hybrid protein EGFP–EBFP was efficiently cleaved into two monomers, EGFP and EBFP, of equal molecular size (Fig. 1B, lane 2). In cells transfected by pGDB alone, no cleavage was detected (Fig. 1B, lane 1). In addition, the efficiency of cleavage corresponded directly to the amount of pCMV-Rip used in co-transfection (data not shown). This experiment demonstrated that the DEVD linker sequence inserted between EGFP and EBFP was recognized efficiently by CPP32 protease activated during cellular apoptosis. To show further that TNF activation of CPP32 protease could be detected by this assay, we transfected pGDB into HeLa cells. Cells were treated by TNF and cycloheximide 24 h after transfection. Cells were then harvested 16 h after TNF/cycloheximide treatment for western analysis. Our results showed that in the presence of TNF and cycloheximide, EGFP–EBFP hybrid protein was efficiently cleaved in floating cells (apoptotic cells) (Fig. 1B, lane 4) but not in attached cells (non-apoptotic cells) (Fig. 1B, lane 5). Addition of the CPP32 inhibitor Z-VAD (Alexis Biochemicals) into the culture medium completely inhibited the cleavage of the EGFP–EBFP hybrid protein (Fig. 1B, lane 6). This result further shows that activated CPP32 recognized and cleaved the DEVD linker region between EGFP and EBFP.

To demonstrate the disruption of fluorescence energy transfer between EBFP and EGFP due to activation of CPP32 during

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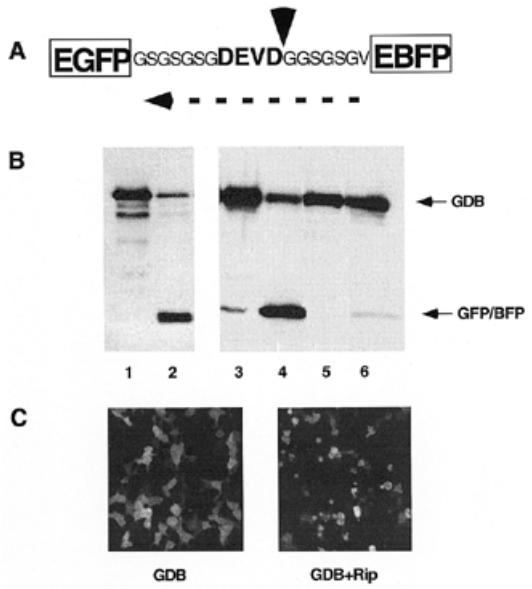

Figure 1. Detection of CPP32 protease activation. (**A**) Structure of EGFP–EBFP hybrid protein. The linker sequence is shown with DEVD highlighted. Dashed arrow indicates the FRET direction. Arrow above DEVD points to CPP32 protease cleavage site. EBFP (F64L, S65T, Y66H) was amplified by PCR from pEBFP (Clontech) and ligated into the *Sal*I–*Not*I sites of pCI-neo (Promega) to construct pCI-EBFP. EGFP (F64L, S65T) was then amplified by PCR from pEGFP-N1 (Clontech) and ligated into the *Nhe*I–*Sal*I gap of pCI-EBFP to construct the pGDB. Oligo used for EGFP PCR amplification also contained the DEVD linker sequence highlighted. (**B**) Western analysis of CPP32 cleavage. Lanes 1 and 2 contain results from 293 cells. Lane 1, pGDB only; lane 2, pGDB plus pCMV-Rip. Lanes 3–6 contain results from pGDB transfection of HeLa cells. Lane 3, no TNF or cycloheximide added; lane 4, TNF plus cycloheximide, floating cells; lane 5, TNF plus cycloheximide attached cells; lane 6, TNF plus cycloheximide plus Z-VAD. TNF concentration is 10 ng/ml. Cycloheximide concentration if 10 μg/ml. Z-VAD concentration is 20 μM. (**C**) Picture of Rip induced apoptosis. Fluorescence microscope with excitation wavelength between 420 and 490 nm was used. Only EGFP emission at 520 nm was monitored.

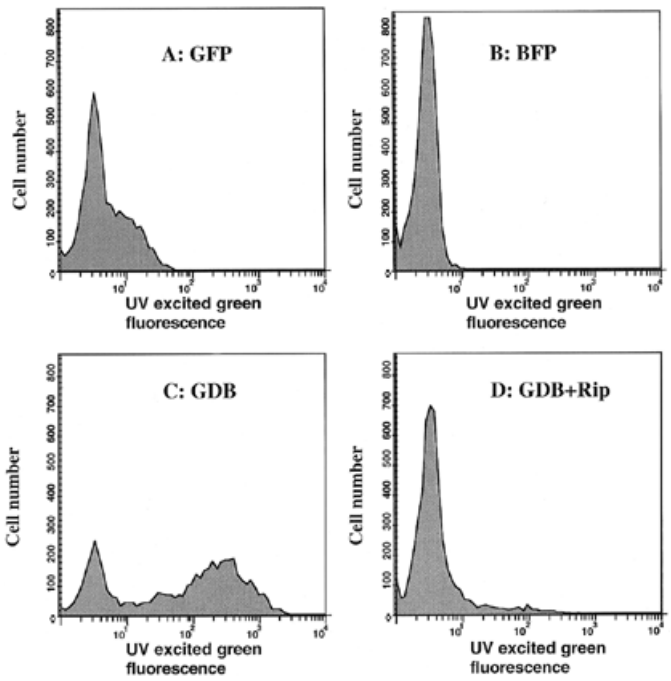

Figure 2. FACS analysis of FRET effect. 293 cells were harvested 24–36 h after transfection. Analyses were performed on a MOFLO cytometer (Cytomation, Fort Collins, CO), equipped with two argon-ion lasers. Laser 1, used for triggering and scatter detection, 488 nm excitation line (ILT 5500A, Salt Lake City, UT); laser 2, used for fluorescence detection in two channels using 450/65 and 530/40 bandpass filters, 351 excitation line (Innova 300C, Coherent, Santa Clara, CA). Only 530/40 detection channel data is shown. (**A**) Cells expressing EGFP only. (**B**) Cells expressing EBFP only. (**C**) Cells transfected by pGDB. (**D**) Cells co-transfected by pGDB and pCMV-Rip.

apoptosis, we performed FACS analysis on cells co-transfected by pGDB and pCMV-Rip. Expression vectors of EGFP or EBFP alone were used as control in these experiments. Results are shown in Figure 2. When excited by UV light at 351 nm wavelength, cells expressing EBFP alone showed characteristic emission at 440 nm. Cells expressing EGFP alone also showed characteristic emission at 510 nm when excited at 488 nm wavelength (data not shown). No GFP emission was detected at 510 nm when cells expressed EGFP or EBFP alone were excited by UV light at 351 nm wavelength (Fig. 2A and B). When cells transfected by pGDB alone were excited at 351 nm wavelength, strong GFP emission was detected at 510 nm (Fig. 2C). This result demonstrated that our 18 amino acid linker region containing DEVD is sufficient for fluorescence energy transfer between EBFP and EGFP in the hybrid protein. Co-transfection of pCMV-Rip effectively decreased the FRET effect (Fig. 2D), which is consistent with our previous observation that Rip was able to activate CPP32 to cleave the hybrid protein EGFP-EBFP at the DEVD linker site (Fig. 1B).

A major advantage of using a FRET assay to monitor programmed cell death is that no cell staining is needed. Live cells can be monitored continuously during the course of apoptosis. Since different classes of caspases are activated by different apoptosis signals (4,7), the FRET assay can also be used to monitor the activation of specific apoptosis pathways by changing the protease recognition site in the linker region. It is also plausible that other protease cleavage sites, such as ICE recognition sequence YVAD, can be used to replace DEVD so that one can monitor the ICE protease activity in cells. Availability of a different colored mutant GFP, such as yellow fluorescent protein (YFP), will provide more flexibility in generating a variety of constructs. For example, co-transfection of both EBFP–DEVD–EGFP and EBFP–YVAD–EYFP constructs into the same cell will allow monitoring of two different kinds of protease activities simultaneously. Use of a high-speed cell sorter renders this assay suitable for high throughput screening of apoptosis-inducing peptide/compounds.

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