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 nanometers 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 (EFM) 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 chemotherapeutic drugs, are able to induce apoptosis by activating CPP32 (4–7). Substrates of CPP32 share a consensus consensus 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
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.

REFERENCES