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Live cell detection of caspase-3 activation by a Discosoma-red-fluorescent-protein-based fluorescence resonance energy transfer construct

Lucy M. Elphick [a], Annika Meinander [b,c], Andrey Mikhailov [b], Morgiane Richard [a], Nick J. Toms [d], John E. Eriksson [b,e], George E.N. Kass [a,*]

[a] School of Biomedical and Molecular Sciences, University of Surrey, Guildford GU2 7XH, UK
[b] Turku Centre for Biotechnology, P.O.B. 123, FIN-20521 Turku, Finland
[c] Department of Biochemistry and Pharmacy, Åbo Akademi University, FIN-20521 Turku, Finland
[d] Peninsula Medical School, St. Luke's Campus, Heavitree Road, Exeter EX1 2LU, UK
[e] Department of Biology, University of Turku, FIN-20014 Turku, Finland

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Abstract

A probe consisting of Discosoma red fluorescent protein (DsRed) and enhanced yellow fluorescent protein (EYFP) linked by a 19-amino-acid chain containing the caspase-3 cleavage site Asp-Glu-Val-Asp was developed to monitor caspase-3 activation in living cells. The expression of the tandem construct in mammalian cells yielded a strong red fluorescence when excited with 450- to 490-nm light or with a 488-nm argon ion laser line as a result of fluorescence resonance energy transfer (FRET) from donor EYFP to acceptor DsRed. The advantage over previous constructs using cyan fluorescent protein is that our construct can be used when excitation wavelengths lower than 488 nm are not available. To validate the construct, murine HT-22 hippocampal neuronal cells were triggered to undergo CD95-induced neuronal death. An increase in caspase-3 activity was demonstrated by a reduction of FRET in cells transfected with the construct. This was manifested by a dequenching of EYFP fluorescence leading to an increase in EYFP emission and a corresponding decrease in DsRed fluorescence, which correlated with an increase in pro-caspase-3 processing. We conclude that CD95-induced caspase-3 activation in HT-22 cells was readily detected at the single-cell level using the DsRed–EYFP-based FRET construct, making this a useful technology to monitor caspase-3 activity in living cells.

Keywords: Green fluorescent protein; FRET; Apoptosis; CD95; Ischemia; Cytochrome *c*; Caspase

During central nervous system ischemia, glutamate receptor overactivation (excitotoxicity) is established to play a key role in inducing neuronal injury [1]. However, emerging data suggest that the CD95 death receptor (also known as Fas, APO-1) may also play an important role in inducing delayed neuronal death [2–6]. The CD95 death receptor is reported to be expressed in many tissues, including the brain [7,8]. Its natural ligand, CD95L, activates the CD95 receptor that is directly linked to caspase-8 and caspase-10 via the Fas-associated death domain. Apoptosis may then proceed via caspase-8-mediated activation of caspase-3 which can be amplified by induction of a mitochondrial pathway involving the caspase-8-catalyzed truncation of Bid, followed by the release of cytochrome *c* and other apoptogenic proteins from mitochondria [9,10].

Cellular and tissue caspase activity can be readily quantitated ex situ by incubating a cell or tissue extract with a

* Corresponding author. Fax: +44 1483 300374.
E-mail address: g.kass@surrey.ac.uk (G.E.N. Kass).

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tetrapeptide substrate linked to a cleavable fluorescent tag, such as Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarine (DEVD–AFC)[1] [11]. The disadvantage of this approach is that a large number of cells are required to generate the cell extract and the acquired data relate only to cell populations. Cell-permeable caspase-specific substrates can be used instead to generate information from living cells at the single-cell level but their peptide-based nature is restricting their cell permeability and requires prolonged incubation periods. More recently, efforts have been made to design green fluorescent protein (GFP)-based tandem constructs where caspase activity can be detected in living cells at the single-cell level as a result of the caspase of interest cleaving a recognition substrate peptide introduced to link cyan fluorescent protein (CFP) with GFP or yellow fluorescent proteins (YFP) [12–17]. The resulting cleavage of the substrate linker peptide leads to the loss of fluorescence resonance energy transfer (FRET).

The excitation of CFP requires special excitation filter blocks when using conventional fluorescence microscopes and is only poorly achieved by the 457-nm blue line of argon ion lasers found in flow cytometers and confocal laser microscopes since it lies outside the 430- to 440-nm excitation maxima of the fluorescent protein. Therefore, we modified here the tandem construct approach to generate an optimized EYFP–DsRed fluorescent protein pair containing the caspase-3 cleavage site Asp-Glu-Val-Asp. The expression of this construct in the murine hippocampal HT-22 neuronal cell line (derived from HT-4 hippocampal neuronal cell line [18]) enabled us to follow in situ caspase-3 activation by FRET in response to CD95-induced apoptosis.

Materials and methods

Materials

Cycloheximide (CHX), Triton X-100, DMSO, Hoechst 33258, and saponin were purchased from Sigma–Aldrich Ltd. (Poole, UK). Digitonin was obtained from VWR Int. (Poole, UK). FuGENE transfection reagent and bovine serum albumin (BSA) were obtained from Roche (Lewes, UK). Monoclonal anti-CD95 antibody (Jo2), anti-cytochrome *c* antibody (clone 6H2.B4), and active caspase-3 detection kits were purchased from BD Pharmingen (Oxford, UK). Anti-Bax antibodies (B-9 and N-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-cytochrome *c* oxidase (subunit IV) antibody was obtained from Molecular Probes (20E8-C12) (Eugene, OR, USA). Secondary HRP- and FITC-conjugated polyclonal antibodies were obtained from DAKO Ltd. (Cambridge, UK).

Cell culture and apoptosis assays

HT-22 cells were maintained (humidified 5% CO_2 atmosphere, 37 °C) in DMEM containing 10% FBS, 2 mM L-glutamine, and 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin solution and passaged every 3–4 days. For viability assays, 8×10^3 cells were seeded into 24-well plates and incubated overnight prior to treatment. Following media renewal, cells were treated in DMEM containing 2% FBS with 200 ng ml^{-1} anti-CD95 monoclonal antibody (Jo2), plus 1 μg ml^{-1} CHX where appropriate. Aliquots of the media were then removed at the indicated time points and assayed for lactate dehydrogenase (LDH) activity according to the manufacturer's instructions (Roche). The release of LDH from cells into the culture medium was used as an indicator of cell death and was expressed as percentage of the total (Triton X-100-releasable) LDH from the culture. Morphological evidence for chromatin condensation was obtained using the fluorescent chromatin dye Hoechst 33258 (2μg ml^{-1}) as previously described [11].

Plasmid construction

A 19-amino-acid peptide chain encoding the caspase-3 cleavage site, GS(*Bsp*EI)GGSGSG**DEVD**NGSGSGS (*Bam* HI), was added in front of the start codon of pDsRed2-N1 (Clontech, CA, USA) by PCR cloning. The cDNA for the peptide sequence along with that of pDsRed2-N1 was then digested with *Bsp*EI and *Eco*RI and subcloned into the multiple cloning site of pEYFP-C1 (Clontech) to generate pFRET-casp3 (Fig. 1A).

Detection of caspase-3 activation by FRET microfluorimetry

HT-22 cells were transfected with pFRET-casp3 by using FuGENE 5 transfection reagent (Roche) according to manufacturer's instructions. Following 72 h incubation, the transfected cells were treated with the apoptosis-inducing drug staurosporine (STS, 1 μM) or vehicle control (dimethyl sulfoxide) for 18 h. After trypsinization, cells were washed and resuspended in PBS and transferred to a black 96-well plate (Costar) before FRET measurement using a Spectra Max Gemini XS microfluorometer (Molecular Devices) (excitation, 488 nm; emission 525–550 nm for EYFP and 570–595 nm for DsRed). The FRET ratios were obtained by dividing the integrated DsRed signal (λ_{em} 570–595 nm) by the integrated EYFP signal (λ_{em} 525–550 nm).

Detection of caspase-3 activation by FRET microscopy

In situ caspase-3 activity was analyzed using the pFRET-casp3 construct. Cells were transfected with pFRET-casp3 by using FuGENE 5 transfection reagent (Roche) according to manufacturer's instructions. Following 72 h incubation,

[1] *Abbreviations used:* FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; CFP, cyan fluorescent protein; DsRed, Discosoma red fluorescent protein; DEVD, Asp-Glu-Val-Asp; BSA, bovine serum albumin; CHX, cycloheximide; LDH, lactate dehydrogenase: HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; STS, staurosporine; PBS, phosphate-buffered saline.