Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis

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Background: The caspase-mediated proteolysis of many cellular proteins is a critical event during programmed cell death or apoptosis. It is important to determine which caspases are activated in mammalian cells, and where and when activation occurs, upon receipt of specific death stimuli. Such information would be useful in the design of strategies to regulate the activation of caspases during apoptosis.

Results: We developed two novel fluorescent substrates that were specifically cleaved by caspase-1 or caspase-3. For *in vitro* studies, four-amino-acid recognition sequences, YVAD for caspase-1 and DEVD for caspase-3, were introduced between blue fluorescent protein (BFP) and green fluorescent protein (GFP), expressed in bacteria and purified. For *in vivo* studies, YVAD and DEVD were introduced between cyan fluorescent protein and yellow fluorescent protein, and expression was monitored in live mammalian cells. The proximity between fluorophores was determined using fluorescence resonance energy transfer. Purified substrates were cleaved following exposure to purified caspase-1 and caspase-3. In Cos-7 cells, caspase-1 and caspase-3 substrates were cleaved upon induction of apoptosis with staurosporine, a protein-kinase inhibitor, whereas caspase-3 but not caspase-1 substrate was cleaved upon treatment of cells with the DNA-damaging agent mitomycin c.

Conclusions: These substrates allow the spatial activation of specific members of the caspase family to be deciphered during the initiation and execution phase of programmed cell death, and allow activation of specific caspases to be monitored both *in vivo* and *in vitro*. This technology is also likely to be useful for high-throughput screening of reagents that modulate caspase activity.

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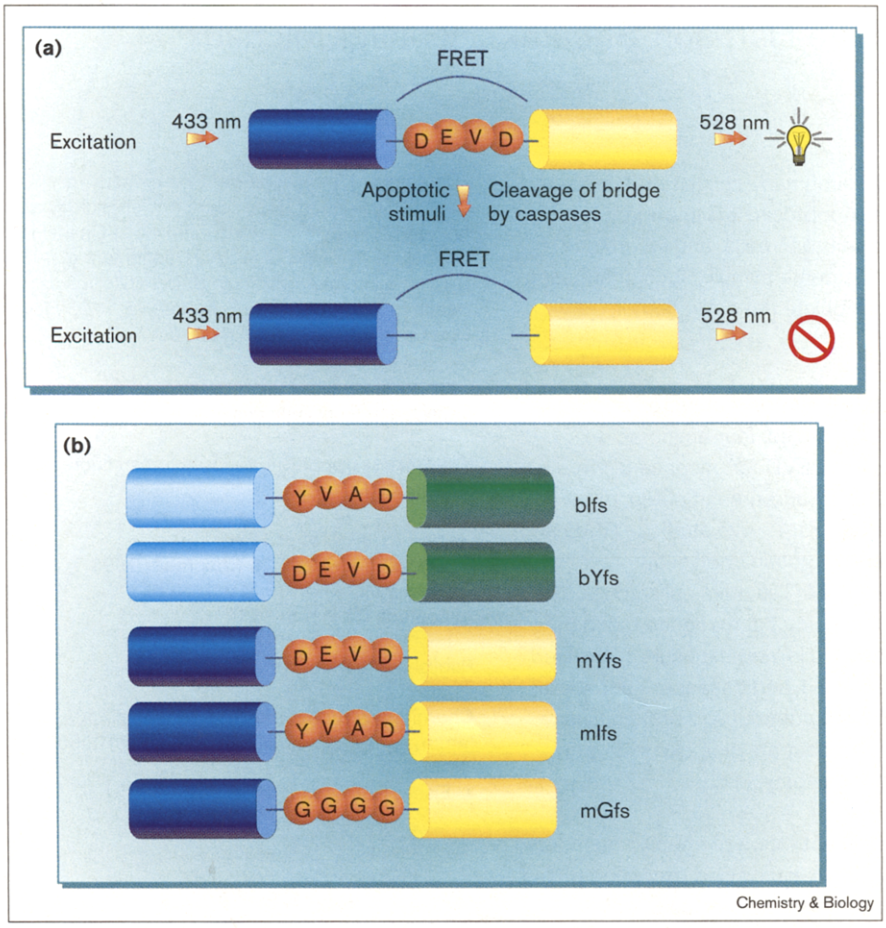
Introduction

Apoptosis or programmed cell death is a normal physiological process of cell suicide, which is of crucial importance for the development and maintenance of all multicellular organisms. It can be initiated by many different stimuli such as withdrawal of growth factors, DNA damage and binding of certain ligands to cell-surface receptors. Mutation studies carried out in the nematode *Caenorhabditis elegans* led to the identification of the *ced-3* gene as essential for programmed cell death [1]. Later, the protein encoded by *ced-3* was identified to be a mammalian homolog of interleukin-1β-converting enzyme (ICE) [2], which has been shown to be a member of a family of cysteine proteases termed caspases (for cysteinyl aspartate-specific proteinase) [3]. ICE (also called caspase-1) and other members of this family share an absolute requirement for aspartic acid in the P1 position of their substrates [4]. Caspase-1 is expressed as an inactive 45 kDa precursor in resting cells, but during apoptosis it is cleaved to form two subunits of 10 and 20 kDa that together constitute active protease [5]. The catalytically active form of caspase-1 is a tetramer consisting of a $(p20–p10)_2$ homodimer that cleaves the 31 kDa IL-1β precursor (pro-IL-1β) at Asp116–Ala117 to generate the 17.5 kDa mature form of IL-1β [6]. Similarly, YAMA, or caspase-3, has been shown to be expressed as a 32 kDa precursor that is cleaved into 17 and 12 kDa subunits upon initiation of apoptosis by various death stimuli [7].

Activated caspases not only cleave other pro-caspases, but also cleave and inactivate various essential cellular proteins, as well as DNA, through activation of specific deoxyribonucleases called CAD [8]. Caspases have distinct preferences for their substrates. Caspase-3 cleaves the DNA-repair enzyme poly (ADP-ribose) polymerase (PARP) into signature apoptotic fragments [7,9], whereas lamin A is cleaved by caspase-6 (Mch2α) and not by caspase-3 [10]. The location of caspases within viable cells and their redistribution upon induction of apoptosis has also been studied. The precursor of caspase-3 has been shown to be localized in mitochondria and cytoplasm in normal cells but, in the presence of apoptotic stimuli, it is lost from mitochondria [11].

Figure 1



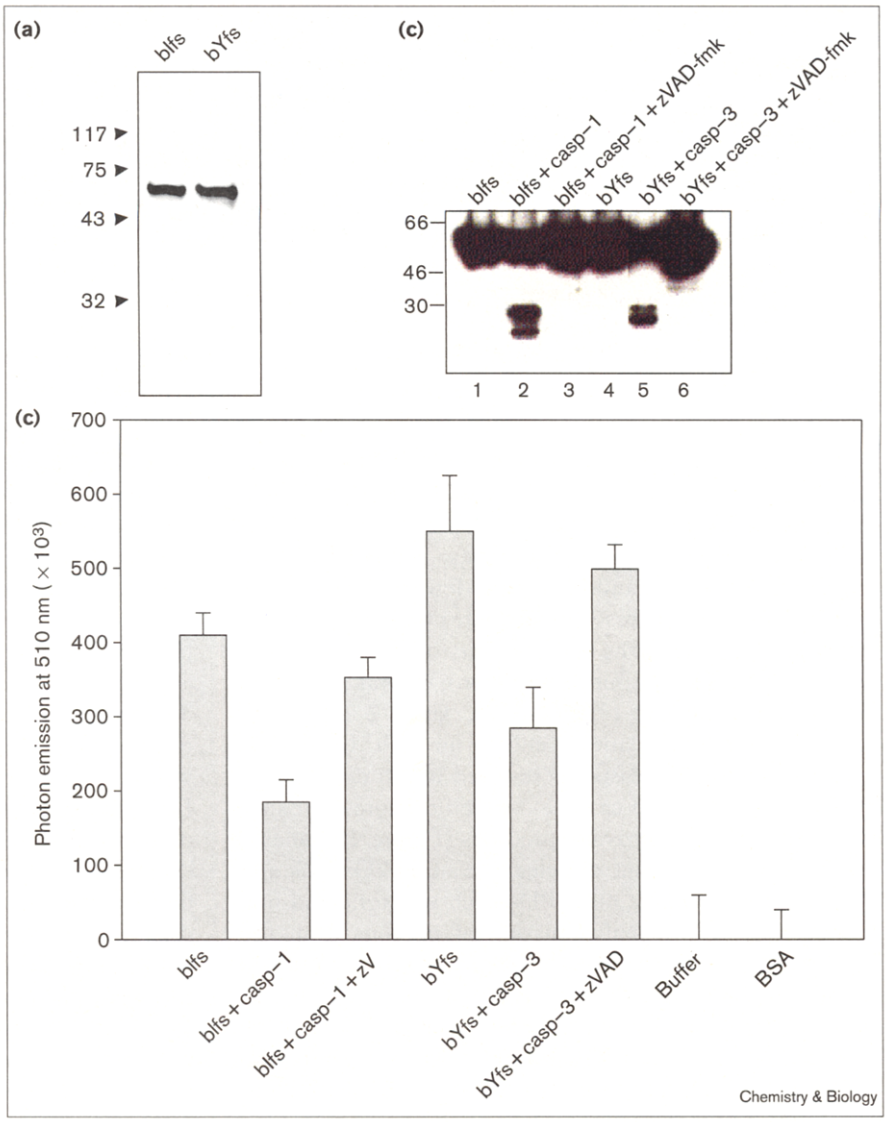
The principle of measuring caspase activity using FRET. Two mutant GFP molecules (BFP and GFP or CFP and YFP) are joined by a short peptide bridge containing either an ICE (caspase-1)- or YAMA (caspase-3)-specific cleavage site. **(a)** When the mutant GFPs (in the case illustrated here) CFP and YFP are within 10–100 Å of one another and excitation energy is supplied at the maximum absorbance of the donor (CFP, 433 nm), CFP can transfer its excitation energy nonradiatively to the acceptor (YFP), which will fluoresce at its emission maximum (528 nm). When caspases are activated, the link between these two mutant GFPs is broken and the distance between them increases to >100 Å, resulting in a loss of measurable FRET. **(b)** BFP/GFP ICE (blfs) and YAMA (bYfs) FRET caspase substrates. Also shown are CFP/YFP ICE (mIfs), YAMA (mYfs) and nonspecific glycine (mGfs) substrates.

Green fluorescent protein (GFP), a protein of 238 amino acids and ~27 kDa, has been used extensively to determine the localization of various proteins in many mammalian and other eukaryotic cells [12–14]. Mutants of GFP with enhanced emission and altered excitation and emission properties have been used effectively to monitor the movement of proteins [14–15] and protein–protein interactions [12] in live mammalian cells. Two different mutants of GFP, Ser65→Tyr (S65T), which emits bright green fluorescence with an excitation peak of 489 nm and an emission peak of 511 nm, and BFP, a blue fluorescent protein that has excitation and emission peaks of 389 nm and 445 nm, respectively, have been used recently to examine protein–protein interactions *in vivo* [12,14] using fluorescence resonance energy transfer (FRET). Another set of GFP mutants has recently been identified — cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) [16] — that can also be used to examine protein–protein interactions or changes in protein conformation *in vivo*. The combination of BFP–GFP or CFP–YFP fluorophores can be used for FRET studies as the emission spectrum of BFP/CFP (donor) overlaps the excitation spectrum of GFP/YFP (acceptor; Figure 1a) [12,16–17]. Following excitation of the donor (BFP/CFP), its excitation energy can be transferred nonradiatively to the acceptor (GFP/YFP), if the distance separating these two molecules is between 10 and 50 Å.

We have employed a novel strategy to study the activation of specific caspases in live mammalian cells (see Figure 1a). The fluorescent substrates are designed in such a way that the two fluorophores (BFP and GFP or CFP and YFP) are linked physically via a four-amino-acid caspase-recognition sequence (Figure 1a,b). In the present study, fluorescent substrates bYfs (bacterially expressed YAMA-specific fluorescent substrate) and bIfs (bacterially expressed ICE-specific fluorescent substrate) were expressed in bacteria by employing the BFP–GFP pair, whereas in mammalian cells the CFP–YFP pair was used for the synthesis of fluorescent substrates mIfs (mammalian ICE specific fluorescent substrate), mYfs (mammalian YAMA-specific fluorescent substrate) and mGfs (mammalian glycine-containing fluorescent substrate; Figure 1b).

Figure 2

Western blot and FRET analysis of caspase activity *in vitro*. An anti-GFP monoclonal western blot demonstrating purified BFP/GFP ICE (blfs) and YAMA (bYfs) substrates, either **(a)** alone or **(b)** following 12 h exposure to purified ICE or YAMA caspases. **(c)** FRET photon emission from purified BFP/GFP ICE (blfs) and YAMA (bYfs) substrates, either alone or following 12 h exposure to purified ICE or YAMA caspases. A decrease in FRET, measured as described in Figure 1, indicates caspase-mediated cleavage of the mutant GFP FRET substrates.



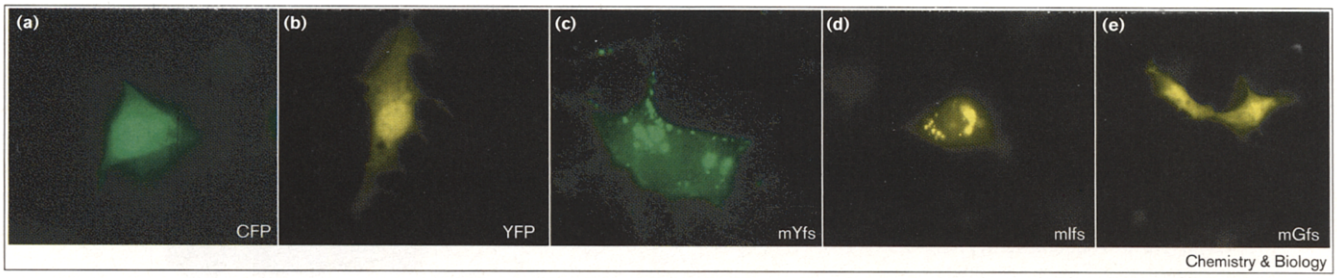
In mammalian cells, upon excitation at ~433 nm, a significant increase in emission (due to FRET) at ~528 nm would occur only if the two fluorophores CFP and YFP are within 10–50 Å of one another or linked physically together by the four-amino-acid bridge. Upon treatment with different apoptosis-inducing agents, cells would activate different members of the caspase family, resulting in cleavage of the respective fluorescent substrates within the four-amino-acid recognition sequence. This would result in a separation of the two fluorophores leading to a decrease in FRET. Similarly, purified fluorescent substrates bYfs and bIfs (Figure 1b) would have significant emission (FRET) at ~511 nm upon excitation at ~389 nm because the two fluorophores BFP and GFP are essentially linked via a four-amino-acid caspase-recognition sequence. Following digestion with purified caspases, there would be a decrease in FRET emission. Here we demonstrate that FRET emission from purified fluorescent substrates was decreased upon digestion with caspase-1 and caspase-3. Similarly, treatment of mammalian cells with staurosporine and mitomycin c resulted in a significant caspase-specific decrease in FRET emission, suggesting that specific caspases are activated during apoptosis.

Results

Purification of GFP–BFP fluorescent substrates and FRET measurement

Two recombinant fluorescent substrates bIfs and bYfs were expressed in bacterial cells in which four-amino-acid recognition sequences for caspase-1 (YVAD) or caspase-3 (DEVD) were sandwiched between GFP and BFP molecules (Figure 1b). These fluorescent substrates were puri-

Figure 3



Transient *in vivo* expression of CFP, YFP, mYfs, mIfs and mGfs in Cos-7 cells. Expression of either **(a)** CFP or **(b)** YFP results in diffuse cytosolic distribution. In approximately 50% of cells, expression of mYfs, mIfs or mGfs resulted in a diffuse distribution similar to that seen in (a) and (b). In the rest of the cell population, **(c)** mYfs, **(d)** mIfs and **(e)** mGfs accumulated into cytosolic vesicular structures of unknown identity.

fied and separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels, and analyzed using western blotting. On hybridizing the blots with anti-GFP monoclonal antibodies, bands of 55 kDa corresponding to bIfs and bYfs were detected, which confirmed that intact fluorescent substrates had been isolated (Figure 2a). To verify the susceptibility of bIfs and bYfs to caspases, an excess of purified bIfs or bYfs was incubated with purified caspase-1 (Figure 2b, lanes 2 and 3) or caspase-3 (Figure 2b, lanes 5 and 6) for 12 h. Incubation with their respective caspases resulted in the digestion of 55 kDa caspase-1- and caspase-3-specific fluorescent substrates into 27 kDa cleavage products (Figure 2b, lanes 2 and 5). To confirm that the cleavage of 55 kDa bIfs and bYfs into two subunits of 27 kDa each was reflected in a change in FRET signal, emission of photons from the digested, as well as the undigested, bIfs and bYfs was measured. As expected, the cleavage of bIfs and bYfs was associated with a decrease in FRET signal (Figure 2c). The *in vitro* cleavage of bIfs and bYfs was inhibited by the pan-caspase inhibitor z-Val–Ala–Asp–fluoromethylketone (zVAD-fmk; Figure 2b,c).

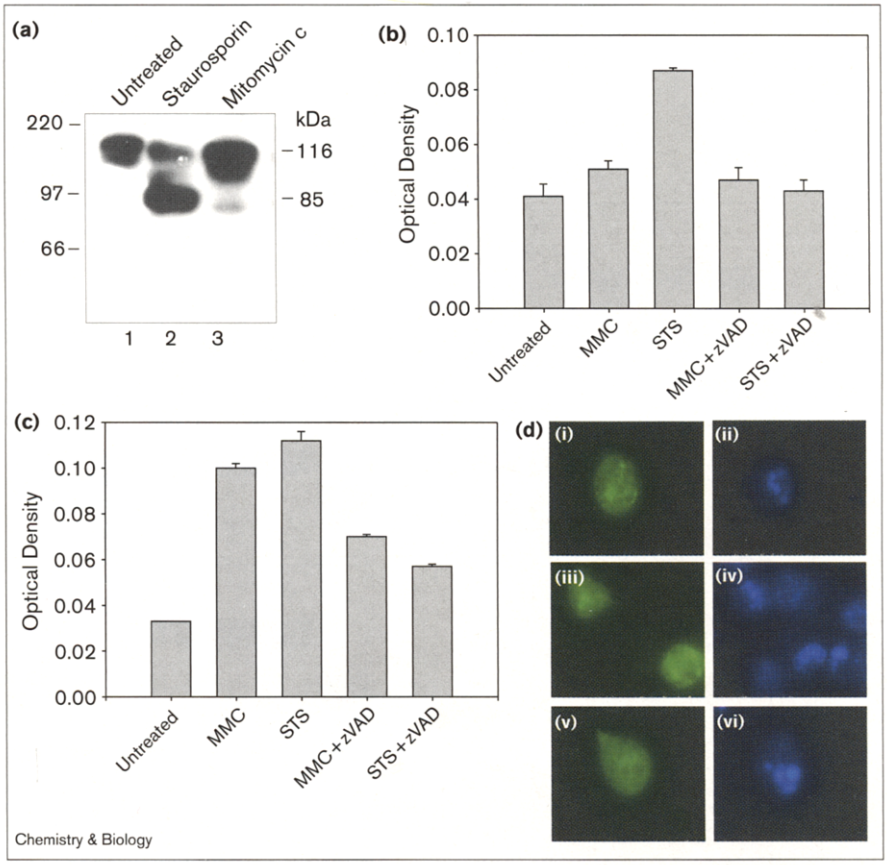
Expression of CFP–YFP fluorescent substrates in mammalian cells

For expression in mammalian cells, we chose CFP and YFP instead of BFP and GFP (Figure 1b). CFP and YFP are humanized versions of GFP whose excitation and emission spectra are red-shifted relative to BFP and GFP, and they are therefore considerably brighter in mammalian cells. Three amino-acid recognition sequences, YVAD (for caspase-1), DEVD (for caspase-3) and GGGG (control), were introduced between CFP and YFP to create three fluorescent substrates called mIfs, mYfs and mGfs, respectively (Figure 1b). mGfs was used as a control fluorescent substrate because to our knowledge there is no caspase in cells that has a recognition sequence of four glycine residues. Cos-7 cells transfected with constructs expressing CFP, YFP or mGfs fluorescent substrates demonstrated uniform distribution of fluorescence in ~50% of cells (Figure 3a,b,e). Although approximately 50% of mIfs- and mYfs-expressing cells had a uniform distribution of fluorescence in cells identical to mGfs, the rest of the cells demonstrated fluorescence in spherical vesicles, which were predominantly localized in the cytoplasm and rarely in nuclei (Figure 3d,e).

To determine if Cos-7 cells undergo apoptosis upon treatment with death-inducing agents, cells were treated with the protein-kinase inhibitor staurosporin and the DNA-damaging agent mitomycin c. It has been amply demonstrated that induction of apoptosis leads to the cleavage of the endogenous death substrate PARP [7,9]. Cells were treated with staurosporin and mitomycin c and western blot analysis was performed using anti-PARP monoclonal antibody. Staurosporin treatment for 5 h resulted in cleavage of the majority of 116 kDa PARP into a characteristic 85 kDa fragment (Figure 4a, lane 2), whereas mitomycin treatment for 5 h resulted in considerably less cleavage (Figure 4a, lane 3). This clearly indicates that both staurosporin and mitomycin c induce apoptosis in Cos-7 cells, which can be seen as signature cleavage of PARP.

Commonly used synthetic fluorogenic substrates YVAD-AMC and DEVD-AMC were employed to verify the specificity of the caspases upon induction of apoptosis (Figure 4b,c). The fluorogenic substrate YVAD-AMC is specifically cleaved by caspase-1, whereas members of the caspase-3 subfamily cleave DVED-AMC. YVAD-AMC or DEVD-AMC was incubated with extracts of cells treated with apoptotic agent staurosporin or mitomycin c for 5 h. Incubation of the substrates with an extract of cells treated with mitomycin c resulted in cleavage of DEVD-AMC (Figure 4b), but not YVAD-AMC (Figure 4c). Staurosporine treatment therefore led to activation of both caspase-1 and caspase-3 subfamily members, whereas mitomycin c treatment led to activation of members of the caspase-3 subfamily.

Figure 4



Induction of apoptosis upon treatment of Cos-7 cells with apoptosis-inducing agents. **(a)** Measurement of endogenous caspase activity. Cos-7 cells were treated with staurosporin or mitomycin c for 5 h and western analysis was performed using anti-PARP antibodies. The 116 kDa endogenous PARP is cleared into an 85 kDa fragment. **(b)** The cleavage of caspase-1-specific synthetic fluorescent substrate YVAD-AMC, on treatment with staurosporine (STS) and mitomycin c (MMC) for 5 h. An increase in optical density indicates production of AMC fluorescence due to cleavage of the synthetic fluorescent substrate. **(c)** The cleavage of caspase-3-specific synthetic fluorescent substrate DEVD-AMC, on treatment with staurosporine and mitomycin c for 5 h. **(d)** Induction of apoptosis in cells expressing (i) mIfs, (iii) mYfs and (v) mGfs as seen by disintegration of nuclei by DAPI staining (ii, iv and vi).

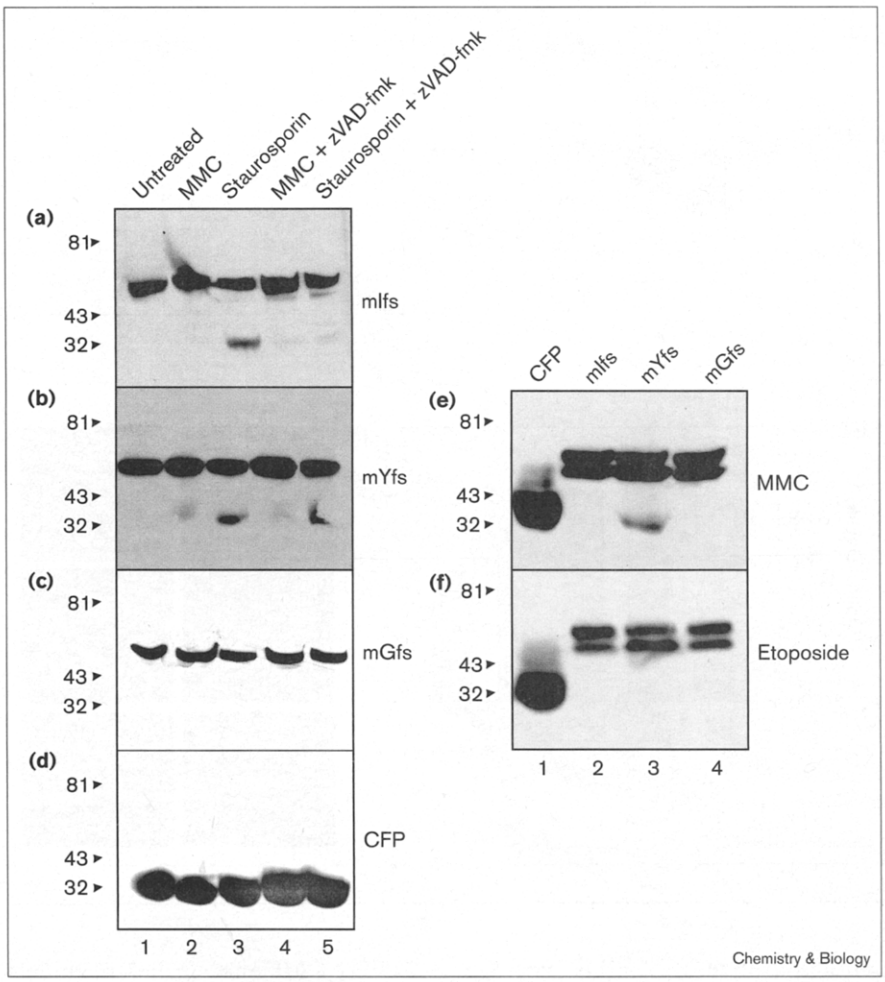
It was important to determine if the distribution of the three fluorescent substrates was affected when cells undergo apoptosis. Cos-7 cells expressing mIfs, mYfs or mGfs were treated with staurosporine for 5 h. Cell nuclei were stained with the DNA-binding stain DAPI for visualization of fragmentation of nuclei, a hallmark of apoptosis (Figure 4d, panels ii, iv and vi). All of the three fluorescent substrates were uniformly distributed throughout the cells during apoptosis (Figure 4d, panels i, iii and v). The proportion of cells undergoing apoptosis after a 5 h treatment with staurosporin was found to be ~63% as visualized by fragmentation of nuclei.

Cleavage of CFP–YFP fluorescent substrates during apoptosis

To determine if the fluorescent substrates mIfs, mYfs and mGfs are cleaved in mammalian cells upon receipt of a death stimulus, Cos-7 cells were treated with the protein-kinase inhibitor staurosporine and the DNA-damaging agent mitomycin c. Treatment of cells with staurosporin for 5 h resulted in cleavage of 55 kDa mIfs and mYfs into 27k Da fragments corresponding to CFP and YFP (Figure 5a,b, lane 3). Similar treatment did not bring about cleavage of 55 kDa mGfs into 27 kDa fragments or further cleavage of 27 kDa CFP into smaller fragments (Figure 5c,d, lane 3). Addition of zVAD-fmk, a broad spectrum, cell permeable, tripeptide inhibitor of caspases, in cell-growth medium resulted in complete repression of the cleavage of mIfs and mYfs into 27 kDa fragments (Figure 5a,b, lane 5). This indicates that staurosporine treatment results in activation of both caspase-1 and caspase-3, which cleave at the recognition sequence to produce two fragments of ~27 kDa each.

To determine whether specific caspases are activated upon the induction of apoptosis by a DNA-damaging agent, cells were treated with mitomycin c for 5 h, and analyzed using western blotting. None of the fluorescent substrates was cleaved to form of 27 kDa cleavage product (Figure 5a,b,c,d, lane 2), indicating that 5 h of mitomycin c treatment does not result in the activation of either caspase-1 or caspase-3. To determine whether a longer duration of mitomycin c treatment is required to activate caspases, mIfs-, mYfs- and mGfs-expressing cells were treated with mitomycin c for 12 h, and analyzed using western blotting (Figure 5e). Cleavage of the 55 kDa substrate occurred in mYfs-expressing cells, whereas the 27 kDa product was not observed in

Figure 5



Activation of specific caspases upon induction of apoptosis with DNA-damaging agents. **(a,b,c,d)** Cells were treated with mitomycin c for 5 h, and analyzed using western blotting. **(e)** Cells were treated with mitomycin c for 12 h and analyzed using western blotting. **(f)** Cells treated with etoposide, another DNA-damaging agent.

mIfs- and mGfs-expressing cells upon a longer duration of mitomycin c treatment (Figure 5e). As a control, the cells were treated with etoposide, another DNA-damaging agent (Figure 5f). Treatment with etoposide for 12 h did not result in the formation of the 27 kDa product. The exposure of cells to mitomycin c for 12 h therefore led to activation of caspase-3 but not caspase-1. Etoposide treatment for 12 h did not bring about activation of caspase-1 or caspase-3 (Figure 5f).

Measurement of FRET

To determine if the induction of apoptosis in cells was followed by a change in the FRET signal of the fluorescent substrates mIfs, mYfs and mGfs, Cos-7 cells were treated with staurosporin and mitomycin c for 8–10 h. Cells were washed and the emission of photons from live cells was measured using the FRET filter set. Cells expressing mIfs and mYfs demonstrated 33 and 36% decrease in emission of photons on treatment with staurosporin, respectively (Figure 6). The photon emission from cells expressing mGfs and CFP remained unchanged (Figure 6). Similarly, cells expressing mYfs demonstrated a decrease of ~34% in emission of photons on treatment with mitomycin c, whereas photon emission from cells expressing mIfs, mGfs and CFP remained unchanged (Figure 6). Addition of zVAD-fmk to cells treated with staurosporin and mitomycin c demonstrated an increased emission of photons, similar to that of untreated cells, indicating a lack of cleavage of the substrates.

Discussion

Activation of specific caspases is a critical element of apoptotic process. Induction of apoptosis by specific death stimuli leads to the activation of distinct sets of caspases. Here, we demonstrate the design of GFP-based caspase tetrapeptide recognition sequence containing substrates (GTRCOSs), which were cleaved upon induction of cell death. This strategy not only enabled us to monitor the activation of particular caspases upon induction of cell death with specific death stimuli, but also allowed us to monitor caspase activation in live mammalian cells. In addition, large-scale purification of these

fluorescent substrates made it possible to confirm the presence of activated caspases *in vitro*.

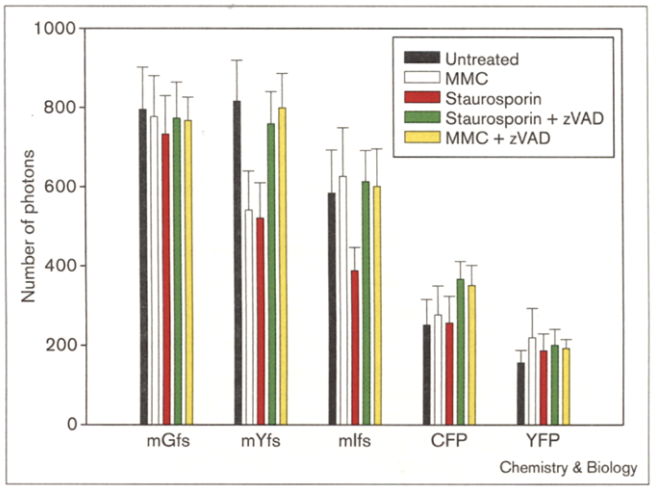
Caspase-3 has been reported to become activated upon staurosporine treatment of GM701 cells [18]. The activation of caspase-3 was monitored by cleavage of its substrate PARP; this cleavage was shown to be inhibited by zVAD-fmk and overexpression of Bcl-2 [18]. Furthermore, it was demonstrated that Rat-1 cells expressing the caspase-1 inhibitor CrmA were as sensitive to staurosporine treatment as those that did not express CrmA [18]. On the basis of these results, it was suggested that staurosporine-induced apoptosis does not depend on caspase-1 in Rat-1 or GM701 cells [18]. It is possible, however, that redundant caspase pathways exist in cells. By employing GTRCOS we have been able to show that indeed both caspase-1 and caspase-3 are activated on staurosporine treatment of Cos-7 cells and this activation was inhibited by the broad spectrum caspase inhibitor zVAD-fmk. These findings suggest that the FRET-based approach to measuring caspase activity is a more sensitive assay for caspase activation.

The expression of fluorescent substrates showed a uniform distribution in mammalian cells because of the lack of any specific organelle-targeting sequence. Vesicular fluorescence was observed in 50% of mIfs- and mYfs-expressing cells, mostly localized in the cytoplasm (Figure 3c,d). At the moment we do not know the reason why such distinctive patterns of distribution are shown by these fluorescent substrate expressing cells. Experiments are underway to determine if the caspases and their respective fluorescent substrates localize, resulting in the vesicular fluorescence pattern. Western analysis of mammalian cells expressing mIfs or mYfs did not show a complete digestion of these 55 kDa substrates into 27 kDa fragments (Figure 4a,b). These fluorescent substrates are under the strong Cytomegalovirus (CMV) promoter, which generates high levels of transcripts corresponding to full-length fluorescent substrates in cells induced to undergo apoptosis. Such overwhelming amounts of substrate may not undergo complete digestion by newly activated caspases, resulting in the presence of uncut, full-length substrates at any given time.

The bands on the western blot of the cleavage of purified bIfs and bYfs with caspase-1 and caspase-3 were smeared (Figure 2b). We observed that purified, bIfs and bYfs are very stable in the Tris buffer in which they are eluted and stored (Figure 2a) but tend to show aberrant mobility when suspended in HEPES buffer (compare Figure 2a with 2b).These substrates are probably more stable in Tris buffer than in HEPES.

GTRCOSs could be utilized for the study of spatial and/or temporal changes in protease activity, the identification of specific caspases and to determine the location of activity of caspases by targeting GTRCOSs to various cellular organelles. Large-scale production of GTRCOS by overexpression in bacteria would help in identifying and designing inhibitors that are caspase specific or organelle specific.

Figure 6



Measurement of caspase activity *in vivo*. Cos-7 cells expressing either mGfs, mYfs, mIfs, CFP or YFP were treated with staurosporin or mitomycin C for 8–10 h, and the extent of FRET of the caspase substrates was monitored. No change in FRET was seen in cells expressing CFP, YFP or mGfs. In Cos-7 cells expressing mYfs, both mitomycin C and staurosporin caused a decrease in the FRET signal, signifying cleavage. In mIfs-expressing cells only staurosporin caused activation of caspases.

The strategy presented here has enabled us to determine the activation of specific caspases upon treatment of cells with different cell death stimuli. This method also demonstrated the activation of specific caspases in a single living cell, which has not yet been described. The method described here could be adapted to decipher the execution pathway for various other apoptotic stimuli.

Significance

Apoptosis is a genetically regulated cellular suicide mechanism that plays a crucial role in development and in the defense against disease. One of the key regulators of mammalian apoptosis is the caspase family of proteases. Caspases are cysteine proteases sharing a conserved active site that cleaves proteins at a highly specific site. Mammalian caspases now number 16 and can be subdivided into the ICE subfamily, caspase-2 subfamily and caspase-3 subfamily, each of which attack different cellular substrates. Caspases promote apoptosis through proteolytic degradation of cellular components, a process that involves autocatalysis of the caspases themselves. Defects in the caspase pathway are now known to underlie ischemic injury in stroke and neuronal degeneration in amyotrophic lateral sclerosis, and are likely to

play a major role in the development and treatment of cancer. The finding that caspases play such a central role in these diseases is leading to the rapid development of drugs targeted to regulation of caspase activity. The assay described here will allow the screening, both at the single cell level and via high-throughput methods, of candidate caspase inhibitors that could be applied clinically to modulate disease pathology. The caspase substrates described in this paper should allow stimuli and cell-type-dependent apoptotic pathways to be characterized.

Materials and methods

Construction of GFP–BFP fluorescent substrate expressing vectors

The pS65T vector (Clontech) was digested with restriction enzymes *Eco*47III and *Xho*I to release a 0.7 kilobase DNA fragment corresponding to the GFP-coding region. The GFP-coding region was subcloned into pCAL-N prokaryotic expression vector (Stratagene) digested with restriction enzymes *Sma*I and *Xho*I (termed as pNCG). The cDNA of BFP (kindly provided by Dr Roger Tsien) was amplified by the polymerase chain reaction (PCR) with the following primers. For Yama substrates: (forward: 5′-ATG ACT CGA GCT GAT GAG GTA GAT GGC GGC ATG GGT AAA GGA GAA GAA CTT -3′; reverse: 5′-CAA AAG CTT CTA GGA CTT GTA TAG TTC ATC CAT GCC-3′) containing *Xho*I and *Hind*III restriction sites were used, whereas for ICE substrates, primers (forward: 5′-ATG ACT CGA GCT TAC GTC GCT GAT GGC GGC ATG GGT AAA GGA GAA GAA CTT -3′; reverse: 5′-CAA AAG CTT CTA GGA CTT GTA TAG TTC ATC CAT GCC-3′) with *Xho*I and *Hind*III restriction sites were used. All PCR amplifications were for 30 cycles (1st cycle, 5 min 94°C, 2 min 56°C, 3 min 72°C; 29 cycles, 1 min 94°C, 2 min 55°C, 3 min 72°C; last cycle, 1 min 94°C, 10 min 72°C) with *Pfu* polymerase (Stratagene). PCR products were digested with *Xho*I and *Hind*III restriction enzymes and subcloned into pNCG vector digested with *Xho*I and *Hind*III, termed as pNCI (for ICE substrate, bIfs) and pNCY (for Yama substrate, bYfs), respectively.

Purification of (GFP–BFP) fluorescent substrates

BL21(DE3) cells were transformed with pNCI and pNCY constructs followed by growth in 250 ml of LB broth with ampicillin and chloramphenicol antibiotics. Cells were induced to express recombinant fluorescent substrates by addition of 1 mM of IPTG for 3 h followed by harvesting and lysis of cells in binding buffer (50 mM Tris, pH 8; 150 mM NaCl; 10 mM β-mercaptoethanol; 1 mM $MgCl_2$ and 1 mM $CaCl_2$). Calmodulin affinity resin was suspended in binding buffer and bacterial cell lysate was added followed by incubation at 4°C for 2 h. Affinity resin was washed using ten volumes of binding buffer and eluted in ten volumes of elution buffer (50 mM Tris, pH 8; 10 mM β-mercaptoethanol; 2 mM EGTA). Purified fluorescent substrates, bIfs and bYfs were digested with purified caspase-1 and -3 in cleavage buffer (20 mM HEPES, pH 7.5 and 2 mM DTT) for 12–16 h at 37°C.

Construction of (YFP–CFP) fluorescent substrate expressing vectors

The cDNA of YFP (Packard) was amplified by PCR with the following primers: forward: 5′-TCC GCT AGC GCT ACC ATG CAC CAC CAC CAC CAC CAC ATG GTG AGC AAG GGC GAG GAG-3′; reverse: 5′-TCG AGA TCT GAG TCC GGA CTT GTA CAG CTC GTC CAT GCC-3′). A 0.7 Kb PCR product was digested with *Nhe*I and *Bgl*II restriction enzymes and subcloned into the pS65T vector (Clontech) digested with restriction enzymes *Nhe*I and *Bgl*II (termed as pNT). For the YAMA fluorescent substrate, a cDNA of CFP (kindly provided by R. Tsien) was amplified by PCR with the following primers: forward: 5′-ATG CAA GCT TCG GAT GAG GTA GAT GGC GGC ATG GTG AGC AAG GGC GAG GAG-3′; reverse: 5′-CAA GAA TTC TTA CTT GTA CAG CTC GTC CAT-3′), while for ICE substrates, primers were (forward: 5′- ATG CAA GCT TCG TAC GTC GCT GAT GGC GGC ATG GTG AGC AAG GGC GAG GAG-3′; reverse: 5′-CAA AAG CTT CTA GGA CTT GTA TAG TTC ATC CAT GCC-3′). For the G4 fluorescent substrate, the cDNA of CFP was PCR amplified using the following primers: forward: 5′-ATG CAA GCT TCG GGA GGA GGA GGA GGC GGC ATG GTG AGC AAG GGC GAG GAG-3′; reverse: 5′-CAA GAA TTC TTA CTT GTA CAG CTC GTC CAT-3′). PCR products were eluted, digested with *Hind*III and *Eco*RI restriction enzymes and subcloned into pNT vector digested with *Hind*III and *Eco*RI. These constructs were termed as pNTYC for the YAMA substrate mYfs; pNTIC for the ICE substrate mIfs and pNTGC for the control fluorescent substrate mGfs.

Transfection of mammalian cells and western blotting

Cos-7 cells were transfected transiently with various plasmid constructs using Lipofectamine (Boerhinger Manheim). In six-well plates, 2×10^5 cells per well were seeded on glass cover slips in appropriate growth medium and incubated at 37°C until cells were 60–70% confluent. DNA (2 mg) was added to 200 ml of growth medium lacking serum and antibiotics followed by addition of 10 ml lipofectamine reagent. This mixture was incubated at room temperature for 45 min and then 800 μl medium lacking serum and antibiotics were added. This DNA-lipofectamine mixture was added to cells and incubated for 4 h at 37°C. Cells were rinsed with media containing serum and antibiotics and fresh media was added followed by incubation for another 24 h. The expression of various fusion proteins was confirmed by western blot analysis using anti-GFP monoclonal antibodies (Boehringer Mannheim). The cells were lysed and separated on 10–20% gradient SDS–PAGE. Proteins were transferred onto PVDF membrane and detected by using enhanced chemiluminiscence (ECL kit, Amersham).

FRET quantitation

For detection and quantitation of FRET due to GFP–BFP fluorescent substrates, the following filter sets were employed. For detection of GFP fluorescence, an excitation filter of 480/20 nm, a dichroic beamspliter of 500 nm and an emission filter of 515/30 nm; for BFP fluorescence, excitation filter of 390/20 nm, a dichroic beamsplitter of 420 nm and an emission filter of 450/40 nm and for FRET studies, excitation filter 390/20 nm, dichroic beamspliter 420 nm and an emission filter of 515/30 nm. Purified protein (20 μl) was taken on glass slide and cover slip was placed on it, followed by sealing with rubber cement. Slides were viewed under microscope and emission of photons per second was recorded using photon counter. As a control, bovine serum albumin was suspended in buffer and the photon emission was recorded. The emission of photons from buffer alone was used as background and was deducted from values obtained using bIfs and bYfs substrates.

Microscopy and image analysis

Cells were treated with 5 μg/ml mitomycin c or 3.14 μM of staurosporine for 12 h. 50 mM of zVAD-fmk or 10 μM of DEVD-CHO/YVAD-CHO was used for inhibition of caspase activation. Cells were washed with phosphate buffered saline (PBS) and mounted on a glass slide in Vectashield mounting medium (Vector Labs). For detection of CFP, cells were viewed with a filter set having an excitation filter of 425/40 nm, a dichroic beamspliter of 460 nm and an emission filter of 495/30 nm. YFP expression was detected by filter set having an excitation filter of 512/20 nm, a dichroic beamsplitter of 530 nm and an emission filter of 560/40 nm. The filter set combination used for FRET studies was: excitation filter 425/40 nm, dichroic beamspliter 460 nm and an emission filter of 560/40 nm. The images were captured using a slow-scan cooled CCD camera (exposure 1.3–2 s) and were artificially colored using Adobe Photoshop.

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