Single-cell Fluorescence Resonance Energy Transfer Analysis Demonstrates That Caspase Activation during Apoptosis Is a Rapid Process

ROLE OF CASPASE-3*

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Activation of effector caspases is considered to be the final step in many apoptosis pathways. We transfected HeLa cells with a recombinant caspase substrate composed of cyan and yellow fluorescent protein and a linker peptide containing the caspase cleavage sequence DEVD, and we examined the cleavage kinetics at the single-cell level by fluorescence resonance energy transfer (FRET) analysis. Caspase activation in response to tumor necrosis factor-α, staurosporine, or eto- poside resulted in cleavage of the linker peptide and subsequent disruption of the FRET signal. The time to caspase activation varied among individual cells, dependent on the type of treatment and concentration used. However, once initiated, disruption of the FRET signal was always rapid (≤15 min) and largely independent of these parameters. In contrast, FRET probe cleavage was significantly slower in the caspase-3-deficient MCF-7 cells, particularly at low concentrations of the pro-apoptotic agents. Under these conditions, MCF-7 cells required up to 90 min for the FRET probe cleavage, whereas MCF-7/Casp-3 cells displayed rapid cleavage kinetics. Interestingly, we could still observe comparable cell death rates in MCF-7 and MCF-7/Casp-3 cells. Our results suggest that caspase activation during apoptosis occurs in an “all or nothing” fashion. Caspase-3 is required for rapid cleavage kinetics when the onset of apoptosis is slow, suggesting the existence of caspase-3-dependent feedback loops.

Apoptosis is an evolutionary conserved, cellular process that plays an important role during development, but it is also involved in tissue homeostasis and in the pathophysiology of proliferative and neurodegenerative disorders (1, 2). A family of intracellular cysteine proteases, the caspases, are responsible for most biochemical and morphological alterations during apoptosis (3). Caspases reside in the cytosol as dormant pro-enzyme forms that can be activated by proteolytic cleavage at specific aspartate residues (3). Caspases involved in apoptosis can be subdivided into initiator and effector caspases. Effector caspases such as caspase-3, -6, and -7 cleave multiple cellular substrates during the death process. These cleavage events result in degradation and reorganization of cellular structures, inactivation or activation of signal transduction pathways, alterations in gene transcription, and inhibition of DNA repair (3–5).

Initiator caspases predominantly function to activate effector caspases. Activation of initiator caspase-8 and -9 requires the binding of adaptor proteins to specific interaction motifs within their prodomain, leading to their oligomerization and autoactivation (6–10). For example, activation of caspase-9 occurs via binding of the adaptor protein apoptotic protease-activating factor-1 to the caspase recruitment domain (7, 11). The association of caspase-9 and apoptotic protease-activating factor-1 and subsequent “apoptosome” formation is triggered by the pro-apoptotic factor cytochrome c (12, 13). This factor normally resides in the mitochondrial intermembrane and intracristal space where it participates in electron transport during respiration. During apoptosis, cytochrome c and other pro-apoptotic factors are released from the intermembrane space because of a significant increase in mitochondrial outer membrane permeability (14).

Recent time-lapse confocal microscopy experiments in cells expressing cytochrome c-green fluorescent protein (GFP)2 fusion proteins suggested that the release of pro-apoptotic factors during apoptosis occurs rapidly and completely (15–17). Apart from stimuli that may activate both necrotic and apoptotic pathways, the release kinetics during apoptosis are similar and largely independent of the type of stimulus (16, 17). Cleavage kinetics following caspase activation have also been observed at the single-cell level using the fluorescence resonance energy transfer (FRET) technology and recombinant caspase substrates composed of GFP variants linked by peptides contain-
ing the caspase cleavage sequence, DEVD (18, 19). This sequence is found in many cytosolic and nuclear caspase substrates and is cleaved by several effector caspases including caspase-3 and -7 (20). Caspase-3 is believed to play a central role in the execution of apoptosis, because this enzyme is required for oligonucleosomal DNA fragmentation and promotes the activation of other effector caspases (21–25).

Little is known about potential differences in the extent and kinetics of effector caspase activation during apoptosis and its functional consequences. In the present study, we demonstrate that caspase activation resembles an “all or nothing” type of response that is only marginally influenced by the type of stimulus or concentration used. We also show that efficient activation of caspases can occur in the absence of caspase-3, albeit leading to significantly slower cleavage kinetics. Finally, we address the issue whether differences in cleavage kinetics lead to differences in cells death.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human tumor necrosis factor-α (TNF-α), cycloheximide (CHX), etoposide, propidium iodide, and embryo-tested mineral oil were purchased from Sigma, Staurouporin, STS, and leupeptin from Alexis (Grünenbach, Germany). The caspase substrate acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) and the broad spectrum caspase inhibitor V-ALa-A-A-D-pNA for caspase-8, LEHD-pNA for caspase-7, YVAD-pNA for caspase-2, DEVD-pNA for caspase-7, and the broad spectrum caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-fmk) were purchased from Bachem (Heidelberg, Germany). All other chemicals in analytical grade were of Roche Diagnostics, Karlsruhe, Germany, or Merck.

**Expression and Purification of a Recombinant FRET Probe**—The recombinant FRET probe BFP-DEVD-GFP, containing a 18-amin acid linker region identical to the plasmid myc-CFP-DEVD-YFP (18), was expressed as an NH2-terminus His6-tagged fusion in Escherichia coli using plasmid pTrcHisB. Bacteria were cultured in Luria broth at 30 °C, and expression was induced by 0.5 mM isopropylthiogalactoside at A600nm = 0.5. Cells were harvested by centrifugation at 5000 g for 10 min and washed in NaH2PO4 buffer (50 mM, pH 8.0). Bacteria then were resuspended in 5 mM NaH2PO4 (50 mM, pH 8.0) buffer containing 300 mM NaCl, 10 mM imidazole, 0.1 mM EDTA, 5 units/ml benzonase, 0.1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepsatin A, 1 mM leupeptin, and 5 mM aprotinin and subsequently lysed by repeated cycles of freezing and thawing plus sonication (Sono- plus, Bandelin Electronic, Berlin, Germany). Cell debris was removed by centrifugation (100,000 g for 1 h). BFP-DEVD-GFP was purified from the supernatant by fast protein liquid chromatography on a monoS column (Amersham Biosciences). Membranes were stripped in standard stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, pH 7.5) and blocked with 5% non-fat dry milk in TBST (15 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.1% Tween 20) at room temperature for 2 h. Membranes were incubated as follows: a rabbit polyclonal anti-caspase-3 antibody (H-25, 1:1000; kindly provided by Dr. D. W. Nicholson, Merck Frosst, Point Pleasant, NJ), a mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody (clone C2 1/2), a mouse monoclonal anti-active p18 caspase-9 antibody (MF445, 1:1000; BD PharMingen); a rabbit polyclonal anti-active caspase-9 antibody (1:1000, BD Pharmingen); a rabbit polyclonal anti-active p20 caspase-7 antibody (1:1000, New England Biolabs, Beverly, MA) or the polyclonal active p12 caspase-7 antibody (AB445, 1:1000, kindly provided by Dr. D. W. Nicholson, Merck Frosst, Point Claire-Dorval, Quebec, Canada); a mouse monoclonal anti-GFP antibody (1:1000, CLONTECH Laboratories, Palo Alto, CA); a mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody (clone C2 10–12, 1:2000, BD Pharmingen); a mouse monoclonal anti-α-spectrin antibody (clone 6H11 G2/1, Chemicon International Inc., Temecula, CA); or a mouse monoclonal anti-α-tubulin antibody (clone DM 1/2, 1:5000, Sigma). Membranes were washed with TBST six times for 10 min and incubated with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (Promega, Madison, WI) for 1 h. Blots were washed and developed using the ECL chemiluminescence detection reagent (Amersham Biosciences). Membranes were stripped in standard stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, pH 6.8) at 60 °C for 20 min, washed twice in TBST for 10 min, and reprobed.

**Preparation of Whole Cell Extracts and Western Blotting**—Cells were collected at 200 × g for 5 min and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerin, 2% (v/v) SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM/l pepsatin A, 1 mM leupeptin, and 5 mM aprotinin). Cell homogenates were centrifuged at 15,000 × g and 4 °C for 15 min. Protein content was determined with the Pierce Micro-BCA Protein Assay (KMF, Cologne, Germany). An equal amount of protein (20–40 µg) was loaded onto SDS-polyacrylamide gels (12% or gradient gels). Proteins were separated at 120 V for 1.5 h and then blotted to nitrocellulose membranes (Protein BA 83; 2 µm; Schleicher & Schuell) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v)) at 20–25 V for 45–60 min. The blot was incubated with 5% non-fat dry milk in TBST (15 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.1% Tween 20) at room temperature for 2 h. Membranes were incubated as follows: a rabbit polyclonal anti-caspase-3 antibody (H-277, 1:1000, Santa Cruz Biotechnology); a rabbit polyclonal anti-caspase-9 antibody (1:1000, BD Pharmingen); a rabbit polyclonal anti-active p20 caspase-7 antibody (1:1000, New England Biolabs, Beverly, MA); or a mouse polyclonal active p12 caspase-7 antibody (1:1000, kindly provided by Dr. D. W. Nicholson, Merck Frosst, Point Claire-Dorval, Quebec, Canada); a mouse monoclonal anti-GFP antibody (1:1000, CLONTECH Laboratories, Palo Alto, CA); a mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody (clone C2 10–12, 1:2000, BD Pharmingen); a mouse monoclonal anti-α-spectrin antibody (clone 6H11 G2/1, Chemicon International Inc., Temecula, CA); or a mouse monoclonal anti-α-tubulin antibody (clone DM 1/2, 1:5000, Sigma). Membranes were washed with TBST six times for 10 min and incubated with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (Promega, Madison, WI) for 1 h. Blots were washed and developed using the ECL chemiluminescence detection reagent (Amersham Biosciences). Membranes were stripped in standard stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, pH 6.8) at 60 °C for 20 min, washed twice in TBST for 10 min, and reprobed.

**Measurement of Caspase Activity**—After treatment with TNF-α/CHX, etoposide, staurosporine, or vehicle, cells were lysed in 200 µl of lysis buffer (10 mM Heps, pH 7.4, 42 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM EGTA, 1 µM phenylmethylosulfonyl fluoride, 0.1 mM dithiothreitol, pH 7.5, and 10 µM of the caspase substrate Ac-DEVD-AMC). Accumulation of fluorescent AMC fluorescence was monitored over 120 min using a HTS fluorescent plate reader (PerkinElmer Life Sciences) (excitation 380 nm, emission 465 nm). Fluorescence of blanks
containing no cell lysate was subtracted from the values. Protein content was determined using the Pierce Coomassie Plus Protein Assay reagent. Caspase activity was expressed as change in fluorescent units per μg of protein and hour.

**RESULTS**

**Characterization of the Recombinant FRET Probes**—In the present study we employed two recombinant FRET probes, BFP-DEVD-GFP and CFP-DEV-D-YFP. Both FRET probes composed of two spectrally distinct, FRET-compatible GFP mutants fused covalently with an identical 18-amino acid peptide linker containing a single DEVD caspase cleavage sequence (18). The DEVD sequence is found in many cellular caspase substrates, including PARP, catalytic subunit of DNA-dependent protein kinase, protein kinase C-θ, and βII-spectrin (26–31), and is the optimal tetrapeptide sequence for caspase-3 and -7 (20). To test whether caspases are capable of cleaving the recombinant FRET probes, the purified BFP-DEVD-GFP protein was incubated in vitro with different recombinant active caspases and subsequently subjected to SDS-PAGE. As GFP and its variants are still fluorescent in the gel, the extent of cleavage could be judged by UV illumination. Complete cleavage of the probe into its GFP and FRET constituents was observed when the FRET probe was incubated with caspase-3. Significant cleavage was also detected in the case of caspase-7, -8, and -9, whereas incubation with caspase-6 resulted in only minor cleavage of the BFP-DEV-D-GFP protein (Fig. 1A). Subsequent Coomassie staining indicated no alternative products of other sizes (data not shown). Therefore, caspase cleavage outside the linker-region could be excluded.

To determine in vivo the kinetics of caspase activation during apoptosis, we generated HeLa D98 cells stably expressing the recombinant FRET probe, CFP-DEVD-YFP. Protein CFP-DEVD-YFP does not require UV illumination and was therefore chosen for subsequent experiments. Western blot analysis of the stably transfected HeLa D98 cells treated with the apoptosis-inducing protein kinase inhibitor staurosporine (3 μM) demonstrated that the active caspase-3 subunits p20 and p17 were first visible after 3 h and were more pronounced after 6 and 8 h of treatment. This correlated well with cleavage of endogenous cytosolic or nuclear caspase substrates as evidenced by the accumulation of the 85-kDa PARP cleavage product and the caspase-generated 150- and 120-kDa α-spectrin breakdown products (31–33). Also, the CFP-DEVD-YFP construct was cleaved into its myc-CFP and YFP constituents after 3 h and was more pronounced after 6 and 8 h of treatment (Fig. 1B). Our results demonstrate that the cleavage of the recombinant FRET probe occurred within the same time frame as the activation of procaspase-3 and the cleavage of endogenous caspase substrates in bulk analyses. Hence, this system allows us to monitor reliably caspase activation at the single-cell level.

To provide evidence that indeed caspases cleave the FRET probe in vivo, stably transfected HeLa D98 cells were treated with 3 μM STS in the presence of the two protease inhibitors Z-VAD-fmk and calpeptin. Z-VAD-fmk is a cell-permeable, broad spectrum caspase inhibitor. Calpeptin is a potent, cell-permeable calpain inhibitor. Western blot analysis of cellular extracts after 6 h of treatment showed no inhibition of cleavage in the presence of calpeptin, whereas Z-VAD-fmk completely blocked the degradation of the FRET probe as well as the activation of caspase-3 (Fig. 1C).

**Single-cell FRET Analysis of Caspase Activity in HeLa D98 Cells**—In the next set of experiments, we investigated the changes in the CFP/YFP emission ratio of the recombinant FRET probe CFP-DEV-D-YFP at the single-cell level during apoptosis. Rapid caspase activation could be observed in the stably transfected HeLa D98 cells during an exposure to 3 μM staurosporine. Fig. 1D demonstrates CFP/YFP ratio changes within a single HeLa cell monitored every 60 s for a period of 4 h. The cell initially showed a stable base-line CFP/YFP emission ratio. Cleavage of the FRET probe resulted in an increased CFP/YFP emission ratio (Fig. 1D), indicating a decrease in resonance energy transfer. Of note, ratio changes were completed in less than 10 min, suggesting that the entire caspase substrate was cleaved within this short period. Control experiments in transfected HeLa D98 cells exposed to staurosporine in the presence of the broad spectrum caspase inhibitor Z-VAD-fmk (200 μM) did not show a change in the CFP/YFP emission ratio (n = 24 cells; data from two experiments (>12 h), not shown). Likewise, transfection of HeLa D98 cells with the mutated FRET construct CFP-DEVD-YFP that cannot be cleaved by caspases did not reveal significant changes in the CFP/YFP emission ratio in response to staurosporine until late morphological changes such as secondary necrosis occurred (data not shown).

Fig. 1E shows pseudo-colored CFP/YFP ratio images and phase contrast images from a field of stably transfected HeLa D98 cells during an exposure to 3 μM staurosporine. After an initial period during which no changes in the CFP/YFP emission ratio had occurred, the first cell completed a rapid ratio change at 180 min. Three more cells subsequently underwent ratio changes within a 20-min time period (Fig. 1E). Bright field images of the same cells taken 80 min after completion of the FRET probe cleavage showed progressive cell shrinkage and membrane blebbing. Hence, activation of DEVDases can be detected at the single-cell level in stably transfected HeLa D98 cells using the recombinant FRET probe.

**Kinetics of Caspase Substrate Cleavage Are Independent of the Type of Pro-apoptotic Stimulus**—To establish whether the kinetics of caspase substrate cleavage depend on the type of apoptotic stimulus, we exposed HeLa D98 cells to three distinct pro-apoptotic agents as follows: (i) TNF-α (200 ng/ml) plus CHX (1 μg/ml) to stimulate a cell death pathway that involves activation of death receptors and caspase-8 as initiator caspase (6); (ii) STS (3 μM), a protein kinase inhibitor that stimulates a transcription-independent cell death and utilizes the mitochondrial apoptosis pathway (34); and (iii) etoposide (10 μM), a topoisomerase II inhibitor and DNA-damaging agent that also utilizes the mitochondrial apoptosis pathway (35). For each agent, we used concentrations that evoked significant, near-maximal apoptosis in HeLa D98 cells (data not shown). First, we noted temporal differences in the onset of FRET disruption. In the case of TNFα or staurosporine, the onset of effector caspase activation occurred early (Fig. 2A, upper and middle panels, respectively) and could be detected after an average time of 275 ± 22 min (n = 23 cells in two 8-h experiments) and 280 ± 19 min (n = 39 cells in two 11-h experiments), respectively. The onset of effector caspase activation was more delayed in cells treated with etoposide (Fig. 2A, lower panel), averaging 885 ± 44 min (n = 32 cells in two 22-h experiments). These values correlated with the time course of caspase-3-like protease activity in bulk analyses determined by measuring...
the cleavage of the fluorogenic caspase substrate Ac-DEVD-AMC by cytosolic extracts (Fig. 2B and Fig. 3A).

Despite these differences in the lag time until onset of the FRET disruption, we did not detect significant differences in the cleavage kinetics once the changes in CFP/YFP emission ratio were detected (Fig. 2A). For a quantification of single-cell kinetics, the values of the CFP/YFP emission ratio were fitted for each cell with the sigmoidal Boltzmann equation (see “Experimental Procedures”). In this analysis, \( dx \) determines the width of the turnover and, hence, represents a measure of the cleavage kinetics. Statistical evaluation of the \( dx \) data employing one-way analysis of variance and subsequent Tukey’s test confirmed these results and yielded no significant differences in the cleavage kinetics induced with the three treatments (Fig. 2C).

**Kinetics of FRET Probe Cleavage in Response to STS or TNFα/CHX Are Only Minimally Affected by Drug Concentration**—The previous experiments suggested that the kinetics of FRET probe cleavage in HeLa D98 cells were independent of the type of stimulus using concentrations of the pro-apoptotic...
agents that elicited near-maximal responses in these cells. In the next set of experiments we compared the influence of maximal and submaximal drug concentrations on the FRET probe cleavage. HeLa D98 cells were exposed to 30 or 200 ng/ml TNF-α (plus 1 μg/ml CHX). In bulk analyses, treatment with 30 ng/ml TNF-α resulted in a moderate, yet steady increase in caspase-3-like protease activity, peaking at 12 h (Fig. 3A, left panel). In contrast, exposure to 200 ng/ml TNF-α led to a more rapid and pronounced increase in caspase-3-like protease activity peaking at 6 h (Fig. 3A, right panel). Lower enzyme activity was observed for all time points with 30 ng/ml TNFa.

Similar results were obtained in bulk analyses of cultures treated with low (0.1 μM) or high (3 μM) concentrations of staurosporine (0.1 μM, peak at 12 h with a cleavage activity of 19.0 ± 0.7 A.U. h⁻¹ μg protein⁻¹; 3 μM, peak at 6 h with a cleavage activity of 76.2 ± 2.3 A.U. h⁻¹ μg protein⁻¹; vehicle-treated controls, cleavage activity of 6.2 ± 0.9 A.U. h⁻¹ μg protein⁻¹; n = 4 cultures per treatment and time point).

FRET analysis of single cells exposed to 30 or 200 ng/ml TNF-α revealed that the onset of FRET disruption was significantly delayed when the cells were exposed to the low TNF-α concentration (Fig. 3, B and C). The average time until the probe cleavage was observed shifted from 275 min with the higher TNF-α concentration to 612 min with the lower TNF-α concentration (Fig. 3C). Similar results were obtained in cells exposed to low and high staurosporine concentrations (Fig. 3D). Accordingly, lowering the drug concentration reduced the percentage of cells that underwent FRET disruption from 86 to 24% after 8 h of treatment in case of TNF-α (n = 46 and 115 cells in four experiments) and from 48 to 11% after 8 h of treatment in case of staurosporine (n = 53 and 117 cells in four experiments).

However, once initiated, the kinetics of FRET probe cleavage in individual cells were very fast (<15 min) and only marginally affected by the drug concentration (Fig. 3, B and E). Fitting of the CFP/YFP ratios of individual cells with the sigmoidal Boltzmann equation and subsequent analysis of dx values revealed only slightly decreased dx values for the higher drug concentrations. The level of statistical significance was reached in case of the two STS concentrations (Fig. 3E). However, this appeared to be of little functional significance as the time from onset of FRET disruption to maximal cell shrinkage was not significantly altered (0.1 μM STS, 47 ± 3 min; 3 μM STS, 57 ± 6 min; n = 57 and 39 cells; p > 0.1).

Efficient FRET Probe Cleavage in MCF-7 Cells Lacking Caspase-3—To establish the requirement of caspase-3 for the rapid FRET substrate cleavage during apoptosis, we utilized a well established model system, the caspase-3-deficient MCF-7 breast adenocarcinoma cell line (22). We noticed that parental MCF-7 cells transfected with the FRET construct and analyzed by Western blotting also showed significant cleavage of the recombinant FRET probe into its myc-CFP and YFP constituents after treatment with 3 μM staurosporine (Fig. 4A). Treatment of parental MCF-7 cells with staurosporine indeed led to an accumulation of the active caspase-7 p20 subunit (Fig. 4A) (36, 37). The activation of this effector caspase occurred within the same time frame as the cleavage of the FRET probe. As reported previously (33), parental MCF-7 cells failed to generate the 120-kDa alpha-spectrin breakdown product selectively generated by caspase-3 but accumulated significant amounts of the 150-kDa breakdown product that are produced by several effector caspases (Fig. 4A). Evidence has been provided that caspase-7 may be directly activated by caspase-9 (4, 21, 38). Treatment with STS also led to an activation of initiator caspase-9 in MCF-7 cells as demonstrated by the decrease in procaspase-9 expression and a corresponding increase of the active p18 caspase-9 subunit (Fig. 4A).

Subsequently, we transfected caspase-3-deficient MCF-7
cells and MCF-7 cells expressing human caspase-3 (MCF-7/Casp-3) with the recombinant FRET probe. Cells were exposed to vehicle (PBS) for 10 h. Cleavage of fluorogenic Ac-DEVD-AMC by cytosolic extracts was monitored for 1 h using a fluorescent plate reader. Data are means ± S.E. from n = 4 cultures per treatment. a.u., arbitrary fluorescence units. *, p < 0.05, difference from vehicle-treated controls. Experiments were performed twice with similar results. B. CFP/YFP emission ratios of individual HeLa D98 cells expressing myc-CFP-DEVD-YFP in response to 200 ng/ml (diamonds) or 30 ng/ml (open triangles) TNF-α plus (1 μg/ml CHX). Data from three representative cells and the appropriate fitted sigmoidal Boltzmann functions are shown. C and D, scatter plot showing the onset of FRET disruption in cells treated with low and high concentrations of pro-apoptotic agents. Symbols represent first quartile, median, and third quartile (from top to bottom). Data are from two experiments per treatment and concentration. *, p < 0.05 (t test). E, evaluation of cleavage kinetics. $dx$ determines the width of the turnover of the sigmoidal Boltzmann function. Data are means ± S.E. from n = 13–61 cells per treatment. Data were analyzed by Mann-Whitney U test. *, p < 0.05.

response to 30 and 200 ng/ml TNF-α (Fig. 4C). This difference in the kinetics of FRET probe cleavage was also reflected by the fact that MCF-7 cells required a significantly longer time to undergo cell shrinkage (MCF-7/Casp-3 exposed to 3 μM STS, 32 ± 5 min after onset of the FRET probe cleavage; MCF-7 exposed to 3 μM STS, 166 ± 11 min; MCF-7 exposed to 0.1 μM STS, 379 ± 33 min; n = 18, 17 and 41 cells; p < 0.001 compared with MCF-7/Casp-3 cells). Interestingly, caspase-3 overexpression did not accelerate the period to the onset of the FRET probe cleavage following staurosporine treatment (MCF-7 cells, 247 ± 17 min; MCF-7/Casp-3 cells, 361 min ± 41 min; STS 3 μM; data from 6 and 9 transient transfection experiments; p > 0.1). The percentage of cells that underwent effector caspase activation after 8 h of treatment was also not increased in the MCF-7/Casp-3 cells (MCF-7 cells, 64%; MCF-7/Casp-3 cells, 57%; in the 6 and 9 experiments).

Finally, we addressed the question whether differences in the kinetics of caspase substrate cleavage may result in a differential sensitivity to cell death. We therefore performed flow cytometric analyses of propidium iodide uptake into
MCF-7 and MCF-7/Casp-3 cells in response to 0.1 and 3 μM staurosporine. Significant cell death could be observed in both cell lines after 18 h of staurosporine treatment, with only slightly accelerated cell death rates in MCF-7/Casp-3 cells.

**DISCUSSION**

In the present study we employed single-cell FRET analyses to monitor the activation of DEVD-preferring caspases within individual cells during apoptosis. *In vitro*, the 18-amino acid linker region was efficiently cleaved by effector caspase-3 and -7 but also by initiator caspase-8 and -9. These findings are in agreement with previous specificity studies on recombinant caspases (20, 39, 40). Caspase-6 and in particular caspase-2 were less efficient in cleaving the recombinant FRET probe (20, 39, 40), suggesting that the FRET probe monitors the activity of the two major but not all effector caspases. We cannot exclude the possibility that part of the FRET probe cleavage was attributable to the activity of initiator caspase-8 and -9. However, the differential kinetics of FRET probe cleavage in MCF-7 compared with MCF-7/Casp-3 cells suggest that the majority of the activity was due to caspase-3. It should also be noted that caspase-8 may function as an effector caspase during apoptosis (41). Our data indicate that the FRET probe cleavage is a surprisingly rapid and efficient process that is only marginally influenced by the type or severity of the apoptotic stimulus. Bulk studies suggest that treatment of cells with different apoptotic stimuli may result in differential kinetics of FRET probe cleavage. Our results demonstrate that these differences are rather caused by differences in the lag time between onset of stimulus and onset of caspase activation. Differences in the magnitude of effector caspase activation observed in bulk analyses are likewise caused by differences in the percentage of

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**Fig. 4.** Kinetics of effector caspase activation in MCF-7 and MCF-7/Casp-3 cells. A, Western blot analysis of MCF-7 cells expressing the recombinant caspase substrate myc-CFP-DEVD-YFP. Caspase-3-deficient MCF-7 cells were treated with 3 μM STS for the indicated times. Control received the vehicle (Me2SO). Cleavage of the recombinant FRET probe and α-spectrin, the appearance of the active caspase-7 subunit p20, and activation of procaspase-9 were detected by immunoblotting. α-Tubulin served as control for equal loading of the samples. B, CFP/YFP emission ratios of individual MCF-7 and MCF-7/Casp-3 cells expressing myc-CFP-DEVD-YFP in response to 3 μM STS or 0.1 μM STS, respectively. Traces of three representative cells and respective fitted sigmoidal Boltzmann functions are shown. C, evaluation of cleavage kinetics. dx determines the width of the turnover of the sigmoidal Boltzmann function. Data are means ± S.E. from n = 10–50 cells from n = 2–9 experiments. *, p < 0.01 (U test). D, flow cytometric analysis of cell death rates in response to STS determined by propidium iodide uptake. MCF-7 and MCF-7/Casp-3 cells were treated with the indicated concentrations of STS or vehicle (Me2SO) for 16 h. Experiment was performed twice with similar results. ctrl, control.
cells actually undergoing effector caspase activation. Rapid FRET probe cleavage (∼10 min) has been detected previously in response to staurosporine in COS-7 cells transiently transfected with the myc-CFP-DEVD-YFP probe (18), suggesting that rapid cleavage kinetics are common to many cell types. Interestingly, rapid cleavage of the recombinant FRET probe occurred both in the cytosol and in the nucleus (Fig. 1E (18)). This finding is in agreement with previous reports (42, 43) showing that nuclear pores open rapidly during apoptosis and supports the concept that the activation of effector caspases is a rapid and efficient process, encompassing the entire cell.

The release of pro-apoptotic factors such as cytochrome c from the mitochondrial intermembrane space into the cytosol during apoptosis is believed to be a very rapid and coordinated event (16, 17). It is likely that a critical accumulation of pro-apoptotic Bcl-2 family members in the cytosol or outer mitochondrial membrane coordinates the rapid release of pro-apoptotic factors (14) and downstream from this the rapid activation of effector caspases. A rapid, complete release of pro-apoptotic factors may be required for an efficient apoptosis some formation or to efficiently override an apoptosis inhibitory block achieved by inhibitor-of-apoptosis proteins and molecular chaperones (44–47). In future studies, it will be interesting to investigate at the single-cell level whether cytochrome c release is always followed by caspase activation and to determine the temporal relationship between the two events. Our data suggest that rapid FRET probe cleavage is also involved in death receptor-induced apoptosis. In many cell types, activation of death receptors is directly linked to the mitochondrial apoptosis pathway via caspase-8 cleavage of the BH3-only family member Bid (48, 49). Interestingly, MCF-7/Casp-3 cells do not require this positive feedback signal, thereby representing so-called type I cells (50). Here, the successful formation of another multiprotein complex, the death-inducing signaling complex, may be the rate-limiting step for the activation of DEVDases.

FRET disruption was significantly slower in MCF-7 cells that lack caspase-3 compared with MCF-7/Casp-3 cells. Apart from the actual lack of this enzyme, it has been shown that caspase-3 activation represents a positive feedback loop for the activation of procaspase-9 (51, 52). Lack of this feedback loop may cause a slower activation of caspases in MCF-7 cells, particularly at submaximal concentrations of the pro-apoptotic agents. Caspase-3 is also important for the processing and activation of effector caspase-2 (51) and -6 (21) and downstream from this for the activation of caspase-8 (21). Caspase-7, the major effector caspase in MCF-7 cells (36–38, 53), is activated largely independent of caspase-3 (21). It localizes mainly to the light membrane fraction (54). It is conceivable that diffusion processes may also play a role in the slower kinetics of FRET disruption in MCF-7 cells. Interestingly, we did not detect a decreased percentage of MCF-7 cells that underwent FRET probe cleavage in response to staurosporine compared with the MCF-7/Casp-3 cells. It is important to note in this context that the release of cytochrome c after activation of the mitochondrial apoptosis pathway is believed to be a caspase-independent process (16, 55), a finding that we have also observed in MCF-7 cells (17). Interestingly, the release of the pro-apoptotic factor Smac/DIABLO from mitochondria has been suggested to be a caspase-dependent process (56). Smac/DIABLO facilitates effector caspase activation during apoptosis by neutralizing the activity of inhibitor-of-apoptosis proteins (57, 58). Other studies (59, 60) have shown that Smac release can precede cytochrome c release and subsequent caspase-3 activation. The release of Smac from mitochondria could represent an important regulatory step for the (kinetics of) effector caspase activation. Its release could be significantly slowed down in MCF-7 cells, particularly at submaximal pro-apoptotic stimuli. This could lead to the significantly slower kinetics of FRET disruption observed under these conditions.

The slower cleavage kinetics of caspases in MCF-7 compared with MCF-7/Casp-3 cells were of functional significance. For example, MCF-7 cells required a longer time to undergo cell shrinkage after onset of the FRET probe cleavage. Likewise, MCF-7 cells do not exhibit oligonucleosomal DNA fragmentation (22). However, fluorescence-activated cell sorter analyses demonstrated no pronounced differences in the total cell death rate between the two cell types after treatment with staurosporine, both at maximal and submaximal staurosporine concentrations. Activation of effector caspases may therefore represent a "point-of-no-return" in mammalian cells with multiple caspases being able to compensate for each other. However, alternative cell death pathways activated by the release of pro-apoptotic factor from mitochondria may also mediate cell death. These include a mitochondrial dysfunction program with subsequent ATP depletion and free radical production directly due to the loss of cytochrome c (61–63). Nerve growth factor-deprived sympathetic neurons, for example, can only be rescued to the time point of mitochondrial dysfunction in the presence of caspase inhibitors (64). The pro-apoptotic activity of apoptosis-inducing factor and endonucleases may also come into play once the activation of effector caspases is blocked (65–67).

Further single-cell studies are required to answer the question at which stage in the apoptotic cascade cells are able to escape the death process. Studies in trophic factor-deprived neurons have provided evidence that cytochrome c release is a reversible event (64, 68). In our experiments, all cells that underwent FRET probe cleavage showed subsequent cell shrinkage (average lag time in HeLa D98 cells, 42 ± 2 min), but it still remains unclear whether all these cells are indeed irreversibly damaged. We monitored HeLa D98 cells exposed to low dose staurosporine (0.1 μM) up to 10 h after the FRET probe cleavage had occurred. Interestingly, only 44 and 60% of these cells were disrupted into apoptotic bodies or underwent secondary necrosis 7 and 10 h after the onset of the FRET probe cleavage (n = 48 and 30 cells, respectively). In mutants of the nematode Caenorhabditis elegans, it has been demonstrated that cells are able to escape cell death after caspase activation (25, 69). However, C. elegans expresses only one protein of the caspase family for the execution of apoptosis. Likewise, evidence is lacking whether activation of apoptosis in C. elegans also involves a mitochondrial organellae dysfunction program (70). Further single-cell studies that allow recovery of cells from pro-apoptotic stimuli over a prolonged period are necessary to establish whether mammalian cells are indeed able to escape cell death once effector caspases are activated.

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