The Kinetics of Translocation of Smac/DIABLO from the Mitochondria to the Cytosol in HeLa Cells†

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Running Title: Kinetics of Smac/Diablo Release from Mitochondria
Summary

Smac, (second mitochondrial activator of caspases), is released from the mitochondria during apoptosis to relieve inhibition of caspases by the IAPs (Inhibitor of Apoptosis Proteins). The release of Smac antagonizes several IAPs and assists the initiator caspase-9 and effector caspases (-3, -6, -7) in becoming active; ultimately leading to attack on cellular structural targets and death of the cell. Translocation of Smac along with cytochrome c and other mitochondrial pro-apoptotic proteins represent important regulatory checkpoints for mitochondrial-mediated apoptosis. Whether or not Smac and cyt. c translocate by the same mechanism is not known. Here, we show that the time required for Smac efflux from the mitochondria of cells subjected to staurosporine induced apoptosis is approximately four times longer than the time required for cytochrome c efflux. These results suggest that Smac and cytochrome c may exit the mitochondria by different pathways.

Introduction

Apoptosis (programmed cell death) plays key roles in both normal development and pathophysiology. Given the central importance of programmed cell death, apoptotic pathways are highly regulated, containing many checkpoints. Failures of regulatory checkpoints are pathogenic, the effects of which range from neurodegeneration to cancer(1,2).

Apoptosis proceeds by two major signaling pathways, known as the “intrinsic” (mitochondrial) and “extrinsic” (death receptor) pathways. One early event in mitochondrial-mediated apoptosis is the activation of the Bcl-2 family of proteins(3). They are important in controlling the release of regulatory mitochondrial proteins, which are sequestered in the mitochondrial intermembrane space^1 (IMS) until signaled. There is substantial debate about the mechanisms for release of mitochondrial proteins in apoptosis(3-5).
When apoptosis is initiated, Smac in its processed form of 184 amino acids (21 kDa) is released from the mitochondria to interact with various IAPs, including XIAP, c-IAP1, and ML-IAP(6-8). Smac performs its function of antagonizing XIAP by competing for the same binding site as that of caspase-9 on the BIR3 domain of XIAP, as well as binding to the linker peptide between the BIR1 and BIR2 domains, sterically inhibiting access to the caspase-3 and –7 binding site(9).

The detailed mechanism of Smac’s translocation from the mitochondria to the cytosol remains unknown and the kinetics of Smac release from a single cell have not been measured. While the specific Bcl-2 family members which cause the release of mitochondrial proteins remain controversial, one key question is: do Smac, cytochrome c and other mitochondrial proteins exit the mitochondria on the same time scale and via the same pathway? Studies on cell populations indicate that Smac and cyt. c exit the mitochondria on the same general time scale, and a single transport mechanism has been suggested. However, such studies are not sufficiently sensitive to resolve the temporal relationship between cyt. c and Smac release or address the duration of release in individual cells undergoing apoptosis. In several experiments, cyt. c release is shown to be caspase independent, while the release of Smac is not(10,11). This has led some researchers to suggest that Smac release may be a downstream event of cyt. c release. A single cell analysis of cytochrome-c-GFP stably expressed in HeLa cells has been performed by Goldstein et al. and the kinetics of this proteins release characterized(12). Here, we report a detailed kinetic analysis of the release of Smac-EYFP from the mitochondria into the cytosol of individual HeLa cells. These data allow direct comparisons of cyt. c and Smac transport kinetics, and thereby can probe whether a single mechanism describes transport of both proteins.
Experimental Procedures.

*Generation of the recombinant plasmid pSmac-EYFP*

The creation of a fusion protein between Smac and the fluorescent protein EYFP was chosen as the method for studying the kinetics of Smac’s apoptotic release from the mitochondria. The plasmid pEYFP-N1 (Clontech) were used to perform the subcloning.

The plasmid pcDNA3.1(-) (Invitrogen) with full-length Smac inserted between the \(XhoI\) and \(BamHI\) sites in the multiple cloning site was a gift from Prof. Yigong Shi and was used as the template for the described PCR reaction. Primers, Smac1 and Smac2 (Keystone Labs), were chosen to anneal 5’ and 3’ ends of the full-length Smac gene in the plasmid pcDNASmac3.1(-). The primers were engineered to include \(XhoI\) and \(BamHI\) sites, respectively, which were employed in later steps of the subcloning procedure. Biotin tags were also included to provide an easy assay for completion of restriction digestion.

A PCR reaction with Smac1, Smac2 and pcDNASmac3.1(-) as the template was performed under the following conditions: 1x 94°C for 1min, 25 x (94°C for 1min; 48°C for 90 sec; 72°C for 90 sec), 1 x 72°C for 10min). Amplification of the target sequence of the correct size was confirmed by gel electrophoresis. The PCR product was subjected to restriction digestion with \(XhoI\) and \(BamHI\). The DNA was digested with HK Phosphatase (Epicentre Technologies). The digested Smac fragment was ligated to digested pEYFP-N1 using T4 DNA ligase (Epicentre Technologies) overnight at 16°C. The resulting plasmid was transformed into the *E. coli* strain XL2- Blue (Stratagene) and transformants were selected on medium containing 30μg/mL Kanamycin. The correct DNA sequence was confirmed using DNA sequencing analysis.
Generation of a stable cell line expressing Smac-EYFP

The plasmid pSmac-EYFP was transfected into HeLa cells using GenePORTER (Gene Therapy Systems, San Diego, CA). Cells were analyzed for fluorescence 48 hours post-transfection under a fluorescence microscope. All cells were incubated at 37°C, 5% CO₂.

Geneticin (Gibco) was added to the medium at a concentration of 1mg/mL. Over three days, geneticin was diluted to a final concentration of 100 µg/mL. Surviving cells were grown for 1 week, and one part of the cell population was seeded into 96 well plates at a density of 0.3 cells/well and propagated at 100 µg/mL geneticin. When grown to confluence, wells with cells exhibiting the desired fluorescence pattern were propagated. After 2 weeks, 40 colonies exhibiting the desired pattern of fluorescence were expanded. Briefly, each fluorescent colony was picked and transferred to a solution of trypsin, and incubated for 15min at 37°C. Cells were then transferred to a 24 well dish and grown in DMEM containing 10% FCS and 1mg/mL geneticin. When grown to confluence, cells showing the desired fluorescence pattern were propagated and a frozen stock of the stable cell line was created.

Confocal Microscopy.

All confocal microscopy was performed on a Zeiss LSM 510 confocal microscope. Time resolved images were obtained using a Plan NeoFluar 40x 1.3NA oil immersion objective, and all other images were obtained on a C Apo 1.2 NA water immersion objective.

EYFP was excited with a 514 nm laser line from a 25mW Argon laser with an AOTF setting of 1% and emission collected from 530 to 600 nm. DAPI nuclear stain (Molecular Probes) was excited with a 364 nm laser line from an 80 mW Argon laser with an AOTF setting of 4% and emission collected from 385 nm to 470 nm. MitoFluor Far Red (Molecular Probes)
was excited with a 633 nm Helium/Neon laser line from a 5 mW laser with an AOTF setting of 15% and emission collected on a 650 long pass filter. LysoTracker (Molecular Probes) was excited with a 364 nm laser line from a UV laser with an AOTF setting of 15% and emission collected from 385 to 470 nm.

For DAPI nuclear staining experiments, DAPI (Molecular Probes) was diluted 1:1000 in a 1:1 solution of sterile water and phosphate-buffered saline (PBS) to a final concentration of 100 nM. After fixing, 1 mL DAPI solution was added to fixed cells in the 60 mm dish and incubated for 20 minutes at room temperature. Cells were then rinsed briefly with PBS and mounted.

For Mito-Tracker experiments, DMEM + 10% FCS containing 500 nM MitoFluor Far Red (680) (Molecular Probes) was added to cells. Cells were incubated for 45 minutes at 37°C and 5% CO₂ and medium was replaced with phenol-red free DMEM supplemented with 25mM HEPES and 10% FCS.

Cells with stable expression of the Smac-EYFP fusion protein were stained with DAPI and fixed at 0, 2, 4, 6, and 16 hours after incubation with 2.14 µM staurosporine. Several images were taken of representative populations of cells at each time point. Approximately 300 cells were scored at each time point. Cells were scored for apoptosis by three factors: morphology, fluorescence, and nuclear DNA fragmentation. A flat cell with many projections on the surface toward other cells was noted to exhibit normal cell morphology, whereas a cell with a rounded appearance, lacking projections was scored as exhibiting an abnormal morphology. A cell with uniform nuclear stain was scored as a normal cell nucleus, and a cell with broken or globular staining was marked as an apoptotic nucleus. Fluorescence was scored as punctate, diffuse, or not observed. The fraction of cells exhibiting an apoptotic phenotype was calculated by taking
the ratio of cells with abnormal features to the total number of cells at that time point. Smac-EYFP release as judged by a diffuse fluorescence pattern was determined for each time point by taking the ratio of the number of cells exhibiting diffuse fluorescence to the total number of cells exhibiting fluorescence at that time point.

**Time resolved Confocal Microscopy.**

Twenty-four hours before imaging, cells were plated to a black covered 15mm Delta T culture dish (Bioptechs) and grown overnight at 37°C under a 5% CO₂ atmosphere to be 50% confluent at the time of imaging. Cells were washed in phenol-red free DMEM supplemented with 25mM HEPES and 10% FCS. The dish was inserted into the stage adapter (Delta T Stage Adapter, Bioptechs). Cells were treated with staurosporine (2.14 µM). The experimental conditions were held constant at 37ºC with a 5% CO₂ atmosphere for the entire imaging period. Time sequences were collected by taking five optical slices at a 2 µm interval once every two minutes. Imaging and cell preparation was identical for a control data set, where no staurosporine was added to the cells. Images were analyzed using the LSM 510 software (v2.8) by projecting optical slices using maximum intensity mode.

**Results**

*Characterization of HeLa cell line stably expressing Smac-EYFP.*

To examine the translocation kinetics of Smac from the mitochondria to the cytosol, we generated a cell line expressing a fusion of Smac and EYFP (enhanced yellow fluorescent protein). Details of the cell line construction are presented in the Experimental Procedures. The sub-cellular location of the fusion protein was confirmed using the fluorescence pattern of EYFP
as a localization diagnostic. The punctate pattern of fluorescence emitted by Smac-EYFP and the observed co-localization of the fusion protein with a dye that specifically associates with mitochondrial membranes (MitoFluor Far Red (680), Molecular Probes) shows that the Smac-EYFP, like endogenous Smac (6), is localized in the mitochondria (see Figure 1).

In the Smac-EYFP cell line created, a minority of the cells expressed the fusion protein at observable levels. To increase the probability of observing a release event the fluorescent cells were enriched using fluorescence activated cell sorting. After sorting, ~90% of the cells fluoresced and exhibited a punctate pattern of emission emanating from the mitochondria.

**Ordering of Smac Release Relative to other Apoptotic Markers**

During apoptosis, characteristic changes to the cellular structure occur. These include: cell shrinkage, membrane blebbing, changes to the plasma membrane structure, chromatin condensation and fragmentation of nuclear DNA (13). We sought to establish the order of Smac-EYFP release among some other apoptotic events. Thus, on a population of cells, we investigated the time dependence of cell shrinkage, Smac release (as judged by the observed fluorescence pattern) and DNA fragmentation after induction of apoptosis. Cells were fixed, stained with DAPI, and imaged on a confocal microscope at 0, 2, 4, 6, and 16 hours after incubation with 2.14 µM staurosporine. At each time point, cells were scored for morphology, release of Smac-EYFP (punctate or diffuse fluorescence pattern) and DNA fragmentation. See Experimental Procedures for a complete description of scoring criteria. Abnormal cell morphology was the leading indicator of apoptosis, with a rapid increase in the number of rounded, shrunken cells apparent between 2 and 4 hours after apoptosis induction and another sharp increase between 4 and 6 hours after induction (Figure 2). Cells exhibiting a diffuse
fluorescence profile became apparent between 2 and 4 hours after induction of apoptosis, but the percentage of cells exhibiting an apoptotic profile increased more slowly than cell shrinkage. The apoptotic character of the nucleus proved to be the lagging indicator of apoptosis. The fraction of apoptotic nuclei began to increase between 4 and 6 hours (Figure 2). By 16 hours after induction of apoptosis, nearly all cells were rounded, exhibited diffuse fluorescence and fragmented nuclear DNA.

**Single Cell Kinetics of Smac-EYFP Translocation**

Upon translocation of Smac-EYFP to the cytosol, the pattern of fluorescence changes from punctate to diffuse corresponding with a change in effective concentration of the fluorophore from high to low (see Figure 3a). Using time resolved confocal microscopy, this translocation event was monitored in live cells in real time. In each experiment, a field of cells was monitored at 37°C and 5% CO2. The apoptotic stimuli used was staurosporine (2.14 µM), a well known kinase inhibitor that induces programmed cell death via the mitochondrial pathway(14). In three separate experiments, multiple cells were observed releasing Smac-EYFP. The length of each experiment ranged from 3-6 hours with pre-incubation of the cells for 1-2 hours before imaging began. Five optical slices that span the depth of the cell were imaged every two minutes. The optics were optimized so that photobleaching was negligible over the course of the experiments. The projected fluorescence along the z-axis was used for analysis. To determine the duration of release, regions were drawn around individual cells and the brightness of the region computed using Image Pro. Brightness was determined by summing the number of pixels with an intensity value between 150 and 255 on a scale of 0 to 255 where 0 represents a black pixel and 255 represents the brightest pixel. Pixels with intensity values
between 1-149 were also summed. A plot of pixel summation vs. time allows analysis of both the decay of the punctate (bright) fluorescence as well as the growth of the diffuse (dim) fluorescence as a function of time (see Figure 3b for a representative kinetic profile). The induction time between exposure to staurosporine and release of Smac-EYFP varied in each individual cell monitored, but the duration of release of Smac-EYFP was consistently found to be about 19 ± 3 minutes. In some cells, it appears that nearly all the Smac-EYFP is released from the mitochondria, in other cases only 60-80% of the protein is released.

**Discussion**

The release of pro-apoptotic factors from the mitochondria is a key step in a cell’s final commitment to cell death. Despite this, the detailed mechanism(s) for the release of Smac (and other pro-apoptotic mitochondrial proteins) remains unknown. In addition, whether pro-apoptotic factors such as Smac and cytochrome c translocate *via* the same pathway is not known. Smac release provides the fatal blow of the “Cyt. c-Smac punch”, thus the idea that their release may be coupled is reasonable(15). However, whether or not such an event is in fact coordinated is not known. Some studies indicate that cyt. c and Smac exit the mitochondria at the same time(10,16), but these are a result of population rather than single cell analyses, and thus cannot accurately assess the temporal ordering of events in individual cells.

In this work, we have determined that the translocation of Smac (tagged with the fluorescent protein EYFP) from the mitochondria to the cytosol of HeLa cells occurs in 19 ± 3 minutes. Previous work by Goldstein *et al.* reported(12) that in HeLa cells undergoing apoptosis, the duration of cyt c-GFP release is 5 ± 2.5 minutes (see Figure 3c). Thus, our results show that Smac release does not occur on the same time scale as cytochrome c release in this cell
type. While cyt. c-GFP release is rapid and complete, the release of Smac-EYFP requires nearly four times as long and its release is frequently only 60-80% complete. These data would suggest that the mechanism for Smac translocation may be different from that for cyt. c release. Whether commencement of cyt c and Smac release is coincident in each cell undergoing apoptosis cannot be ascertained here; however, microscopy experiments to test whether or not this is the case are underway.

Evidence supporting the idea that Smac and cyt. c exit the mitochondria via different pathways has emerged (10,11). Adrain et al. find that while Bcl-2 appears to regulate both cyt c and Smac efflux from the mitochondria, cyt. c release is caspase independent, while the release of Smac is blocked in the presence of the broad based caspase inhibitor Z-VAD-fmk(10). This result indicates that Smac release requires active caspases. Based on all previous models for mitochondrial apoptotic pathways, this result suggested that Smac release may be a downstream event of cytochrome c release. Adrain et al. proposed a model of caspase regulated release of Smac from the mitochondrial IMS. In this model, cyt. c release precedes Smac release and promotes apoptosome assembly and caspase-9 activation. In the absence of Smac, caspase activity is attenuated by XIAP. According to their model, this may trigger, in some as yet undefined way, a caspase-mediated “attack” on the outer mitochondrial membrane, which then allows the efflux of Smac and relief of caspase inhibition to occur(10).

Contrary to this idea, new results indicate that the Bcl-2 family could be involved in the direct activation of caspases, such as caspase-2, prior to the release of mitochondrial proteins(17-20). For instance, caspase-2 activity is found to occur upstream of mitochondrial release of cyt. c or Smac in cell stress related apoptosis(19,20). Further, in certain cell lines, cyt c.release, Smac release and, notably recruitment of Bax to the mitochondria are inhibited by RNA
interference of caspase-2(17). These data support a model wherein cell stress initiates caspase-2 activation, which then causes permeabilization of the outer mitochondrial membrane OMM in a manner that is still not well defined, but likely involves the truncation of Bid and Bax recruitment to the OMM. While caspase-2 may be responsible for initiating release of mitochondrial proteins, it is still unclear whether this event will cause the concomitant release of cyt. c and Smac through the same pore. Further, the observation in several studies(10,11) that cyt. c release is caspase independent while Smac release requires active caspases requires further understanding.

Three largely debated mechanisms for the efflux of the mitochondrial protein cyt. c include 1) a pore comprised of Bax subfamily members, 2) a novel pore formed by the Bax subfamily in conjunction with the Voltage Dependent Anion Channel (VDAC) or 3) rupture of the outer mitochondrial membrane as a result of a permeability transition(21). During a permeability transition, the \(\Delta\Psi_m\) is lost, the permeability transition pore (PTP) opens, the mitochondrial matrix swells and finally the outer membrane ruptures allowing release of proteins into the cytosol.

Given the differences in size and shape of Smac and cyt. c, it is necessary to consider the possibility that these proteins diffuse through the same pore, but the kinetic of diffusion for the larger, elongated Smac dimer are retarded. Simple diffusion models where cyt. c is considered a sphere and the Smac dimer a prolate ellipsoid cannot account for the fourfold difference in the translocation kinetics of cyt. c and Smac. Based on electrophysiological data, Tsujimoto (22) proposed a novel Bax-VDAC channel has a pore size of approximately 120 Å. According to our calculation for diffusion through an orifice of a heterogeneous medium(23), release through this size channel would only marginally affect the rate of Smac release relative to
that of cyt. c. Solid evidence supporting a particular pore size for the proposed “Bax-only”
channels does not exist, making more detailed calculations for this case less meaningful. Such
models, while oversimplified, provide an indication that the translocation kinetics of Smac
suggest a distinctly different release mechanism for Smac than for cytochrome c.

Several researchers have found that cyt. c release precedes mitochondrial depolarization.
Madesh et al. observed that substantial tBid induced cyt. c release begins before the onset of
mitochondrial depolarization in permeabilized HepG2 cells(16). In another study involving live
HeLa cells, loss of mitochondrial transmembrane potential, ∆Ψm, was found to require at least
12 minutes, and begins after a significant fraction of cyt. c has been released(24). Given these
results and other experimental evidence(12), a mechanism for cyt. c release that is distinct from a
permeability transition has been widely argued. It will be interesting to determine whether or not
Smac release precedes, coincides or occurs after mitochondrial depolarization in HeLa cells.
Such experiments are in progress.

Currently, there is no evidence to suggest that Smac release should precede cyt. c release.
Here, we show that in HeLa cells the time required for Smac release is four times that measured
for cyt. c release. In two separate studies(16,24), mitochondrial depolarization began after (but
within 5 minutes) of the onset of cyt. c release and required ~12 minutes. Thus, given the
current measurements of Smac efflux, it appears that Smac release, requiring 19 ± 3 minutes,
occurs during or after mitochondrial depolarization.
References.


Footnotes

1. The abbreviations used are: IMS, intermembrane space; IAP, inhibitor of apoptosis; BIR, baculoviral IAP repeat-containing; cyt. c, cytochrome c; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; Smac, second mitochondria-derived activator of caspases; DAPI, 4’,6-diamino-2-phenylindole, dihydrochloride; zVAD-fmk, N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone; OMM, outer mitochondrial membrane; VDAC, voltage dependent anion channel; PTP, permeability transition pore.
2. This model might be expected to prevail in the case of efflux through large pores of the type expected when a permeability transition occurs.

**Figure Legends**

Figure 1. Co-localization of Smac-EYFP and a mitochondrial membrane specific dye. (a) HeLa cell line expressing Smac-EYFP was incubated as described in “Experimental Procedures” with MitoFluor Far Red (680). (b) the punctate pattern of Smac-EYFP fluorescence is shown in green and (c) the majority of Smac-EYFP expressed co-localizes with MitoFluor Far Red (colocalization appears yellow). No colocalization with lysotracker (shown in blue) was observed.

Figure 2. Percentage of cells exhibiting an abnormal, rounded morphology, Smac-EYFP release and DNA fragmentation. Smac-EYFP release occurs after cell shrinkage, but before DNA fragmentation.

Figure 3. The kinetics of translocation of Smac-EYFP from the mitochondria to the cytosol of HeLa cells during apoptosis. (a) Time lapse images of a HeLa cell releasing Smac-EYFP. Staurosporine (2.14 µM) induced apoptosis was initiated 186 minutes before the first image displayed. (b) The sum of the bright (squares) and dim(circles) pixels in a region drawn around a cell releasing Smac-EYFP as a function of time. (c) Comparison of the time required for release of Smac-EYFP and cytochrome c-GFP in HeLa cells induced to undergo apoptosis using staurosporine.
Figure 1.
Figure 2.
Figure 3.
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