On the Evolutionary Conservation of the Cell Death Pathway: Mitochondrial Release of an Apoptosis-inducing Factor during *Dictyostelium discoideum* Cell Death

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Mitochondria play a pivotal role in apoptosis in multicellular organisms by releasing apoptogenic factors such as cytochrome *c* that activate the caspases effector pathway, and apoptosis-inducing factor (AIF) that is involved in a caspase-independent cell death pathway. Here we report that cell death in the single-celled organism *Dictyostelium discoideum* involves early disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) that precedes the induction of several apoptosis-like features, including exposure of the phosphatidyl residues at the external surface of the plasma membrane, an intense vacuolization, a fragmentation of DNA into large fragments, an autophagy, and the release of apoptotic corpses that are engulfed by neighboring cells. We have cloned a *Dictyostelium* homolog of mammalian AIF that is localized into mitochondria and is translocated from the mitochondria to the cytoplasm and the nucleus after the onset of cell death. Cytoplasmic extracts from dying *Dictyostelium* cells trigger the breakdown of isolated mammalian and *Dictyostelium* nuclei in a cell-free system, and this process is inhibited by a polyclonal antibody specific for *Dictyostelium discoideum* apoptosis-inducing factor (DdAIF), suggesting that DdAIF is involved in DNA degradation during *Dictyostelium* cell death. Our findings indicate that the cell death pathway in *Dictyostelium* involves mitochondria and an AIF homolog, suggesting the evolutionary conservation of at least part of the cell death pathway in unicellular and multicellular organisms.

INTRODUCTION

Programmed cell death (PCD) is a genetically regulated physiological process of cell suicide that is central to the development and homeostasis of multicellular organisms (Raff, 1992; Steller, 1995; Jacobson *et al.*, 1997; Vaux and Korsmeyer, 1999). The basic machinery that controls the

onset of PCD in roundworms (*Caenorhabditis elegans*), insects (*Drosophila melanogaster*), and vertebrates (mammals) appears to be present in all cells, at all times. Crucial aspects of PCD appear to be conserved, including both the genes encoding the basic cell death machinery, and the morphological and biochemical features of apoptosis, the most frequent phenotype of PCD (Jacobson *et al.*, 1997; Horvitz, 1999; Song and Steller, 1999; Vaux and Korsmeyer, 1999).

Mitochondria play a pivotal role in PCD in mammalian cells, in particular through the permeabilization/disruption of their outer membrane, with (or followed by) the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) (Kroemer *et al.*, 1995; Green and Reed, 1998; Petit *et al.*, 1998; Goldstein *et al.*, 2000; Martinou *et al.*, 2000), leading to the release of cytochrome *c* (Liu *et al.*, 1996) and apoptosis-inducing factor

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Abbreviations used: AIF, apoptosis-inducing factor; BSA, bovine serum albumin; DIF-1, differentiation-inducing factor-1; PCR, polymerase chain reaction; $\Delta\Psi_m$, mitochondrial transmembrane potential; PCD, programmed cell death; PPIX, protoporphyrin IX; PBS, phosphate-buffered saline.

(AIF) into the cytosol (Susin *et al.*, 1999). The cytochrome *c* takes part in the activation of caspases, which are major effectors of PCD (Thornberry and Lazebnik, 1998), whereas AIF is involved in a caspase-independent cell death pathway (Susin *et al.*, 1999).

Although it was initially assumed that PCD arose with multicellularity and would have been counterselected in unicellular organisms (Raff, 1992; Vaux *et al.*, 1994; Evan *et al.*, 1995; Steller, 1995) several recent findings indicate that a process of PCD also operates in single-celled eukaryotes (Ameisen, 1996). This has now been described in six species of unicellular eukaryotes, whose phylogenetic origins arose 1 to 2 billion years ago. These are the free-living slime mold *Dictyostelium discoideum* (Cornillon *et al.*, 1994); the kinetoplastid parasites *Trypanosoma cruzi* (Ameisen *et al.*, 1995), *Trypanosoma brucei rhodensiense* (Welburn *et al.*, 1996), and *Leishmania amazonensis* (Moreira *et al.*, 1996); the free-living ciliate *Tetrahymena thermophila* (Christensen *et al.*, 1995); and the dinoflagellate *Peridinium gutanense* (Vardi *et al.*, 1999).

The cell death phenotype in unicellular eukaryotes is similar (*D. discoideum*) (Cornillon *et al.*, 1994) or almost identical (*T. cruzi*) (Ameisen, 1996) to the PCD phenotype of multicellular animals or plants. However, nothing is currently known about the cell death machinery or the genetic control of PCD operating in these single-celled eukaryotes, apart from the fact that developmental cell death appears to be caspase-independent in *D. discoideum* (Olie *et al.*, 1998). Thus, we cannot say how different or similar the processes are in unicellular and multicellular animals.

This report shows that cell death in *Dictyostelium* has several features in common with mammalian cell PCD, including a loss of mitochondrial $\Delta\Psi_m$ followed by the exposure of cell surface phosphatidyl serine, the loss of nuclear DNA, and the engulfment of dying cells by neighboring cells. We have cloned and characterized one of the putative effectors involved in DNA degradation during cell death, a *Dictyostelium* homolog of mammalian AIF (DdAIF). It is released into the cytosol and targeted to the nucleus during cell death mediated by protoporphyrin IX (PPIX) and during developmental cell death induced by differentiation-inducing factor-1 (DIF-1). Cytoplasmic extracts from dying *Dictyostelium* cells triggered the partial degradation of isolated mammalian and *Dictyostelium* nuclei in a cell-free system. This process was prevented by immunodepletion with the use of a polyclonal anti-DdAIF antibody. These findings indicate that the cell death pathway of *Dictyostelium* amoebae involves mitochondria and an AIF homolog, and therefore may have evolved from the same ancestor as the cell death pathway of mammalian cells.

MATERIALS AND METHODS

Cells and Culture Conditions

D. discoideum cells, from the cloned Ax-2 strain (Watts and Ashworth, 1970), were grown in suspension in HL5 medium (Susman, 1987) on a gyratory shaker (150 rpm) at 22–23°C in a water-saturated atmosphere. Cultures were grown in 50-ml flasks with proper oxygenation (culture volume was 1/5 of the total). Conditioned media were prepared by starving a suspension of 4×10^7 *Dictyostelium* cells/ml in Soerensen buffer (100 mM Na_2HPO_4 , 735 mM KH_2PO_4 , 17 mM phosphate, final solution pH 6.8) for 24 h, on a gyratory shaker (150 rpm) at 22°C. A cell-free supernatant was prepared 22 h after initiation of starvation, by centrifuging the

starved cell suspension at $700 \times g$ for 5 min. These supernatants were immediately frozen and kept at -20°C . The exocytotic vesicles were prepared from cells starved for 22 h by centrifugation at $700 \times g$ then membranes were discarded by centrifugation at $1500 \times g$, exocytotic vesicles were collected by centrifugation at $6,500 \times g$. Experiments on developmental cell death were performed in 50-ml plastic flasks (Kay *et al.*, 1987). Briefly, logarithmically growing *Dictyostelium* cells were washed twice with Soerensen buffer (pH 6.0), incubated in the absence or presence of 3 mM cAMP (Sigma, St. Louis, MO) at 10^6 cells/ml for 8 h, and then treated with DIF-1 [1-(13,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone; Molecular Probes, Eugene, OR] (100 nM for 16 h).

Flow Cytometry Analysis

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was measured by incubating cells (5×10^5 /ml) with 3,3'-dihexyloxycarbocyanine iodide [$\text{DiOC}_6(3)$; Molecular Probes; final concentration 2.5 nM (Petit *et al.*, 1995) with modifications (Rottenberg and Wu, 1998)] or 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1) staining, which was performed as previously described (Kuhnel *et al.*, 1997). Cells were analyzed with the use of a FACS Vantage (BD Biosciences, San José, CA), gating the forward and side scatter to exclude debris. The fluorescence was excited with an argon laser (excitation wavelength 488 nm) and collected in FL-1 [band pass 530 ± 30 nm for $\text{DiOC}_6(3)$ and JC-1 green] or FL-2 (band pass 585 ± 20 nm for JC-1 red), after suitable compensation. A minimum of 5×10^3 events was acquired in list mode and analyzed with Cellquest software (BD Biosciences).

Phosphatidylserine (PS) exposed on the outer plasma membrane was measured by staining cells with annexin V-fluorescein isothiocyanate (FITC) (1 $\mu\text{g}/\text{ml}$, 10 min, 4°C; Immunotech, Marseille, France). Relative DNA content was assessed with the use of propidium iodide (1 mg/ml) with 2% saponin.

Viability of the *Dictyostelium* cells was assessed with the use of TO-PRO-3 (Molecular Probes) at 1 $\mu\text{g}/\text{ml}$ final in FL4 (661 ± 16 nm) to avoid any red fluorescence from the accumulated PPIX.

Transmission Electron Microscopy

Dictyostelium cells were fixed in 1.25% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4) for 24 h at 4°C, dehydrated with ethanol at 4°C, and immersed in a 1:1 mixture of propylene oxide and Epon. They were embedded in Epon by polymerization at 60°C for 48 h and examined under the electron microscope (Ryter and de Chastellier, 1977).

Scanning Electron Microscopy

Cell suspensions were fixed for at least 24 h in a 1.25% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate (pH 7.4). Aliquots were filtered through a (diameter 25 mm, 0.2 μm) Anodisc (Whatman, Maidstone, United Kingdom) and the filters were rinsed five times for 10 min in the sodium phosphate buffer. Cells were postfixed for 2 h in 1% osmium tetroxide in sodium phosphate, rinsed five times in Ultrapure water, dehydrated in a graded ethanol series (50, 70, 95, and 100% twice), soaked in isopentyl acetate, and critical point dried in a CO_2 medium with an EMSDCOPE CPD 750 apparatus. The dried cells were sputter-coated with gold-palladium and examined in a JEOL JSM35CF operating at 10 kV, or an S 3000N Hitachi operating at 15 kV.

Preparation of Liposomes

Liposomes were prepared as follows: 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and PPIX from Sigma. Twenty microliters of 100 μM PPIX solution was added to 320 μl of 100 mM POPC in chloroform. The mixture was dried under nitrogen and vacuum. Multilamellar vesicles were obtained by rehydration in 1 ml of phosphate-buffered

saline (PBS), and sonication at 40 W for 3 min with a B-12 Sonifier (Branson, Danbury, CO). Final concentrations were 2 μ M PPIX and 32 mM POPC.

Identification and Cloning of DdAIF

DdAIF was identified by a search for homology with mammalian AIF in the *Cellular Slime Molds* cDNA with the use of TBLASTN (National Center for Biotechnology Information, Bethesda, MD). A cDNA clone named SLB348 was obtained. The 5' end of DdAIF was cloned by polymerase chain reaction (PCR) from a *D. discoideum* cDNA library (kindly provided by D. Fuller, Loomis lab, University of California at San Diego, La Jolla, CA) with the use of gene-specific primers and anchor primers. The PCR product obtained was cloned into a pGEM-T vector (Promega, Madison, WI), sequenced, and fused to SLB348 to obtain full-length DdAIF cDNA. The accession number of DdAIF is AJ272500 (EMBL Nucleotide Sequence Database).

Antibodies, Western Blotting, and Immunofluorescence Microscopy

Polyclonal anti-DdAIF sera were obtained from rabbits immunized with a mixture of two DdAIF peptides (amino acids 221–234 and 465–478, coupled to keyhole limpet hemocyanin). Cytosol and heavy membranes from *Dictyostelium* cells were separated on a 4/20% polyacrylamide gel (Bio-Rad, Hercules, CA) and then transferred to polyvinylidene difluoride (Bio-Rad). The membrane was immunoblotted with rabbit anti-DdAIF (1/1000) and visualized with horseradish peroxidase-conjugated sheep anti-rabbit IgG F(ab')₂ fragment (Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, United Kingdom), followed by enhanced chemiluminescence (Amersham Pharmacia Biotech UK). For immunofluorescence microscopy, cells were fixed in methanol (5 min at -15°C) and permeabilized in 0.1% Triton X-100. They were then incubated with anti-DdAIF antibodies (1/100 in PBS, 1% bovine serum albumin) or with anti-heat shock protein (Hsp)-60 antibodies (Stressgen, Victoria, BC, Canada; 1/200 in PBS, 1% BSA) for 2 h at room temperature. Bound antibodies were visualized with FITC-labeled antirabbit antibodies (Sigma) or tetramethylrhodamine B isothiocyanate-labeled anti-mouse antibodies (Sigma).

Pulsed Field Gel Electrophoresis

DNA was extracted from mammalian cells or *Dictyostelium* cells as previously described (Martin *et al.*, 1995). The pulsed gel electrophoresis was performed with the use of 1.1% agarose gels in 0.5 $\times$ Tris borate-EDTA buffer on a Gene Navigator (Amersham Pharmacia Biotech UK) for 17 h at 226 V with phase A pulse 0.5 s/4 h, phase B 1.0 s/9 h, and phase C pulse 2.0 s/4 h. Gels were stained for 15 min in ethidium bromide with agitation.

Cell-Free Extract Assays

Cytoplasmic extracts of *Dictyostelium* cells and Jurkat cells, nuclei from CEM cells, and the reconstituted cell-free extracts were prepared as described previously (Martin *et al.*, 1995). Cytoplasmic extracts were prepared as follows: cells were washed twice in PBS and incubated on ice for 20 min with cell extract buffer (50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [PIPES], pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol [DTT], 10 μ M cytochalasin B, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). They were lysed by homogenization with a B-type pestle. Lysis was monitored under a phase contrast microscope. Cell lysate was first centrifuged for 5 min at 800 $\times$ g to eliminate nuclei and unbroken cells and then centrifuged at 4 $^{\circ}\text{C}$ for 15 min at 17,000 $\times$ g. The clear cytosol (corresponding to light fraction) was carefully removed and stored at -80°C . CEM nuclei were prepared as follows; CEM cells were washed twice in PBS and once with nuclei

isolation buffer (NB: 10 mM PIPES, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 10 μ M cytochalasin B, and 1 mM PMSF); they were suspended in this buffer, allowed to swell on ice for 20 min, and gently lysed with a Dounce homogenizer. Liberated nuclei were then layered >30% sucrose in NB and centrifuged at 800 $\times$ g for 10 min, followed by washing in NB and suspension in nucleus storage buffer (10 mM PIPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF, and 50% glycerol) at 2×10^8 nuclei/ml. Nuclei were stored at -80°C .

Dictyostelium nuclei were prepared as described by Charlesworth and Parish (1975) with a modified lysis medium composed of 10 mM PIPES pH 7.9, 200 mM NaCl, 300 mM NaF, 1 mM DTT, 1 mM ATP, 0.5 μ M leupeptin, 2.5 μ M pepstatin, 0.53 g of β -glycerophosphate, 200 μ l of saturated solution of vanadate, 0.1 mM PMSF for 100 ml. Cells were lysed, nuclei were purified on sucrose gradients and then stored in nucleus storage buffer.

Cell-free reactions (25 μ l) were performed with the use of 20 μ l of cytoplasmic extract (20–30 mg/ml protein), 1 μ l (2×10^5) of nuclei, and 4 μ l of extract dilution buffer (10 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, and 50 μ g/ml creatine kinase). For flow cytometric analysis of the nuclei, propidium iodide (1 μ g/ml) was added for 30 min and then the nuclei analyzed with the use of the FL3 LP pass filter (FACScalibur 4C; BD Biosciences).

Dictyostelium cytoplasmic extracts (CEs) were immunodepleted by diluting anti-DdAIF serum and nonimmune serum to 1/200 in 0.1 M Tris-HCl pH 9.6 buffer and coating 96-well plates (Maxisorp; Nunc, Wiesbaden, Germany) by incubation overnight at 4 $^{\circ}\text{C}$. The plates were washed four times with PBS. *Dictyostelium* cytoplasmic extracts were incubated for 5 $\times$ 1 h in the wells at 4 $^{\circ}\text{C}$.

RESULTS

Apoptosis-like Phenotype, Cell Shrinkage, Loss of Mitochondrial $\Delta\Psi$ m and Nuclear DNA Content, and Exposure of Phosphatidyl Serine during *Dictyostelium* Cell Death

Exocytosis, a potential mechanism of detoxification, occurs in *Dictyostelium* cultures and leads to the shedding of vesicles (Tatischeff *et al.*, 1998). Vesicles are also released during starvation of suspended *Dictyostelium* cells that subsequently die. Living *Dictyostelium* cells harvested from exponentially growing cultures were incubated for 45 h with the supernatant from a suspension of *Dictyostelium* cells starved for 22 h and containing a high concentration of exocytotic vesicles. Epifluorescence microscopy and flow cytometric analysis demonstrated that >85% of the *Dictyostelium* cells were dead at 43 h (Figure 1a) and 99% at 45 h (Figure 1b), whereas incubation for 45 h in starvation medium alone caused the spontaneous death of <10% of cells (data not shown).

Plasma membrane integrity is a feature that distinguishes apoptotic from necrotic cell death. Necrotic cells versus apoptotic cells were assessed with TO-PRO-3 staining. This stain only enters into necrotic cells, and as shown in Figure 1a only 4% of the cells were stained by TO-PRO-3 at 43 h, whereas 85% exposed their PS residues as shown by annexin V staining. Living *Dictyostelium* cells are round, and there was no significant increase in the cell granulometry (side scatter) of dying *Dictyostelium* cells (Figure 1b). Cell death was associated with several other apoptosis-like features as well as cell shrinkage (transmission light microscopy, Figure 1a), reduced forward scatter and side scatter in flow cytometry (Figure 1b), and loss of mitochondrial $\Delta\Psi$ m measured

Figure 1. Flow cytometric analysis of *D. discoideum* cell death. (a) Epifluorescence microscopy of control *Dictyostelium* cells and dying cells (cell death caused by incubation with supernatant from a suspension of *Dictyostelium* cells starved for 22 h) and stained with 5 nM tetramethylrhodamine ethyl ester for 15 min. Apoptotic versus necrotic cells were analyzed by flow cytometry after double staining with the use of annexin-V FITC (PS exposure) and TO-PRO-3 in control cells (33 h) and dying cells at 43 h. (b) Light scattering properties analysis precluded analysis of the mitochondrial membrane potential with the lipophilic cationic probe JC-1 (1 nM) for normal cells (right) and dead cells (left). The flow cytometric two-parameter histograms combine the green fluorescence (JC-1 green) of the monomeric form of JC-1 and the red fluorescence (JC-1 red) of the aggregates formed inside the mitochondria due to the high transmembrane potential ($\Delta\Psi_m$) (Reers *et al.*, 1991; Smiley *et al.*, 1991). The one-parameter histograms show the drop in $\Delta\Psi_m$ after cell death (blue) and in control cells (red) with a decoupling agent mCICCP, which completely abolishes the $\Delta\Psi_m$ (green). (c) Time course of cell death caused by a 22-h supernatant of starved *Dictyostelium* cells. Red circles, mitochondrial membrane potential of *Dictyostelium* cells measured with DiOC₆(3) (5 nM). Black squares, aberrant exposure of phosphatidylserine moieties at the outer surface of the plasma membrane, as detected with annexin-V FITC. The stars indicated the shrinkage of the cells, whereas the empty circles represent the DNA loss (and/or reduced stainability). The big arrows indicated where the vacuolization take place. (d) One-parameter histogram showing the exposure of PS (in red for 35 h, and in blue for 45 h). (e) Determination of DNA content with propidium iodide staining (1 mg/ml propidium iodide) of control and dying *Dictyostelium* cells. Black, control cell. Red, whole dying cells. The results are representative of three independent experiments.

with tetramethylrhodamine ethyl ester (epifluorescence microscopy; Figure 1a), or with JC-1 probes (flow cytometric analysis; Figure 1b) (Vayssière *et al.*, 1994; Petit *et al.*, 1995; Zamzami *et al.*, 1995). Almost all (85.7%) of the dying cells had a low mitochondrial $\Delta\Psi_m$, compared with <2.5% of control cells. Control experiments showed that carbamoyl cyanide m-chlorophenylhydrazone (mClCCP), an uncoupler of oxidative phosphorylation, abolished the JC-1 dye uptake, demonstrating that this is driven by the $\Delta\Psi_m$ (Figure 1b).

Cell death was also associated with an externalization of PS (Figure 1, c and d) at the plasma membrane in 94% of dying cells at 45 h compared with 8.5% of cells at 35 h (Figure 1d). A kinetic analysis (Figure 1c) revealed that PS was exposed after the loss of $\Delta\Psi_m$, as in mammalian cells (Kroemer *et al.*, 1995; Petit *et al.*, 1995; Zamzami *et al.*, 1995). *Dictyostelium* cells began to lose $\Delta\Psi_m$ 31 h after incubation with the supernatant from 22-h *Dictyostelium* culture containing exocytotic vesicles. Most of the cells (97%) had lost their $\Delta\Psi_m$ after 37 h, whereas the PS exposure had just begun. *Dictyostelium* cells (58.8%) had exposed their PS at 40 h, by at which time they had all lost their $\Delta\Psi_m$ (Figure 1c). At 45 h most of the cells had externalized PS.

We also observed that *Dictyostelium* cell death was associated with a loss of nuclear DNA (59.3 vs. 6% in control cells) (Figure 1e). This loss of nuclear DNA occur slightly later than the exposure of phosphatidyl residues on the external surface of the plasma membrane. The increased vacuolization that is evidenced in Figure 2a also appeared to increase simultaneously with the dramatic mitochondrial drop as soon as the 50% mitochondrial decrease is reached at 37 h (Figure 2c) but could be detected as early as 34 h (Figure 1c, arrow). The cellular condensation was almost parallel to the loss of mitochondrial membrane potential (Figure 1c, stars).

Apoptotic Phenotype of *Dictyostelium* Cell Death and Engulfment of Dying Cells by Neighboring Cells

Transmission and scanning electron microscopy of the dying *Dictyostelium* cells showed that they had a phenotype presenting most of the characteristic features of apoptosis (Figure 2a), including a progressive cell shrinkage (Figure 2, a and c), intense autophagic vacuolization (Figure 2, a and b), and blebbing of the cell surface (Figure 2c). The development of autophagic vacuolization consisted of vacuoles containing damaged mitochondria with an electron-dense matrix and an altered outer mitochondrial membrane (Figure 2b), which was either fully digested or released with the extracellular vesicles.

The dying *Dictyostelium* cells released large vesicles (~500 nm in diameter) that resembled apoptotic bodies (Figure 2c). These bodies sometimes enclosed part of the nucleus and contained large amounts of DNA (Tatischeff *et al.*, 1998). Pseudoapoptotic bodies and/or dying cells were also ingested by neighboring cells, indicating that *Dictyostelium*, a unicellular organism, can perform this function (Figure 2d left), similar to multicellular animals. The engulfed cells or fragments of cells were found in digestive vacuoles, where they were degraded (Figure 2d, left). This is the first time that such engulfment has been reported in unicellular eukaryotic cell death. These observations suggest the existence of *Dictyostelium* homologs of the engulfment machinery as described during apoptosis in multicellular animals.

A nucleus characteristic of a living *Dictyostelium* cell is shown in Figure 2a ($t = 0$). Nucleoli, with highly condensed chromatin, are linked to the nuclear membrane and the nuclear chromatin is uniformly distributed within the nucleus. We observed that spots of condensed nuclear chromatin appeared during *Dictyostelium* cell death, and nucleoli were damaged, shown by the fragmented state of their condensed chromatin (Figure 2b, damaged nuclei). However, the chromatin was not fully fragmented (Figure 6c), and there was no oligonucleosomal DNA degradation as assessed on agarose gel electrophoresis. The mitochondria of *Dictyostelium* underwent metabolic changes during death, as indicated by the drop in mitochondrial membrane potential (Figure 1, a and b). When mitochondria were not found in autophagic vacuoles (Figure 2b) and were present in the cytoplasm, they appeared to be condensed with their outer membrane partly disrupted (Figure 2e), suggesting that intermembrane space proteins might have been released into the cytosol during cell death. The "apoptosis-like" phenotype was not specific of the cell death induced by exocytotic vesicles, because actinomycin D, another potent inducer of apoptosis, produced similar changes (unpublished data).

Identification of PPIX, a Putative Inducer of Light-dependent Cell Death Present in Exocytotic Vesicles

We purified exocytotic vesicles (Figure 3a) or *Dictyostelium* exosomes by differential centrifugation. The vesicles were mainly spherical (30–100 nm in diameter) (Figure 3a) and were enriched in a 17-kDa protein that was identified as ponticulins, as assessed by Western blotting (unpublished data), an integral membrane glycoprotein that binds to F-actin and the nuclear actin assembly (Hitt *et al.*, 1994). High-performance liquid chromatography (HPLC) and spectral analysis of shed vesicles revealed (Figure 3, b and c) that they contained PPIX. This type of porphyrin has previously been reported to cause apoptosis in mammalian cells by a process that requires photoactivation (Noodt *et al.*, 1996). Exogenous PPIX (that enters the cells) has also been found mainly in membranes, including mitochondrial membranes, and it triggers the release of apoptogenic factors such as cytochrome *c* and AIF (Marchetti *et al.*, 1996). PPIX has a red fluorescence, with a major emission at 637 nm and a minor emission peak at 705 nm (Figure 3c). We used this property to assess the intracellular accumulation of PPIX during *Dictyostelium* cell death. The exposure of PS by *Dictyostelium* cells incubated with purified exocytotic vesicles was accompanied by an increase in the red fluorescence intensity, indicating an accumulation of PPIX (Figure 3d). Most of the cells (93.4%) accumulated PPIX and exposed PS at 45 h, whereas 80.4% did so at 40 h and only 8.5% did so at 35 h. We then incubated *Dictyostelium* cells with either purified exocytotic vesicles or with liposomes loaded with purified PPIX. More than 90% of the cells were dying or dead in both cases after incubation for 6 h with exposure to light (15 min at 3 J/cm²), as assessed by flow cytometry (Figure 3e), whereas exposure to light in the absence of purified exocytotic vesicles or liposome loaded with PPIX induced death in <5% of the cells (Figure 3e). Again, cell death was accompanied by cell shrinkage, loss of $\Delta\Psi_m$ (Figure 3e), exposure of PS, and loss of nuclear DNA (unpublished data). Incubation of *Dictyostelium* cells with pure PPIX alone (5 μ M) for a similar time and exposure to light also caused cell death

Figure 2. *Dictyostelium* cell death shows most of the features of apoptosis. Cell death, induced with exocytotic vesicles purified from supernatant from a suspension of starved *Dictyostelium* cells, was monitored by scanning electron microscopy and transmission electron microscopy. (a) Decreased *Dictyostelium* cell size and increased autophagic vacuolization during induced cell death. N, nucleus; Nu, nucleolus; M, mitochondria. (b) Details of dying cells at 46 h. Mitochondria (M) in the cytoplasm and an autophagic vacuole (V) containing damaged mitochondria (DM). Typical nucleus of dying *Dictyostelium* cells (DN, damaged nucleolus). (c) Time course of *Dictyostelium* cell death. Dying *Dictyostelium* cells released pseudoapoptotic bodies and became smaller. (d) Dying *Dictyostelium* cells and pseudoapoptotic bodies engulfed by neighboring cells (left). Autophagic dying cell (right). DV, digestive vacuole. (e) Appearance of mitochondria during *Dictyostelium* cell death. Outer (OM) and inner (IM) mitochondrial membranes are visible on mitochondria in control cells. Dying *Dictyostelium* cells (46 h) may contain mitochondria with a disrupted outer membrane (DOM) and condensed matrix (CM).

(unpublished data). The finding that purified exocytosis vesicles and PPIX required light to cause *Dictyostelium* apoptosis, whereas the 22-h *Dictyostelium* supernatant containing exocytosis vesicles caused death without such exposure indicates that the supernatant contains other unidentified light-independent inducers of *Dictyostelium* cell death.

Identification of a *Dictyostelium* Homolog of Apoptosis-inducing Factor (DdAIF)

DNA degradation during apoptosis can be due either to caspases activation (Nagata, 2000) or to caspase-independent effectors, such as AIF (Susin *et al.*, 1999). One major difference is that caspases are involved in stage II of nuclear

Figure 3. PPIX: a putative inducer of *Dictyostelium* cell death present in the exocytotic vesicles. (a) Transmission electron microscopy and scanning electron microscopy of *Dictyostelium* cells releasing exocytotic vesicles and purified vesicles. V, vesicles; FP, filopodia. (b) HPLC analysis of *Dictyostelium* vesicles. PPIX present in cells or vesicles was quantitatively extracted (Dellinger *et al.*, 1990). The cell or vesicle pellets were suspended in PBS (100 μ l) by vigorous shaking, mixed with 2-chloroethanol (900 μ l) in a glass tube and shaken gently for 30–60 min at room temperature. The extracted PPIX was stored at -20°C until dissolved in the HPLC solvent (0.2 ml) for analysis. (c) PPIX fluorescence spectra. (d) PS exposure and intracellular accumulation of PPIX in *Dictyostelium*. PPIX accumulation was assessed by measuring its intracellular fluorescence by flow cytometry at different times. Cell death was caused by incubation with purified exocytotic vesicles. (e) Liposomes loaded with PPIX caused *Dictyostelium* cell death in the same way as exocytotic vesicles with light exposure. The monoparametric histogram shows the $\Delta\Psi_m$ after incubation with a source of PPIX and exposure to light for 15 min at $1.5 \text{ J}/\text{cm}^2$ (in red), or with a source of PPIX without exposure to light (in black), or with source of PPIX without exposure to light (in green). Control: mCICCP, which completely abolished the $\Delta\Psi_m$ (in blue). The results are representative of three independent experiments. HpB, hematoporphyrin B.

apoptosis, i.e., oligonucleosomal DNA fragmentation (180 bp) and that AIF is involved in stage I of nuclear apoptosis, i.e., perinuclear chromatin condensation and large scale DNA fragmentation (several kbp) (Susin *et al.*, 2000).

We observed a DNA degradation during *Dictyostelium* cell death (Figure 1e). Incubation of *Dictyostelium* cells with peptide inhibitors of caspases (zVAD-fmk, ac-DEVD-CHO, YVAD-fmk), with broad-spectrum cysteine-proteinases/calpain inhibitors (leupeptin, E64), or with a cathepsin inhibitor (FA-fmk) did not prevent the DNA degradation during *Dictyostelium* cell death, suggesting that this process is caspase

and cysteine proteinase independent. Moreover, we did not observe oligonucleosomal DNA fragmentation.

During *Dictyostelium* cell death, the cells show nuclear features of apoptosis similar to those of stage I (Figure 2b), and the DNA is degraded on a large scale (Figure 6c) in a caspase-independent manner, suggesting the involvement of an AIF as nuclear effector.

A TBLASTN search disclosed a homologous partial cDNA clone (clone SLB348, kindly provided by T. Morio, University of Tsukuba, Tsukuba, Japan) in the *Dictyostelium* cDNA database of Japan (University of Tsukuba); which is very

Figure 4. Predicted amino acid sequence of DdAIF and comparison with hAIF. (a) Amino acid sequence of DdAIF. Boxes indicate the MLS, NLSs, and DBM. (b) DdAIF immunodetection: 1) 293T cells transfected with control vector; 2) 293T cells overexpressing DdAIF (AIF); 3) *Dictyostelium* cell extract; 4) *Dictyostelium* cell extract immunodepleted with a nonimmune serum; 5, *Dictyostelium* cell extract immunodepleted with anti-DdAIF antibody; 6) *Dictyostelium* cell extract immunodepleted with anti-DdAIF antibody in the presence of DdAIF-specific peptides (20 μ M peptides). (c) CLUSTAL-W was used to generate an alignment of DdAIF with hAIF. DdAIF is ~33% identical and ~60% similar to hAIF.

similar to mammalian AIF and confirming that AIF seems to be conserved across several phyla (e.g., *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, mammals) (Lorenzo *et al.*, 1999). We obtained the full-length cDNA by cloning the 5' end by PCR from a *Dictyostelium* cDNA library, with the use of gene-specific

and vector-specific primers. The final 1.7-kb cDNA encoded a protein of ~60-kDa (Figure 4a). We generated anti-DdAIF polyclonal antibodies by immunizing rabbits with a mixture of two DdAIF peptides. The molecular weight of the mature form was ~53 kDa, as assessed by Western blotting (Figure 4b). DdAIF shares >30% identity and 60% similarity with

human AIF (Figure 4c). Like mammalian AIF, DdAIF contains a mitochondria localization site (MLS) (Claros and Vincens, 1996) and a putative nuclear localization site (NLS) (Boulikas, 1993). DdAIF also contains a putative helix-turn-helix DNA binding motif (Dodd and Egan, 1990) at its C-terminal end (Figure 4a), suggesting that DdAIF may bind to DNA. All the amino acids believed to interact with the prosthetic groups FAD and NAD are present in DdAIF, as in human AIF (Lorenzo *et al.*, 1999) (Figure 4c).

DdAIF Is Released from Mitochondria during Dictyostelium Cell Death

In mammalian cells, AIF has a mitochondrial localization and upon cell death induction, AIF is released to the cytosol and then targeted to the nucleus (Susin *et al.*, 1999). Immunofluorescence microscopy showed that in living *Dictyostelium* cells, DdAIF, like Hsp 60 (a protein of the mitochondrial matrix), was associated with the mitochondria. On triggering cell death, DdAIF was released into the cytosol and secondarily targeted to the nucleus after the triggering of apoptosis (Figure 5a), whereas Hsp 60 remained localized in the mitochondria. We used cell fractionation to confirm that DdAIF was present in heavy membranes (including mitochondria) in living *Dictyostelium* cells like Hsp 60 (Figure 5b). By cell fractionation, we also confirmed that DdAIF was translocated from the heavy membranes to the cytosol after treatment with PPIX or actinomycin, whereas Hsp 60 was still present in the heavy membranes (Figure 5c). These data show that DdAIF, like mammalian AIF, is translocated from mitochondria to the cytosol and the nucleus during cell death.

DdAIF Is Also Released during Developmental Cell Death Induced by DIF-1

D. discoideum can undergo, upon adverse environmental conditions, a complex developmental process, depending on DIF-1, cAMP secretion, and cAMP responsive gene expression, resulting in the emergence of a multicellular aggregated body (the fruiting body) made up of 20% dead stalk cells forming the stalk wall that supports the 80% viable spores (Soderbom and Loomis, 1998; Brown and Firtel, 1999; Thomason *et al.*, 1999).

Dictyostelium cells treated in the presence of DIF alone, or cAMP plus DIF, exhibit a decrease of their forward and side scatter and show a markedly decreased mitochondrial membrane potential in up to 74 and 83% of cells, respectively (Figure 6a). The loss of mitochondrial $\Delta\Psi_m$ was associated with an externalization of PS. Indeed, only 3% of the control living cells bound annexin-V, whereas 35 and 45% of *Dictyostelium* cells treated with DIF-1 alone or cAMP plus DIF-1 were respectively labeled. Only 12 and 18% of the *Dictyostelium* cells showed plasma membrane damage during developmental cell death versus 1.5% in the control living cells as assessed with TO-PRO-3 staining. These data confirm that DIF-1 and cAMP plus DIF-1 cause mainly an "apoptotic-like" cell death in *Dictyostelium* cells as described by Cornillon *et al.* (1994).

Developmental cell death, as cell death induced by PPIX, was also associated with DdAIF translocation from mitochondria to the cytosol (Figure 6b) and with large-scale DNA fragmentation, as assessed with pulse field gel elec-

trophoresis (Figure 6c). DdAIF is released from mitochondria during developmental cell death, but the amounts are smaller than those released by *Dictyostelium* cells treated with PPIX or actinomycin D (Figure 6b).

DdAIF Is an Evolutionarily Conserved Effector of Nuclear Apoptosis

We used a cell-free system (Martin *et al.*, 1995) to explore the role of DdAIF as a nuclear effector. Mammalian nuclei (isolated from CEM cells) were incubated for 4–5 h with cytoplasmic extracts of *Dictyostelium*. Cytoplasmic extracts from dying cells (cell death mediated by PPIX) caused the condensation and partial degradation of CEM nuclei (86.4% of nuclei with low DNA content), whereas cytosol from living untreated cells did not (20.4% of nuclei with low DNA content) (Figure 7a). Nuclear damage was not prevented by treating the cytoplasmic extracts with various proteinase inhibitors (zVAD-fmk, ac-DEVD-CHO, YVAD-fmk, leupeptin, E64, and FA-fmk) (unpublished data), suggesting that these early chromatin changes caused by cytoplasmic extracts from dying *Dictyostelium* cells did not depend on caspase/proteinase activation (whether in the cytoplasmic extracts or in the nuclei). Concordant with these findings, nuclear chromatin was not fully fragmented by cytoplasmic extracts from dying *Dictyostelium* cells, in contrast to the full fragmentation caused by cytoplasmic extracts from human Jurkat cells treated with an agonistic Fas antibody (Figure 7b). The chromatin changes caused in the cell-free system by the cytoplasmic extract from dying *Dictyostelium* cells seemed to depend mostly on DdAIF, because the condensation and partial degradation of CEM nuclei were prevented by immunodepletion of the cytoplasmic extracts with the polyclonal anti-DdAIF antibody (27% of nuclei with low DNA content), but not with nonimmune serum (81.6% of nuclei with low DNA content) (Figure 7a). However, DdAIF may not be the sole nuclear effector(s) in the cytoplasmic extracts from dying *Dictyostelium* cells, because late (>6 h after incubation) nuclei damage was observed even after immunodepleting the cytosol with the anti-DdAIF antibody. Pulsed field gel electrophoresis demonstrated that cytoplasmic extracts from dying *Dictyostelium* cells caused large-scale DNA fragmentation (50–24 kbp) of mammalian cell nuclei (Figure 7c, lane 2) and that the fragmentation was prevented after immunodepletion with the DdAIF antibody (lane 4) but not with the nonimmune serum (lane 3). This fragmentation did not occur with cytoplasmic extracts from control cells (lane 1). z-VAD.fmk (100 μ M) did not inhibit the DNA fragmentation caused by cytoplasmic extracts from dying cells confirming that the large DNA fragmentation was a caspase-independent process (lane 5).

We also incubated isolated *Dictyostelium* nuclei with cytoplasmic extract from dying *Dictyostelium* cells. Epifluorescence microscopy (Figure 7d) showed that cytoplasmic extracts from dying *Dictyostelium* cells caused the partial degradation of the nuclei with the dissipation of nucleoli, whereas cytoplasmic extracts from living untreated cells did not. Once again, DNA damage was not prevented by treating the cytoplasmic extracts with various proteinases inhibitors (unpublished data). The changes in nuclei caused by the *Dictyostelium* cytoplasmic extracts seemed to depend mostly on DdAIF in the cell-free system, because the partial degradation of *Dictyostelium* nuclei was prevented by immunodepleting the cytoplasmic extracts

Figure 5. Translocation of DdAIF from mitochondria to the cytosol and nucleus during *Dictyostelium* cell death. (a) Cellular distributions of DdAIF (FITC-labeling) and Hsp 60 (tetramethylrhodamine B isothiocyanate-labeling) in living or dying (incubation with 25 μ M PPIX for 16 h) *Dictyostelium* cells was assessed by immunofluorescence microscopy. (b) DdAIF cellular localization studied by Western blotting after cell fractionation. Hsp 60 was used as control of heavy membranes since Hsp 60 is present in the mitochondrial compartment. (c) DdAIF translocation during *Dictyostelium* cell death studied by Western blotting after cell fractionation. The results are representative of three independent experiments.

with an anti-DdAIF polyclonal antibody but not with nonimmune serum (Figure 7, d and e).

Together, our data suggest that DdAIF has a conserved nuclear apoptosis function and is one of the effectors needed for degradation of the nucleus during *Dictyostelium* cell death.

DISCUSSION

In mammalian cells, PCD or apoptosis is a cell suicide process that depends on two major executionary pathways, one

involving proteolytic activation of effector caspase proteases, the other involving mitochondria outer membrane permeabilization, leading to the release in the cytosol of intermembrane space proteins such as cytochrome *c* and AIF (Green and Reed, 1998; Martinou *et al.*, 2000). Although both pathways usually operate together and amplify each other (cytochrome *c* inducing caspase activation, and activated caspases inducing mitochondria permeabilization [Green and Reed, 1998; Marzo *et al.*, 1998; Thornberry and Lazebnik, 1998]), caspase activity, in several instances, is not required

Figure 6. DdAIF is also released from mitochondria during developmental cell death induced by DIF-1. (a) Light scattering properties and mitochondrial membrane potential of control cells, of DIF-1 treated cells, and of DIF-1 plus camp-treated cells. (b) Immunodetection of DdAIF in the cytoplasmic fraction of control (lane 1), 25 μ M PPIX (lane 2), actinomycin D (lane 3), and 100 nM DIF-1 (lane 4)-treated *Dictyostelium* cells. Hsp-60 was used to check the purity of the cytosolic fractions. (c) Pulsed field gel electrophoresis of DNA extracted from control cells (lane 2), PPIX-treated cells (lane 3), or cAMP plus DIF-1-treated cells (lane 4). Molecular weights are in lane 1 (HMW, Invitrogen, Carlsbad, CA). The results are representative of three independent experiments.

Figure 7. DdAIF induces damage of mammalian and *Dictyostelium* nuclei in a cell-free system. (a) CEM nuclei were incubated for 4–5 h with cytoplasmic extracts from untreated *Dictyostelium* cells (control CE), or *Dictyostelium* cells treated with PPIX to cause cell death (dying cells CE). Cytoplasmic extracts of dying cells were also immunodepleted with anti-DdAIF (dying cells CE -AIF) or with nonimmune serum (dying cells CE NIS). Nuclei were stained with 10 μ M Hoechst 33342 and examined by UV fluorescence microscopy (magnification 1000 $\times$). Nuclear apoptosis was also quantified by staining with the DNA-intercalating dye propidium iodide and analyzing the DNA content by flow cytometry. One experiment representative of three is shown. (b) Control cytoplasmic extracts were from untreated Jurkat cells or Jurkat cells incubated with 100 ng/ml anti-Fas antibody (α CD95) for 6 h at 37 $^{\circ}$ C before the preparation of extracts. (c) Pulsed field gel electrophoresis of the DNA extracted from CEM cells incubated either with control CE (lane 1), dying cells CE (lane 2), dying cells CE immunodepleted with nonimmune serum (lane 3), dying cells immunodepleted with anti-DdAIF antibody (lane 4), or dying cells CE preincubated with 100 μ M Z-VAD.fmk (lane 5) and HMW markers (lane 6). One experiment representative of three is shown. (d) Purified *Dictyostelium* nuclei were incubated for 4–5 h with the same cytoplasmic extracts as in a. The nuclei were stained with 10 μ M Hoechst 33342 and examined by UV fluorescence microscopy (Leika DMRB) (magnification 2500 $\times$). (e) Quantification of damaged nuclei induced by cytoplasmic extracts. Damaged nuclei were assessed by the disappearance of the nucleoli. One hundred nuclei were counted in each condition. Histograms are the mean of three independent experiments. Nu, nucleolus; Dnu, dying cells nucleolus.

for the execution of cell death (Borner and Monney, 1999), and AIF has been proposed to be one of the candidates involved in the caspase-independent cell death pathway (Susin *et al.*, 1999).

Our findings indicate that *Dictyostelium* cells can undergo cell death that shares essential features with mammalian cell apoptosis. This involves a loss of $\Delta\Psi_m$, resulting in the release of the *Dictyostelium* homolog of AIF from the mitochondria. The loss of $\Delta\Psi_m$ precedes nuclear shrinkage and extranucleolar chromatin condensation, the dispersion and partial fragmentation of nuclear chromatin, the exposure of phosphatidylserine at the cell surface, and engulfment of

dying cells by healthy or dying neighboring cells. Neither *Dictyostelium* cell death nor its apoptotic features was blocked by the broad caspase inhibitor zVAD-fmk (unpublished data), confirming a previous report that *Dictyostelium* cell death appears to be caspase independent (Olie *et al.*, 1998). The involvement of DdAIF in at least some of the apoptotic features of dying *Dictyostelium* cells, e.g., the nuclear chromatin condensation and partial DNA fragmentation, is suggested by our observation that in a cell-free system, cytoplasmic extracts from dying *Dictyostelium* cells induced similar changes in isolated nuclei, and these were prevented when DdAIF was immunodepleted from the ex-

tracts with the use of an anti-DdAIF antibody. This is, to our knowledge, the first evidence of the existence of phylogenetic conservation between the basic cell death pathway (DNA degradation) that operates in a single-celled eukaryotic organism and part at least of the cell death pathway which operates in cells of metazoan organisms, particularly from mammals.

Dictyostelium is a single-celled organism that can undergo a complex developmental process under adverse environmental conditions that depends on a factor of differentiation (DIF-1), on cAMP secretion, and on cAMP responsive gene expression. These changes involve cell chemotaxis and aggregation and differentiation into two main cell populations, the stalk cells and the spore cells. This results in the emergence of a multicellular aggregated body (the fruiting body) made up of ~20% dead stalk cells that support the 80% of viable spores (Soderbom and Loomis, 1998; Brown and Firtel, 1999; Thomason *et al.*, 1999). Terminal differentiation into stalk cells has been reported to be a caspase-independent form of PCD (Cornillon *et al.*, 1994; Olie *et al.*, 1998), with nuclear chromatin condensation, cytoplasmic vacuolization, and the formation of a rigid cellulose cell wall. The resulting stalk cell corpses retain their structural integrity and are not engulfed by neighboring cells. This process is similar to the developmental cell death in *Myxobacterium* and to the terminal differentiation of bark cells in multicellular plants (Ameisen, 1998). This developmentally regulated PCD has been the only cell death process studied in *Dictyostelium* until now. Our findings show that *Dictyostelium* can undergo a form of "developmental default" cell death, a cell death under conditions that do not lead to either cell aggregation or to stalk cell differentiation. Both processes of cell death share crucial features with metazoan cell apoptosis, in that they involve permeabilization/disruption of the mitochondrial membrane, the release of DdAIF, and DNA fragmentation in large scale. However, cell death induced by PPIX leads to the engulfment of the dying cell by neighboring cells but not during developmental cell death.

DdAIF is very similar to mammalian AIF, in particular in its phylogenetically conserved oxidoreductase domain. This domain appears to be required for the nuclear apoptogenic function of AIF (Lorenzo *et al.*, 1999; Susin *et al.*, 1999). DdAIF also contains an MLS and putative NLSs, as does mammalian AIF. The nuclear apoptosis function of DdAIF is apparently conserved because cytoplasmic extracts from dying *Dictyostelium* cells caused chromatin changes and large-scale DNA fragmentation in a caspase-independent manner in isolated human nuclei in a cell-free system. These changes were similar to those induced by recombinant murine AIF (Susin *et al.*, 1999) and were prevented by immunodepletion of DdAIF from the *Dictyostelium* cytoplasmic extracts.

Our findings are thus consistent with the idea that mitochondria play an evolutionary conserved role in the control of cell suicide (Ameisen *et al.*, 1995; Green and Reed, 1998) and that AIF may be an ancestral and phylogenetically conserved mitochondrial effector of nuclear degradation that could have been recruited to the executionary pathway earlier than caspases (Lorenzo *et al.*, 1999). Such a possibility is also supported by the finding that homologs of caspases (or of proteases that cleave substrates at the same sites as caspases) appear to take part in cell differentiation processes, rather than cell death in *Dictyostelium* (Olie *et al.*, 1998).

Dictyostelium may however represent a particular case in the evolution of apoptosis in single-celled eukaryotes. There are indeed several examples suggesting that molecular effectors recruited to the cell suicide machinery have undergone phylogenetic variation in both metazoan and single-celled eukaryote lineages. For example, although caspases are not needed for cell death in mammals under certain circumstances, caspase activity appears to be crucial in *D. melanogaster* and *C. elegans* (Horvitz, 1999; Song and Steller, 1999; White, 2000), indicating that the recruitment of caspases and mitochondrial effectors may have varied during vertebrate and invertebrate evolution. Apoptosis involving full chromatin and oligonucleosomal DNA fragmentation has also been described in single-celled eukaryotes such as the kinetoplastides (Ameisen, 1996), whose phylogenetic divergence predates that of *Dictyostelium* by several hundred million years, suggesting that these organisms may have effectors of nuclear apoptosis other than AIF.

In summary, although the phylogeny of the eukaryote cell death machinery remains an open question that will require the investigation of cell suicide in other single-celled eukaryotes, our findings provide evidence for the existence of shared ancestry, rather than evolutionary convergence, in some of the molecular mechanisms of apoptosis that operate in mammalian cells and in the single celled eukaryote *D. discoideum*. We further believe that *D. discoideum* is a simple, valuable model system for further studies of the function of AIF and mitochondria in cell death.

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