Developmental Cell Death in *Dictyostelium* Does Not Require Paracaspase*

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Apoptotic cell death often requires caspases. Caspases are part of a family of related molecules including also paracaspases and metacaspases. Are molecules of this family generally involved in cell death? More specifically, do non-apoptotic caspase-independent types of cell death require paracaspases or metacaspases? *Dictyostelium discoideum* lends itself well to answering these questions because 1) it undergoes non-apoptotic developmental cell death of a vacuolar autophagic type and 2) it bears neither caspase nor metacaspase genes and apparently only one paracaspase gene. This only paracaspase gene can be inactivated by homologous recombination. Paracaspase-null clones were thus obtained in each of four distinct *Dictyostelium* strains. These clones were tested in two systems, developmental stalk cell death *in vivo* and vacuolar autophagic cell death in a monolayer system mimicking developmental cell death. Compared with parent cells, all of the paracaspase-null cells showed unaltered cell death in both test systems. In addition, paracaspase inactivation led to no alteration in development or interaction with a range of bacteria. Thus, in *Dictyostelium*, vacuolar programmed cell death in development and in a monolayer model *in vitro* would seem not to require paracaspase.

To our knowledge, this is the first instance of developmental programmed cell death shown to be independent of any caspase, paracaspase or metacaspase. These results have implications as to the relationship in evolution between cell death and the caspase family.

Caspases, a subset of cysteine proteases, play an essential role in apoptotic death in animals (1, 2). The caspase family includes not only caspases but also other members called paracaspases and metacaspases. Paracaspases were identified through their remote homology with caspases, and metacaspases were identified in turn through their remote homology with paracaspases (3). Within eukaryotes, caspases were identified through their remote homology with caspases, and metacaspases were identified and studied in detail *in vitro* using *Dictyostelium* HM44A cells, which upon starvation and addition of the morphogen differentiation-inducing factor (DIF) differentiate as a monolayer from vegetative to “stalk” cells (11), undergoing caspase-independent (12) autophagic vacuolar (13, 14) cell death.

We obtained paracaspase-null (pcp−) clones by homologous recombination in this haploid organism in four distinct *Dictyos-
Dictyostelium Cell Death without Paracaspase

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—D. discoideum axenic strain HMX44A was cloned from HMX44 cells adapted to axenic growth (a kind gift from J. G. Williams). Upon starvation HM44 and HMX44A cells did not morphogenize and produced only little of the differentiation factor DIF-1. Upon the addition of DIF-1, these starved cells differentiated to stalk cells, i.e. dead cells, as a monolayer (11, 13). HMX44A, AX2, and K-AX3 cells were grown in HLA-modified medium (13). The thymidine auxotroph JH10 cells (15) were grown in HLA supplemented with 100 μg/ml thymidine (Sigma). HMX44A, AX2, and KAX3 pcp—cells were selected and grown in HLA supplemented with 10 μg/ml blasticidin (Invitrogen), and JH10 pcp—cells were selected and grown in HLA of all the cultures were at 23 °C.

Preparation of pcp—paracaspase genomic sequence (GenBank™ accession number AF316600) (3) was amplified by PCR from AX2 genomic DNA with forward primer (5′-CCCGGGAATAAATAG-AAAGATAAAA-3′) and reverse primer (5′-TGTTTTTCTCTTCTGAT-3′). The PCR product was cloned into pgEMT easy vector (Promega). The bar cassette of vector pBSK503 (16) flanked by EcoRV restriction sites was inserted into the HpaI site of the paracaspase sequence cloned into pgEMT easy. The DNA was digested with Accl (leading to an extra 400-bp fragment because of an Accl site in the pgEM polylinker) and SacII (a restriction site present in the forward primer) (Fig. 1). The 2280-bp insert was purified from gel fractionation and electrotransfected into HMX44A, AX2, or K-AX3 cells, which were subsequently selected with blasticidin (Invitrogen).

The thymidine auxotroph JH10 cells (15) were transfected with a construct containing the 3.2-kb Thy1 (17) cassette from the pH60 vector (a gift from J. A. Hadwiger). The Thy1 cassette was inserted in the paracaspase/bar vector above the BamHI removal of the bar cassette. The resulting 4080-bp insert was purified and electrotansfected into JH10 cells. Cells were selected for their ability to grow without thymidine.

Resistant cells were cloned by limiting dilution in microplates. Putative pcp—clones were first analyzed by PCR using the primers described above or the forward primer (5′-GATCCCCATGTTGCTTATTGATATGAAATAGAAAGATAAAA-3′) and reverse primer (5′-CTGTTGTTCTTTCTCTGAT-3′). The PCR product was cloned into pgEMT easy vector (Promega). The bar cassette of vector pBSK503 (16) flanked by EcoRV restriction sites was inserted into the HpaI site of the paracaspase sequence cloned into pgEMT easy. The DNA was digested with Accl (leading to an extra 400-bp fragment because of an Accl site in the pgEM polylinker) and SacII (a restriction site present in the forward primer) (Fig. 1). The 2280-bp insert was purified from gel fractionation and electrotansfected into HMX44A, AX2, or K-AX3 cells, which were subsequently selected with blasticidin (Invitrogen).

Southern Blots—10 μg of genomic DNA was digested by HindIII–AccI or Spel. Southern blots were done using Biodyne B transfer membranes (Pall Gelman). Hybridization was performed at 60 °C in ExpressHyb hybridization solution (Clontech) according to the manufacturer’s instructions. Southern blots were probed with a 445-bp HindIII-Seql paracaspase fragment (from a HindIII site 314 bp 5′ of HpaI to a SspI site at 131 bp 3′ of HpaI) and with a plasticidic probe (a 450-bp Xhol-BglII fragment of pUC8BRamBamH1) or with a Thy1 probe (a 1.5-kb ClaI fragment of pJH60).

Development Assays—For development on filters, cells were washed twice with SB (Sorenson buffer 50 times: 100 mM Na2HPO4, 735 mM KH2PO4, pH 6.0) and resuspended in SB. Cells were starved on filter pads (18) (with the exception that cells were starved in SB), the pads were soaked with SB, and 50 μl of cells at 5 × 105 cells were spotted on the filters. The same conditions were used also for development on 1% agarose dishes (19). On both filters and agarose, upon incubation at 23 °C fruiting bodies were obtained in 1–2 days.

For development on bacteria, bacteria previously grown on SM/5 plates (18) were harvested in SB and spread on SM/5 plates. Cells were plated (50 μl of cells at 5 × 105 cells) on the bacteria lawn and incubated for 3–5 days at 23 °C. Plates were examined for plaques formed by Dictyostelium cells and for development with a binocular photomicroscope (Zeiss). Monolayer assays, cell fixation and staining, microscopy, and image processing were done as described previously (14).

Dictyostelium Genome Search—To search the Dictyostelium genome for molecules of the caspase family, we used as probes the caspase domain (amino acids 301–600) of the human caspase 3 (GenBank™ accession number AAG38592), the already known Dictyostelium para-
caspase (GenBank™ accession number AAG38592), the caspase domain (amino acids 251–500) of the Caenorhabditis elegans paracaspase (GenBank™ accession number AAG38591), the S. cerevisiae metacaspase (GenBank™ accession number NP_014840), caspase 3 of Danio rerio (GenBank™ accession number AB047003), amino acids 151–402 of mouse caspase 1 (GenBank™ accession number P29452), and amino acids 1–350 of a Cyanobacteria “pseudocaspase” (NP_485716). These probes were used for blast search on the NCBI site (www.ncbi.nlm.nih.gov/BLAST/), limited to Dictyostelium proteins, default parameters, expect maximum 10, psi-blast on the NCBI site (default parameters, until convergence), wu-blast on the Sanger site (www.sanger.ac.uk/Projects/D_discoideum/blast_server.shtml), on all of the contigs produced by the international consortium, January 2003 update, default parameters), and tblast on the Jena site (genome.imb-jena.de/Dictyostelium), on all available sequences; shotgun Baylor+GCSJ+Sanger, ESTs, GenBank™, mtDNA, RNA, expect 10).

RESULTS

Paracaspase Gene Inactivation Does Not Grossly Alter Dictyostelium Developmental Cell Death—Currently (November 2003), the whole chromosome shotgun approach to Dictyoste-
lium genome sequencing has reached an 8-fold coverage, leading to the probability of finding a given gene estimated to be higher than 0.98 (20). The various Dictyostelium genome search approaches listed under “Experimental Procedures” most often yielded the already known Dictyostelium paracaspase with significant “e” values and sometimes yielded other Dictyostelium sequences, however, with very borderline expect values. Only one of these sequences, the AA010571 (also named a caspase-like domain but considerably truncated and with very low homology. Altogether, the only unambiguous member of the caspase family found in the thoroughly sequenced Dictyostelium genome remains the paracaspase gene AF316600/AAG38592 identified previously (3).

We disrupted this paracaspase gene by homologous recombination in the K-AX3 strain (Fig. 1) and checked the impact thereof on development under three experimental conditions: on filters in starvation buffer, on agar in starvation buffer, and on a lawn of Klebsiella aerogenes. In each of these three conditions, development appeared similar in parent K-AX3 and in two independently obtained pcp—clones (Fig. 2). Testing on a lawn of bacteria checks not only development but also vegetative growth and ability to ingest and process bacteria and to resist to them, all of them clearly not dependent on paracaspase. In particular, stalk development appeared unaltered, suggesting that stalk cell death is unaffected by paracaspase gene inactivation.

Because of mostly unpublished reports that Dictyostelium mutant phenotypes can be strain-dependent, we disrupted the paracaspase gene also in strain AX2 cells (Fig. 1). In this case as well, development as tested on filters and on agar and in particular stalk cell development appeared unaltered by the disruption (data not shown). In both K-AX3 and AX2 cells, the paracaspase gene had been disrupted using a blasticidin resistance cassette. To eliminate possible bias resulting from expression of this cassette, we disrupted the paracaspase gene in another strain, JH10, using the Thy1 cassette and selecting for thymidine prototrophy (Fig. 1) (15). The resulting JH10 pcp—cells tested on filters and on agar also showed unaltered stalk development (data not shown). Thus, in three different Dictyostelium strains, stalk development, thus presumably stalk cell death, did not require an intact paracaspase gene.

To more directly check stalk cell death, K-AX3 stalks were examined after triple staining with fluorescein diacetate (FDA), propidium iodide (PI), and calciofluor. Both parent and pcp—stalks showed the usual pattern of dying vacuolated cells.

L. Eichinger, G. Gloeckner, W. Loomis, R. Sugang, and M.-A. Rajandream, personal communications.
stained with FDA and later with PI and synthesizing cellulose as shown through calcofluor staining (Fig. 3). Thus, by these criteria, stalk cell death in vivo does not require an intact paracaspase gene.

Paracaspase Gene Inactivation Does Not Alter DIF-induced Monolayer Cell Death—Stalk cell death in vivo can be mimicked in vitro in a monolayer system where cell death is induced by starvation and DIF (11). Induction of vacuolated dead cells in the monolayer system was not impaired in both independently derived pcp—K-AX3 clones compared with parent cells (Fig. 4). Very similar results were obtained with parent and pcp—AX2 and JH10 cells (data not shown). In these strains as well, cell death in the monolayer system was not impaired in pcp—cells.

The stages of Dictyostelium cell death have been described in detail (13, 14) using cells of another strain, HMX44A, as a monolayer. Compared with the in vivo situation or with K-AX3 cell monolayers, this system could permit the detection of more subtle alterations of the cell death process. Therefore, we inactivated by homologous recombination the paracaspase gene in Dictyostelium HMX44A cells and the resulting pcp—clone (Fig. 1) was functionally investigated in more detail. Upon induction by starvation and addition of DIF, the morphological stages on the cell death pathway (emergence and demise of paddle cells, cellulose synthesis, paddle-to-round cell transition, actin depolymerization, vacuolization) seen in parent HMX44A cells (14) were also observed in pcp—cells (Fig. 5). To control for any clonal effect, the homologous recombination process in HMX44A cells was repeated in an additional, separate round of transfection and yielded another pcp—clone, which could also be induced to die (data not shown).

Whereas the HMX44A strain was derived from the initial V12M2 Dictyostelium isolate, the AX2, K-AX3, and JH10 strains were derived from the NC4 isolate (see dictybase.org/strain_history.htm). Thus, in pcp—cell clones belonging to four distinct Dictyostelium strains of two distinct derivations, the paracaspase gene was not required for DIF-induced vacuolar cell death in the monolayer system.

Paracaspase Is Not Required for Staurosporine-induced Cell Alterations or for Growth and Development on Some Bacteria—
lar although less marked effects could be seen at 0.1
filopodia often appearing as networks (data not shown). Simi-
the cells acquired a flat morphology with marked extension of
humidified with SB buffer (A); after 42 h on agarose prepared in SB (B),
after 90 h of growth and development on a lawn of *K. aerogenes* (C). The
pattern of development is not detectably affected by paracaspase inac-
tivation. In addition, in C, the right side of each picture shows a similar
bulge of vegetative *Dictyostelium* feeding on bacteria at the periphery of
each “plaque,” indicating that the behavior of K-AX3 clone toward
*K. aerogenes* is the same irrespective of paracaspase inactivation.

Most if not all animal cells would die when subjected to the
protein kinase inhibitor staurosporine, and this death is often
caspase-mediated (21, 22). If staurosporine-induced cell death
requires caspases in animal cells, staurosporine-induced cell
death or other effects might require the caspase-related para-
caspase in *Dictyostelium*. We subjected vegetative K-AX3 cells
to 1 μM staurosporine in HL5 medium for 15 h. Unexpectedly,
the cells acquired a flat morphology with marked extension of
filopodia often appearing as networks (data not shown). Simi-
lar although less marked effects could be seen at 0.1 μM stau-
rosporine. These cells were apparently not dead, because upon
replacement of staurosporine-containing HL5 with fresh HL5,
they reverted to vegetative cell morphology (data not shown).
Thus, in *Dictyostelium* cells and under conditions similar to the
ones that induce death in animal cells, staurosporine did not
induce cell destruction but induced reversible alterations in cell
morphology. We then subjected K-AX3 *pep*– clones and also
parent and *pep*– HMX44A cells to staurosporine and observed
the same alterations (data not shown). Thus, in *Dictyostelium*
cells, under these conditions staurosporine induces reversible
alterations and these do not require paracaspase.

Because of the relationship between cell death and some
defense reactions, we wondered whether paracaspase could be
involved in interactions between *Dictyostelium* and bacteria.
Therefore, we tested a range of bacteria (*Escherichia coli*, *Aero-
monas hydrophila*, *Serratia marcescens*, *Salmonella typhi-
murium*, *Pseudomonas aeruginosa*, *Agrobacterium tumefa-
ciens*, *Xanthomonas campestris* oryzae, *Bacillus megaterium*,
*Shewanella putrefaciens*, *Erwinia carotovora* carotovora, and
*Erwinia chrysanthemi*) for their ability to support vegetative
growth and subsequent plaque development of parental or
*pep*– K-AX3 cells. We found no detectable difference linked to
the absence of the paracaspase gene (data not shown).

**DISCUSSION**

The purpose of this work was to check whether the para-
caspase molecule was necessary for *Dictyostelium* cell death. The
experimental answer seems to be that it is not. To our knowledge
*Dictyostelium* is the only developmentally competent eukaryote
so far, in which through a combination of natural absence of
caspases and metacaspases and experimental inactivation of pa-
caspase, it has been possible to show the persistence of cell
death in the genetic absence of all known caspases and para-
caspases and metacaspases. Strengthening this conclusion, these
results were obtained with four *Dictyostelium* strains derived
from two distinct initial *Dictyostelium* isolates using two selec-
tion agents and two cell death procedures.
though our purpose was to explore the relationship between rosporine-induced alterations, and overall development. Also, showed unchanged cell death but were also not grossly affected lend themselves to a trans-kingdom approach of cell death models exhibiting only one type of cell death. These models same as in parent cells (data not shown) (for details see Ref. 14).

However, there are some limitations to these conclusions. There is still a very remote possibility that a gene encoding a member of the caspase family lies in the few unexplored segments of the Dictyostelium genome. Also, we cannot exclude a role in Dictyostelium cell death of more remote members of the caspase-hemoglobinase fold protease family (4) nor of some other proteases that might have similar enzymatic activity. Clearly, other cysteine proteases could be involved in this cell death. Changes in protease activity have previously been found associated with Dictyostelium cell differentiation including that of stalk cells (23). However, we could not inhibit Dictyostelium cell death using even high concentrations of several calpain inhibitors (data not shown). From another point of view, we cannot exclude the possibility that, in particular through the previously indicated domain differences, animal paracaspases might play a role in some instances of cell death. Dictyostelium cells inactivated for the paracaspase gene showed unchanged cell death but were also not grossly affected as to vegetative growth, co-existence with some bacteria, staurosporine-induced alterations, and overall development. Although our purpose was to explore the relationship between paracaspase and cell death, leading to the conclusion that paracaspase was not required for Dictyostelium cell death, paracaspase clearly did not seem to be required for a number of other key functions in Dictyostelium. We did not investigate further the function of this paracaspase.

These findings have at least two implications. First, in the face of complexity of apoptosis (24) and of multiplicity of non-apoptotic types of cell death, moreover often intertwined within the same dying cell (25), there is a need for "cleaner" cell death models exhibiting only one type of cell death. These models would often belong to non-animal kingdoms and would ideally lend themselves to a trans-kingdom approach of cell death mechanisms (26). Within such cell death models, D. discoideum can be used to explore the mechanism of vascular autophagic cell death. The present results contribute to fully validate it as a model for this caspase-independent cell death.

A second implication bears on the role of the caspase family in cell death mechanisms. To answer this question, two organisms, yeast and Dictyostelium, have been explored that show the favorable experimental situation of expressing only one member of this family. However, yeast cell death was apparently dependent on this caspase-extended family member (8) but Dictyostelium cell death was not. A number of factors may explain these contrasting results. From a molecular point of view, molecular proximity to caspases of the non-caspases should matter. Paracaspases are closer to caspases than metacaspases (3, 4). However, in apparent paradox, the Dictyostelium paracaspase is not involved in cell death (this report), whereas the yeast metacaspase is involved (8). Also, peculiarities of given metacaspases and paracaspases could be important such as the absence of some domains indicated above. From a phylogenetic point of view, the earlier emergence from the main eukaryotic lineage of Dictyostelium compared with yeast (27) and the possibly distinct mode and earlier time of entry into eukaryotes of metacaspases compared with paracaspases (28) may be significant. Also, the circumstances of cell death were quite different, namely development in Dictyostelium and not so in yeast. It could well be, however, that the most important factor is the type of caspase-independent cell death, apoptotic-like in yeast and not so in Dictyostelium. Although clearly more information in more organisms is needed, this report shows that, at least in Dictyostelium, developmental cell death can occur in the absence of any member of the caspase family, making a constitutive link throughout evolution between this caspase family and programmed cell death unlikely. Rather, from the caspase family, only or mostly bona fide caspases were recruited to cell death. This caspase recruitment seems to have occurred when animals emerged in evolution, perhaps mostly as a tool to contribute together with cell death phagocytosis to the disappearance of dead cells, which is remarkably more thorough and rapid in apoptosis in the animal kingdom than in most other types of cell death.

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