Cellular Features of an Apoptotic Form of Programmed Cell Death during the Development of the Ascidian, Boltenia villosa

Author: Bates, William R.

Source: Zoological Science, 21(5) : 553-563

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.553

ZOOLOGICAL SCIENCE **21**: 553–563 (2004)

Cellular Features of an Apoptotic Form of Programmed Cell Death during the Development of the Ascidian, *Boltenia villosa*

William R. Bates*

Department of Biology, Okanagan University College, 3333 University Way, Kelowna, British Columbia, V1V 1V7 Canada

ABSTRACT—The phylogenetic position of ascidians near the base of the chordate tree makes them ideal organisms for evolutionary developmental studies of programmed cell death (PCD). In the present study, the following key features of an apoptotic form of PCD are described in *Boltenia villosa*: fragmentation of DNA, increases in plasma membrane permeability, decreases in mitochondrial activity, production of reactive oxygen species (ROS), and caspase activation. First, evidence is presented for apoptosis of cells within the ovary. Later in development, during the early phase of larval tail resorption at the beginning of metamorphosis, some notochord nuclei showed DNA fragmentation and their cell corpses were rapidly eliminated from the larval body. In striking contrast to the rapid demise of notochord cells, larval muscle cells persisted for more than a week within developing juveniles. Rhodamine 123 and MTT experiments suggest that mitochondria within some of the resorbed larval tail muscle cells were metabolically active for more than a week. Furthermore, resorbed tail muscle cells contained a muscle-specific intermediate filament, termed p58, despite relatively high levels of ROS activity and the ubiquitination of their plasma membranes at day two. Corpses of larval tail muscle cells containing aggregated pigment granules survived within juveniles for more than a month, in contrast to the rapid elimination of notochord cells. Evidence consistent with the formation of larval muscle cell apoptotic bodies is presented. The most surprising result of the present study was that caspase-8, usually associated with apoptotic signaling, was activated in larval endoderm cells that develop into adult structures. When the present results were compared to features of PCD previously reported in other ascidians, significant species differences in PCD were revealed.

Key words: ascidian, development, apoptosis, metamorphosis, evolution

INTRODUCTION

A cell's decision to live or die depends on complex interactions with its environment and on intrinsic cell autonomous pathways. The outcome of every decision is of critical importance in both development and the homeostatic regulation of adult cell populations (Jacobson *et al*., 1997). Ascidians are an excellent whole animal model system to begin to investigate the complex interactions between signaling pathways responsible for cell survival and apoptosis, especially during metamorphosis. It was Alexander Kowalevsky who in 1868 first discovered the close phylogenetic position of ascidians with vertebrates and since then, ascidians have been at the forefront of evolutionary developmental studies of chordates (for example see Holland and Gibson-Brown, 2003). The study of apoptosis in ascidian development can therefore provide us with a deeper understanding of the evolution of apoptosis in chordates.

Many recent papers have described cellular components, including receptors, enzymes, transcription factors, chaperones and organelles, that are shared by apoptotic and cell survival pathways (Abrams, 2002). As discussed by Sperandio *et al*. (2000) and Newmeyer and Ferguson-Miller (2003), cellular features of apoptosis are much more diverse than previously believed. Therefore, detailed comparative studies of PCD in a wide variety of organisms are needed to understand the evolution of PCD mechanisms. Two basic features of cells link together cell survival and apoptotic pathways. First, mitochondria not only produce ATP for cellular metabolism, but they also can release apoptotic signaling factors, for example cytochrome c, that can trigger the activation of caspases for the selective cleavage of proteins within the cell cytoplasm. Cleaved proteins are ubiquinated and subsequently transported to proteasomes where they are degraded. A second basic feature known to regulate cell

* Corresponding author: Tel. +01-250-491-9604;
FAX. +01-250-470-6004.
E-mail: bbates@ouc.bc.ca

survival versus cell death involves the recruitment of specific types of adaptor proteins to the cytoplasmic domains of membrane receptors. A specific example how the recruitment of different adaptor proteins signal cell survival or cell death that may be relevant to the results of the present study is as follows: Members of the tumor necrosis factor (TNF) receptor family can relay either an apoptotic or survival signal to downstream effectors depending on the combinations of adaptor proteins (for example, FADD, RIP, TRAF2) that form labile associations with the cytoplasmic domain of a receptor. In some cases, it has been shown that the signaling required for DNA degradation and protein cleavage is relayed from plasma membrane receptors to directly activate the most proximal member of the caspase family, caspase-8, without the involvement of mitochondria. Recently, Chambon *et al*. (2002) and Jeffery (2002) have shown that caspase is activated during metamorphosis in *Ciona intestinalis* and *Molgula occidentalis*, respectively, using a pan-caspase fluorogenic substrate. In the present study, the possible involvement of caspase-8 activation during ascidian metamorphosis was investigated. Given that caspase-8 is known to be activated by a plasma membrane receptor, as discussed above, the staining of cells with a fluorogenic substrate that is specific for detecting activated caspase-8 in living cells would suggest the possibility that caspase-8 is activated by a ligand-receptor interaction.

MATERIALS AND METHODS

Animals and embryo cultures

Boltenia villosa adults were purchased from Westwind Sealab Supplies, Victoria, British Columbia, Canada and maintained in an Instant Ocean aquarium. Eggs and sperm were surgically obtained from the gonads of several animals, mixed together in filtered seawater for ten minutes followed by extensive washing with large volumes of seawater. Zygotes were transferred to glass Syracuse dishes or plastic culture plates and then cultured at 10 to 11°C until the desired stages of development were obtained.

Immunocytochemisty

The methods for fixation, sectioning and immunochemical staining are described by Bates and Bishop (1996). NN18 and Hsp60 antibodies were purchased from Sigma Chemical Company (USA). Rabbit anti-ubiquitin polyclonal antibody (SPA-200) was purchased from StressGen Biotechnologies Corp (Victoria, BC, Canada). NN18 antibody was previously shown to bind to an ascidian intermediate filament protein by Western blotting (Swalla *et al*., 1991). Hsp60 antibody was previously shown to bind to an ascidian homolog of mitochondrial Hsp60 by Western blotting (Bates and Bishop, 1996). The commercial anti-ubiquitin antibody used in the present study is known to detect a 10 kDa protein based on immunoblot analysis and binds to ubiquitin present in sections obtained from humans, monkeys, rats, hamsters, rabbits, pigs, dogs, sheep, chicken, *Xenopus*, yeast, *Drosophila*, *E. coli* and fish. Therefore, this antibody recognizes an ubiquitin sequence that is common to prokaryotes and eukaryotes (StressGen Biotechnologies technical reports). The anti-fade chemical DABCO (1,4-diazabicyclo- [2.2.2] octane triethylenediamine was purchased from Sigma Chemical Company (USA). Secondary antibodies for indirect immunocytochemistry were purchased from Sigma Chemical Company (USA).

SYTOX and SYTO staining

SYTOX and SYTO dyes were purchased from Molecular Probes, (Oregon, USA) and used as described by Jeffery (2002). These dyes are routinely used to study changes in the permeability of plasma membranes during the early events of apoptosis, as indicated by the extensive list of references supplied by Molecular Probes with these reagents.

The TUNEL method and DAPI staining

The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method and DAPI staining were used to visualize the cleavage of nuclear DNA into oligonucleosome fragments. Specimens for nuclear analysis were fixed in 4% paraformaldehyde at 4°C overnight, washed with large quantities of ethanol, and then embedded in paraplast. Eight micron sections were cut from blocks, dewaxed in xylene, followed by rehydration through an ethanol series into PBS. After the specimens were washed with large volumes of PBS, fifty microlitres of the TUNEL reaction mixture was applied to the surface of the sections, incubated in a humid chamber for 1 hr at 37°C in the dark, and then rinsed three times before mounting on a slide, as described in the instructions with the *In situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Sections were stained with DAPI by diluting 10 μl of the 1 mg/ml stock solution with 10 ml of PBS and then staining sections for six minutes before washing with large volumes of PBS.

Fluorogenic substrates for *in situ* detection of caspase activation

Caspase 8-specific fluorogenic substrate rhodamine 110, bis-(N-CBZ-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid amide: Z-IETD-R110) was purchased from Molecular Probes (Eugene, Oregon, USA) and used at a working concentration of 10 μM. The pan-caspase fluorescent apoptosis marker FITC-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) was purchased from Promega Corporation (Madison, WI, USA) and used at a working concentration of 10 μM.

Detection of alkaline phosphatase activity

Alkaline phosphatase activity was detected using the BCIP method, as previously described by Bates and Jeffery (1987).

Mitochondrial probes to examine cell viability

3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemicals Company and used at a working concentration of 50 μg/ml dissolved in seawater. Mitochondrial respiration reduces MTT resulting in a purple-blue precipitate in vital cells (Hoffman and Weeks, 2001). Rhodamine 123 was purchased from Molecular Probes, Eugene, OR, USA and used at 5 μM concentration. This cationic, cell-permeant fluorescent dye is non-toxic and is rapidly sequestered by active mitochondria.

In vivo detection of reactive oxygen species (ROS)

Dihydroethidium was purchased from Sigma Chemicals. This probe is colourless and nonfluorescent until it is oxidized by superoxide anions into fluorescent ethidium. Larvae were treated at 10 μmol/L in seawater in dark conditions for thirty minutes and then viewed using rhodamine filters, as previously described by Dikalov *et al*. (2002).

Microscopy and Photography

Photographs were taken using an Olympus CH2 fluorescence microscope equipped for obtaining FITC, DAPI and rhodamine images and brightfield images. EliteChrome 400 slides were scanned using an Epson scanner and the images were formatted using Adobe Photodelux and printed using an Epson Photo 825 printer.

RESULTS

Apoptosis within the ovary of *Boltenia villosa*

A decision is made within the ovary of *Boltenia* as to which cells live and which cells die. It was surprising to observe the extent of apoptosis among young developing oocytes and follicle cells that surround the oocytes. Changes in membrane permeability, as demonstrated by the uptake of SYTOX, was evident in oocytes that were freshly released from the ovary (Fig. 1A). In one experiment, eleven percent of the oocytes (eighty-three oocytes examined) were stained after one hour at 11°C. Extra-ovarian follicle cells were also stained with SYTOX. To determine if an increase in the temperature of seawater disrupts oocyte plasma membranes, freshly dissected oocytes were cultured in seawater containing SYTOX at room temperature for six hours. After one hour, thirteen percent (6/45) of the oocytes were stained and when the same clutch of oocytes was examined after being cultured for six hours at room temperature, the number of stained oocytes increased to forty-eight percent. Many SYTOX-positive follicle cells were also evident. An increase in temperature caused cell stress that resulted in changes in plasma membrane permeability to SYTOX. Cell-permeant SYTO 13 was used as a positive control for penetration across the follicle envelope. Elevated temperatures increased the uptake of SYTOX across plasma membranes in oocytes and follicle cells, but did not have a significant effect on test cells.

Further evidence for cell death within the ovary was obtained by staining ovarian sections with DAPI or using the TUNEL method and then scoring for punctate patterns of condensed chromatin that would suggest apoptotic DNA fragmentation. Nuclei of healthy cells appeared evenly stained (using DAPI) or were not stained (using the TUNEL method). Positive controls for DNA fragmentation in sections included UV irradiation of oocytes and the fixation of oocytes with methanol instead of paraformaldehyde. UV treatments and methanol fixation induced extensive, non-specific DNA fragmentation (data not shown). Follicle cells showed evidence of extensive DNA fragmentation using the TUNEL method (Fig. 1B; 96% n=50) or DAPI staining. In contrast, there was little or no evidence for fragmentation in late tailbud larvae (n=one hundred larvae examined) or in swimming larvae (n=eighty larvae examined).

Sequential death of larval cells during metamorphosis

The ascidian larval tail contains epidermal cells, notochord cells, muscle cells and a nerve cord. There are at least two possibilities how tail cells are eliminated during metamorphosis. Larval cells may be somehow coupled together such that they would simultaneously die together in a suicide pact or individual larval cells may die in a specific order at different times during metamorphosis. The first scenario could suggest extensive cell-cell signaling occurs between tail cells to coordinate a group death, whereas the second scenario could suggest a greater degree of autonomous cell death. These two scenarios were investigated by staining larvae and surgically-isolated larval cells with SYTOX, a fluorescent dye known to be impermeable to plasma membranes of healthy cells and by looking at DNA fragmentation.

Notochord cells showed extensive DNA fragmentation at thirty-two hours of development corresponding to the early phase of tail resorption (Fig. 2A and B). By the completion of tail resorption, notochord cells were never observed in sections (more than 300 sections containing notochord cells were examined). Further evidence supporting the idea that notochord cells are rapidly destroyed following tail resorption is that the muscle-specific antibody, NN18, only stained the largest cells in sections obtained at day two of development. Large unstained, vacuolated notochord cells were absent in more than two hundred sections examined.

Tail muscle cells that moved into the body region during tail resorption formed a ring-like pattern around the endoderm cells (Fig. 3A). By twenty-one days of development, the pigmented larval muscle cells always formed two bilaterally symmetrical rows within the body cavity (Fig. 3B). At this time, small cell fragments containing orange pigment granules were evident within the lumen of ampullae (Fig. 3C). The average number of these small, orange-pigmented cell fragments was eight per ampulla. The possible significance of this observation will be discussed later. Changes in membrane permeability were examined in unhatched tailbud larvae, swimming larvae and at various times during metamorphosis. SYTOX staining was first evident in a few scattered muscle cells and notochord cells during tail resorption at thirty-two hours of development (Figs. 4A and B). There was no evidence for staining of cells in the tip region of the tail at the beginning of tail resorption. In contrast to the limited number of SYTOX-positive cells, staining with SYTO 13 resulted in the labeling of all cells (Figs. 4C and D). Larval muscle cells were surgically isolated during the early phase of tail resorption and then briefly cultured in sea water containing SYTOX and then observed. Thirty percent of the isolated muscle cells were stained with SYTOX (Fig. 2C; n=80 isolated muscle cells were examined), in contrast to staining with SYTO13 that stained all of the isolated muscle cells (n=50 examined).

By day four of development, a few scattered larval muscle cells within juveniles showed TUNEL staining in sectioned material (Figs. 2E and F). DNA of notochord cells underwent fragmentation prior to muscle cells and was first evident at thirty hours of development (Fig. 2A). By the time the tail was completely resorbed, notochord cells were completely destroyed and there was no cytological evidence for notochord cell debris. By contrast, dying larval muscle cell fragments survived at least until day twenty-one of development. These results suggest that tail cells die in a cell-specific order, not together in a PCD ‘suicide pact’. Notochord cells die first, followed by larval muscle cells, and the larval epidermal cells persist into adult life. A neural marker is

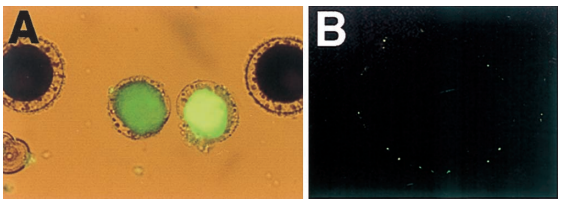

Fig. 1. Apoptosis of oocytes and follicle cells in *Boltenia villosa*. Changes in plasma membrane permeability that accompany early stages of apoptosis permit the transfer of SYTOX fluorescent dye into the cytoplasm. Two SYTOX-labeled oocytes are shown (A). Many follicle cells adhered to the acellular chorion are labeled using the TUNEL method (B).

required to study the fate of the larval nerve cord in *Boltenia villosa*.

Changing patterns of mitochondrial Hsp60 staining during *Boltenia* development and metamorphosis

Hsp60 is a mitochondrial protein involved in protein folding. Fig. 5 shows the staining patterns of Hsp60 in oocytes, tailbud larvae and young juveniles. Hsp60 was mostly restricted to the cortical cytoplasm of oocytes, where most of the mitochondria reside (Fig. 5A). At the tailbud stage, tail muscle cells containing large quantities of mitochondria inherited from the egg cortex were stained with

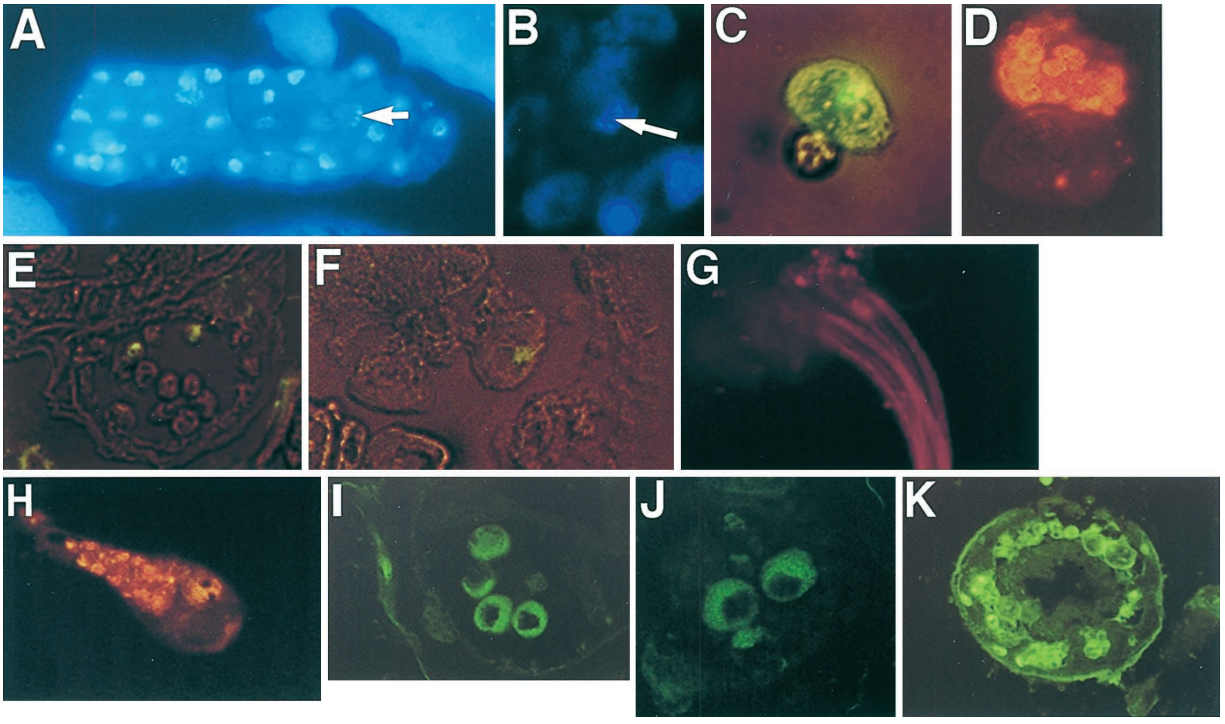

Fig. 2. Larval cell apoptosis during metamorphosis. DAPI-stained sections obtained from thirty-two hour larvae corresponding to the beginning of tail resorption (A and B). Fragmented DNA is evident within notochord cells (see arrows). A larval muscle cell that was surgically isolated from a day four juvenile and immediately stained with SYTOX. Note the nuclear staining (C). A day two juvenile in which resorbed larval muscle cells were surgically extruded through a hole made in the tunic and then stained with rhodamine 123 (D). The accumulation of rhodamine 123 by larval muscle cell mitochondria suggests that resorbed larval muscle cells are vital. TUNEL-labeled larval muscle cells are shown in (E) and (F). ROS activity is shown in the cytoplasm of muscle cells in a tail twitching larva (G) compared to an increase in ROS activity in larval muscle cells after the initiation of tail resorption (H). The intermediate filament protein, p58, persists in the cytoplasm of larval muscle cells at day four (I) and day twelve (J), despite the ubiquination of larval muscle cells at day two (K).

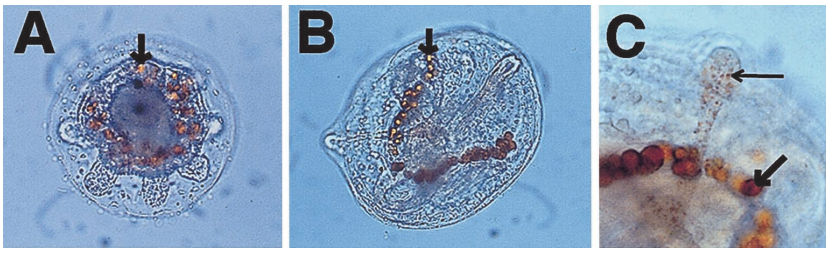

Fig. 3. The slow elimination of larval muscle cell corpses during juvenile development. Light photographs showing the orange pigmented larval muscle cells (see arrow) surrounding the endoderm at day four (A). A one month old juvenile showing two bilaterally symmetrical bands of pigmented larval muscle cells undergoing apoptosis. The arrow points to one band of pigmented larval muscle cells (B). At one month of development, larval muscle cell corpses consisting of orange pigment granules enclosed by plasma membrane are evident, in contrast to the rapid elimination of notochord cells. (C) Small orange pigment granules (see small arrow) that may be derived from apoptotic bodies of larval muscle cells (see larger arrow) are shown in the lumen of ampullae.

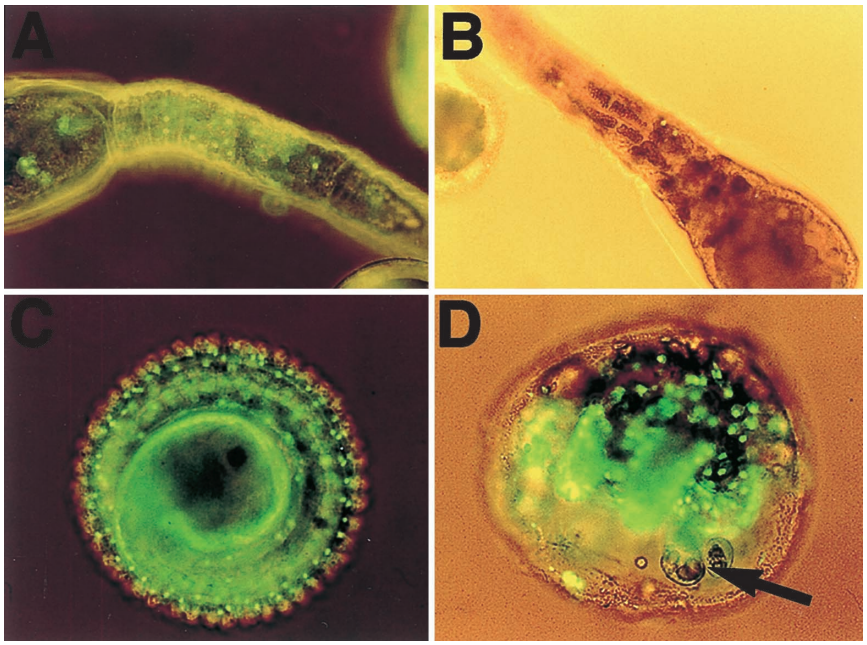

Fig. 4. Changes in plasma membrane permeability during apoptosis demonstrated by the uptake of SYTOX. Larval nuclei labeled with SYTOX. Labeled cells are evident on the surface of a larva (A). Two larval muscle cells are labeled with SYTOX at the beginning of tail resorption (B). The pattern of SYTOX staining of muscle cells was highly variable from larva to larva. For comparison, the nuclear staining of an unhatched larva using the cell-permeant dye SYO13 labels all nuclei (C). A day two juvenile stained with SYTO13 is shown in (D). Stained nuclei of ampullae are in focus and note the extrusion of a larval muscle cell through the epidermis (arrow).

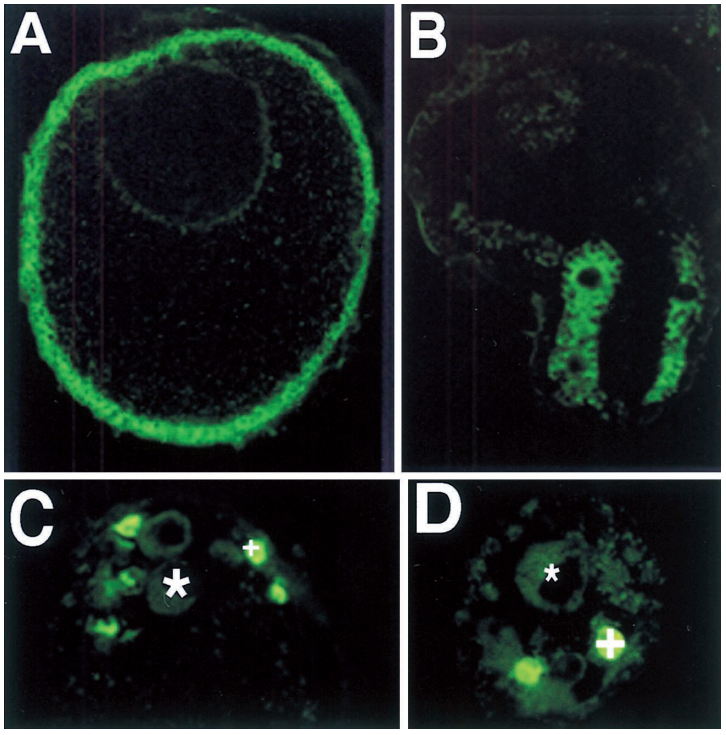

Fig. 5. Hsp60 immunocytochemistry. Most of the mitochondria within an oocyte are situated in the cortical myoplasmic region beneath the plasma membrane and they are stained with anti-Hsp60 antibody (A). During cleavage and subsequent larval development, the myoplasm that contains large quantities of mitochondria is partitioned into larval muscle cells (B). By four day of development (C and D), approximately two days after tail resorption, the larval muscle cells are not stained with Hsp60 antibody (see asterisks), by contrast to the brightly stained small orange pigmented cells (see crosses).

Hsp60 antibody (Fig. 5B). After tail resorption, Hsp60 staining of apoptotic larval muscle cells gradually diminished until by day three many of the larval muscle cells were weakly stained or not stained with this antibody. At day four, very few apoptotic larval muscle cells were stained. By contrast, most of the small orange-pigmented cells were intensely stained with this antibody at day three and day four (Figs. 5C and D; n=100 different juveniles examined for each stage and see Table 1). Furthermore, the average number of small, stained, pigmented cells per day four juvenile was four to eight based on serial sections obtained from thirty day four juveniles. These results suggest that mitochondrial Hsp60 is gradually degraded in apoptotic larval muscle cells, in striking contrast to a population of small, pigmented cells that contained significant quantities of this heat shock protein.

Table 1. Decrease in mitochondrial Hsp60 staining of apoptotic larval muscle cells between day three and day four of development

Section	DAY 3		DAY 4	
	Cell type			
	A	B	A	B
1	3/3	0/1	0/10	4/4
2	4/8	1/3	0/4	2/2
3	0/6	1/2	0/8	4/4
4	3/7	1/1	0/6	3/3
5	0/3	2/2	0/7	1/1
6	0/4	1/1	0/9	1/1
7	1/6	2/2	0/4	2/2
8	2/6	2/2	0/4	4/4
9	5/5	2/2	0/8	2/2
10	2/4	0/0	0/4	7/7
11	3/6	2/2	1/4	2/2
12	1/3	2/3	0/6	2/2
13	2/5	3/3	0/8	4/4
14	1/3	1/1	0/3	2/2
15	0/4	1/2	0/4	3/3
16	1/6	2/2	0/2	6/6
17	4/7	0/0	0/3	2/2
18	2/5	1/1	1/2	3/3
19	3/5	0/0	1/2	3/3
20	3/3	0/0	0/4	1/1
21	5/5	2/2	0/5	1/1
22	1/5	1/1	0/8	2/2
23	2/4	1/1	0/4	4/4
24	2/9	2/2	1/8	2/2
Totals:	50/122 (41%)	30/36 (83%)	4/127 (3%)	67/67 (100%)

Cell type A=large, pigmented apoptotic larval muscle cells; Cell type B=small orange-pigmented cells; Number of stained cells / total number of A or B cells observed per section.

The timing and localization of larval muscle cell ubiquination and the persistence of a muscle-specific intermediate filament protein, p58 in apoptotic larval muscle cells

Next, the timing of ubiquination of various larval cell lineages of *Boltenia* was examined. The first evidence for ubiquination of larval proteins was observed at day two of development after the tail cells were completely resorbed within the larval body. Anti-ubiquitin antibody stained only larval muscle cells (Table 2; Fig. 2K). That is, epidermal and endoderm cells were not stained with the StressGen anti-ubiquitin antibody. The strongest fluorescence was localized in the cytoplasm situated just beneath the plasma membrane of dying larval muscle cells. Despite evidence for ubiquination in larval muscle cells, a muscle-specific intermediate filament protein, p58, persisted within the cytoplasm of larval muscle cells at least until day twelve of development (Figs. 2I and J; Table 2). These results suggest that ubiquination may be selectively triggered within a specific larval cell lineage, in contrast to a pan-ubiquination of all tail cells. Furthermore, most of the ubiquination was associated with the plasma membrane and there appeared to be a selective ubiquination of specific target molecules residing in apoptotic larval muscle cells, as suggested by the presence of p58 in muscle cells stained with the ubiquitin antibody.

Table 2. Timing of ubiquination of larval muscle cells and the persistence of p58 in muscle cells undergoing PCD

A. Anti-ubiquitin antibody staining during metamorphosis:

	Cell Types			
	epidermis	muscle	endoderm	follicle cells
Day 1 (n=30):	–	–	–	+++
Day 2 (n=30)	–	+++++	–	+++++

– not stained; ++++ intense staining

B. Anti-p58 antibody staining of larval muscle cells undergoing PCD within young juveniles:

Day 4: 15/15 stained
Day 7: 15/15 stained
Day 8: 15/15 stained
Day 10: 15/15 stained
Day 12: 15/15 stained

Production of reactive oxygen species (ROS) and changes in mitochondrial activity

Next, the production of reactive oxygen species (ROS) in various larval cell types was examined as ROS activity is known to trigger apoptosis. This idea was tested by examining the production of ROS before and during tail resorption in living larvae using a ROS-specific fluorogenic substrate. Swimming larvae stained with dihydroethidium showed ROS activity within the cytoplasm of tail muscle cells (Fig. 2G). Endoderm, epidermis, notochord cells did not exhibit detectable ROS activity suggested by the absence of fluorescence

(n=thirty larvae examined). Furthermore, ROS activity appeared to increase during tail resorption within the cytoplasm of larval muscle cells, as suggested by an increase in fluorescence (Fig. 2H). Despite significant levels of ROS activity, many larval muscle cells remained vital following tail resorption, as demonstrated by their staining with

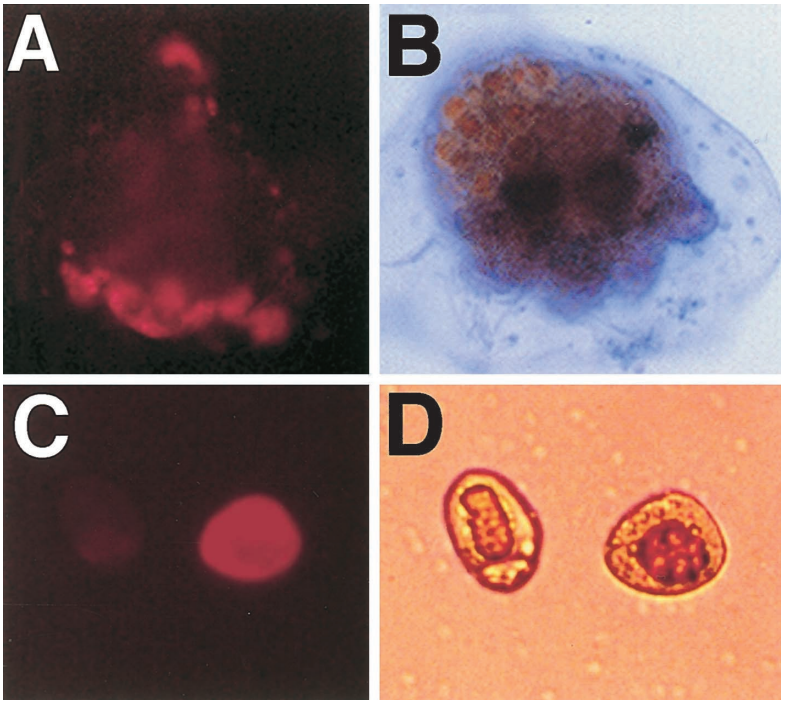

Fig. 6. Changes in mitochondrial activity demonstrated by rhodamine 123 (red) and MTT (purple) staining. Tips of epidermal ampullae were stained with rhodamine 123 (A) and MTT (B) at day three of development. Note the accumulation of rhodamine in the distal-most epidermal cells of the resorbed tail. Darkfield (C) and brightfield (D) images of two larval muscle cells that were surgically removed from day four juveniles and then stained with rhodamine 123 showing one stained (alive) cell and one unstained (dead) cell.

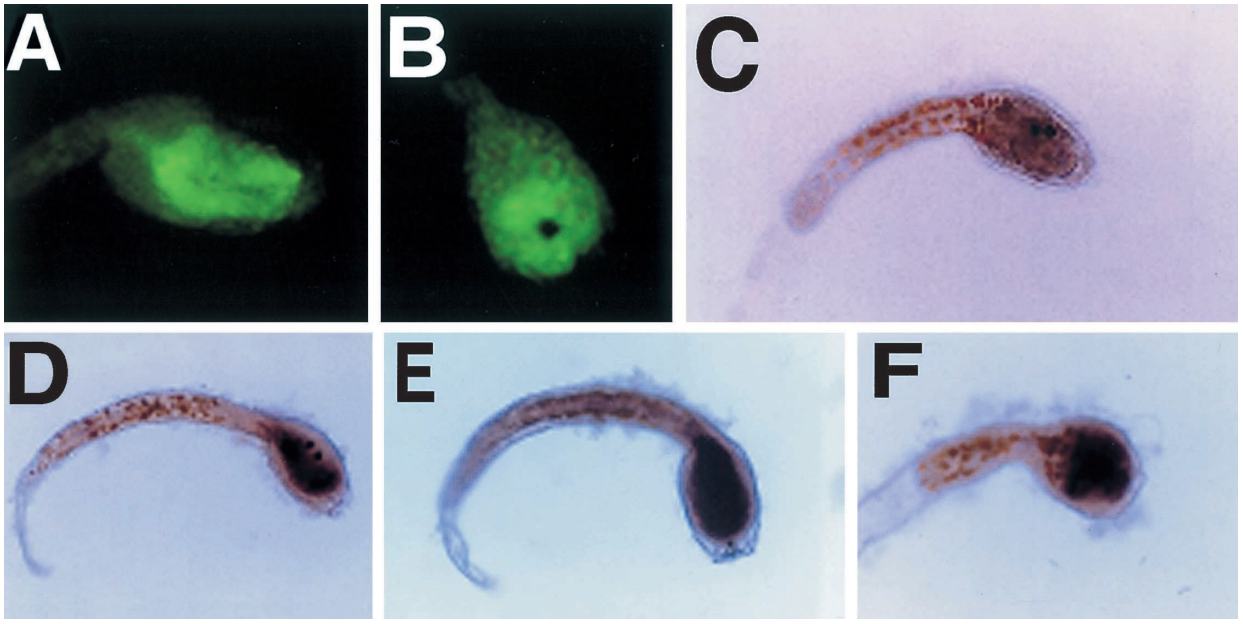

Fig. 7. Activation of caspases in larval endoderm cells. *In vivo* caspase-8 activity is shown restricted to larval endoderm cells at the beginning of metamorphosis (A). *In vivo* pan-caspase activity restricted to endoderm cells following tail resorption (B). An unstained control larva is shown in C, a control larva incubated with seawater containing BCIP to detect AP activity (D), a larva that showed pan-caspase activity was subsequently stained with BCIP is shown in (E) and a larva that showed caspase-8 activity was subsequently stained with BCIP substrate is shown in (F). The expression of caspases in AP-expressing larval endoderm cells was surprising given that these cells differentiate into adult structures.

rhodamine 123 (Figs. 6C and D).

To further investigate changes in mitochondrial viability during muscle cell apoptosis, live specimens at day two, three, four, five and six of development were stained with rhodamine 123. In some cases, larval muscle cells were first surgically removed from the surrounding tunic and then immediately stained with rhodamine, in the event that the tunic prevented the penetration of rhodamine. Other specimens were stained within their tunics for two hours minimum at 11°C, and in some cases overnight. The results suggest there is a gradual reduction in the viability of larval muscle cell mitochondria during metamorphosis. At day two of development, all of the larval tail muscle cells situated within the body cavity were stained with rhodamine 123 (n=thirty examined). At day four, the number of rhodamine-stained cells compared to non-stained cells per juvenile was: 3 out of 8 cells ; 4 out of 12 cells; 1 out of 3 cells; 2 out of 5 cells; 1 out of 3 cells; 6 out of 15 cells; 0 out of 3 cells; 1 out of 8 cells; 0 out of 5 cells; and 2 out of 5 cells. At day six of development, the following ratios of stained:unstained cells was: 2 to 20; 1 to 6; 0 to 8; 0 to 10; 1 to 4; 3 to 14; 4 to 8; 6 to 11; 3 to 6; and 2 to 12. These results suggest that the number of larval muscle cells containing viable mitochondria gradually decreases during metamorphosis. However, compared to rapid demise of notochord cells, it was surprising to find larval muscle cells within the body cavity of day six juveniles that were at least partially metabolically active.

By contrast, mitochondria within ampullar epidermal cells showed a dramatic increase in the uptake of rhodamine 123 and exhibited strong MTT staining (Figs. 6A and B). At day three of development, when approximately thirty percent of the larval muscle cells were stained with either rhodamine or MTT, epidermal cells of all eight ampullae per juvenile were stained using these methods (n=30 juveniles examined for each method). The intensity of MTT staining of ampullae increased by day four of development, in contrast to a weak or absence of staining of resorbed larval muscle cells. These results indicate that mitochondrial activities are down-regulated or up-regulated with PCD or cell differentiation, respectively, during ascidian metamorphosis.

Caspase activation in larval endoderm

Surprisingly, endoderm cells within the body region of competent larvae at thirty-two hours of development showed significant caspase activation, in lieu of the fact that these cells are known to contribute to the formation of adult structures (Figs. 7A and B). Both types of caspase substrates stained endoderm cells and a significantly weaker caspase activation was evident in the tail muscle cells. Caspase-8 staining was more intense than pan-caspase staining and a greater number of endoderm cells showed caspase-8 activation than pan-caspase activation. The most intense caspase-8 staining was localized around the oscellus. Treatment of day two larvae overnight with Z-Asp pan-caspase inhibitor followed by immersion in the caspase-8 substrate resulted in fifteen percent of the larvae stained (n=20), compared to eighty percent of the larva being stained without prior treatment with the pan-caspase substrate (n=20).

The conventional idea is that activation of caspase always results in the degradation of cellular proteins during apoptotic death. Therefore, to determine if caspase activation or the caspase substrates disrupt normal alkaline phosphatase (AP) activity, a major enzyme within endoderm cells, larvae were incubated in the presence of the alkaline phosphatase substrate BCIP. In all cases examined (n=70), all of the endoderm cells exhibited alkaline phosphastase activity comparable to the controls, as judged by the intensity of the colorimetric reaction in wholemount preparations (Figs. 7C and F; n=70). Therefore, caspase activation and treatment with caspase substrates does not appear to cause the degradation of AP. In summary, three observations suggest that caspase activation does not result in endodermal apoptosis: first of all, competent larvae treated with either of the caspase substrates continued to swim for up to three days when cultured at low densities to inhibit metamorphosis; secondly, endoderm cells that showed caspase activation exhibited AP activity at levels comparable to the controls and thirdly, endoderm cells were not stained with the ubiquitin antibody.

DISCUSSION

Apoptosis within the ovary

Apoptosis begins early in *Boltenia villosa* development with the death of oocytes and follicle cells within the ovary. As reviewed by Matova and Cooley (2001), oocyte death has been studied in detail in *C. elegans*, *Drosophilia* and several model vertebrates including *Xenopus*, chicks, rats and humans. These authors suggest that the elimination of oocytes functions to eliminate genetically defective gametes and to maintain germline homeostasis. This is presumably the role of ovarian cell apoptosis in *Boltenia*. The present findings are in general agreement with those of Jeffery (2002) who examined apoptosis in *Molgula manhattensis*, *M. provisionalis*, *M. occidentalis* and *M. occulta*, except that in *Boltenia* apoptosis of test cells was rarely observed. The present study and those reported by Jeffery describe the apoptotic of follicle cells. Chambon *et al*. (2002) observed apoptosis in extra-embryonic cells, but they did not report the apoptotic death of *Ciona* oocytes. Jeffery discussed that PCD can significantly vary between ascidian species. He observed that cell death was less evident in *Ascidia ceratodes* and *Ciona intestinalis*, as compared to molgulids. Species variations in ascidian PCD likely explains the apparent absence of test cell apoptosis in *Boltenia*, as compared to other species examined and likely accounts for additional differences in PCD described in *Boltenia*, as compared to those described by Jeffery (2002) and Chambon *et al*. (2002). For example, despite an examination of more than four hundred *Boltenia* larvae stained with acridine orange at various stages of tail resorption, no evidence was

obtained for a wave of cell death initiated at the tip of the tail, as previously described in *Ciona intestinalis* (Chambon *et al*., 2002). Clearly, it will be important to examine other ascidians for apoptotic waves associated with tail resorption.

The observation that more than half of the oocytes in *C. elegans* die by apoptosis led to the important discovery of a cysteine protease, the caspase CED-3 and its highly conserved role in PCD in a wide variety of organisms (Gumienny *et al*., 1999). The extent of germ-cell apoptosis in *Boltenia* is not as extensive as in *C. elegans*, but given the close phylogenetic kinship of ascidians with vertebrates, future molecular studies that explore in more detail the ascidian oocyte death machine will no doubt fill in a critical phylogenetic gap in the understanding of the evolution of oocyte death in basal chordates.

ROS production and membrane-associated ubiquination of larval muscle cells

ROS generation is a well known activator of apoptosis (Newmeyer and Ferguson-Miller, 2003). Less well known is the important role of ROS production in the induction of tolerance, or preconditioning, in heart and brain cells to subsequent cell stress (Baines *et al*., 1997). Therefore, ROS generation can promote either cell death or cell survival depending on the state of the signaling network within a cell.

The present results showed a cell-lineage specific cleavage of dihydroethidium that was restricted mostly to larval muscle cells. Cleavage of dihydroethidium increased during tail resorption and occurred prior to changes in membrane permeability and nuclear DNA fragmentation within larval muscle cells. The absence of significant levels of dihydroethidium cleavage by notochord cells suggests that the rapid death of these cells may not be promoted by elevated ROS levels within the cytoplasm. Furthermore, the apparent absence of ROS in endoderm cells may rule out a role for preconditioning in this cell lineage that somehow ameliorates the effects of caspase activation. However, the extensive cleavage of dihydroethidium by larval muscle cell cytoplasm is in some ways not surprising given the large quantities of mitochondria within these cells that produce many kinds of free radicals. While the present results could suggest that elevated ROS levels may function as a pre-apoptotic signal in larval muscle cells, additional experiments are needed to further test this hypothesis.

There are at least two forms of ubiquination (Polo *et al*., 2002). In the case of polyubiquination, cytoplasmic proteins are transport to proteasomes for degradation. This would result in a uniform staining of the cytoplasm following immunocytochemistry using anti-ubiquitin antibody. In monoubiquination, molecules associated with the plasma membrane are tagged with a single molecule of ubiquitin. In the present study, anti-ubiquitin antibody heavily labeled the cytoplasmic surfaces of larval muscle cell plasma membranes at day two of development. If most of the larval proteins were ubiquinated, a uniform staining of the cytoplasm would have been observed. Furthermore, it was shown in the present study that an intermediate filament protein, p58, was not significantly degraded despite the ubiquination. Therefore, the present results suggest the possibility that specific targets associated with the larval muscle cell plasma membranes are selectively ubiquinated.

Polo *et al*. (2002) showed that monoubiquination of plasma membranes is involved in the formation of vesicles. Given that the formation of vesicles and apoptotic bodies share several common features, monoubiquination may play an important role in the formation of apoptotic bodies. The ultrastructural results reported by Chambon *et al*. (2002) indicate that apoptotic body formation is a late event in larval muscle cell PCD, therefore, future experiments will test the hypothesis suggested by the present results that monoubiquination may have a role in this process. Furthermore, additional experiments are needed to determine the origin and developmental fate of the small, orange-pigmented cells stained with anti-Hsp60 antibody, as described in the present study. Are these apoptotic bodies derived from dying larval muscle cells or are they mesenchyme cells that contribute to the formation of the adult structures? Ultrastructural studies, now in process, will hopefully answer this question in the near future.

Caspase activation

Jeffery (2002) and Chambon *et al*. (2002) first demonstrated that caspase is activated in the cytoplasm of apoptotic cells of ascidian larvae using the pan-caspase in situ marker, CaspACE FITC-VAD-FMK. This substrate and a caspase-8 specific substrate were used in the present study to examine caspase activation in *Boltenia villosa*. As previously discussed, Chambon *et al*. (2002) inferred the existence of an ‘apoptotic wave’ originating at the tip of the tail using acridine orange staining. By contrast, Jeffery (2002) described apoptotic events occurring in both proximal and distal tail regions in molgulid larvae. These results suggest that there are significant differences in PCD mechanisms in ascidians. An apoptotic wave starting at the tip of the larval tail of *Boltenia villosa* was not observed in the present study, supporting the idea that a diversity of PCD mechanisms have evolved in ascidian metamorphosis. In *Boltenia*, apoptotic cells were scattered within the larval tail. SYTOX staining of cells surrounding the otolith of *Molgula occidentalis* was previously described by Jeffery (2002) and in the present study cells surrounding the sensory vesicle showed intense caspase activity.

The activation of caspase-8 in larval endoderm cells described in the present study raises some interesting questions pertaining to cell signaling. One possibility is that a Fas/TNF-like receptor may be upstream to caspase-8 activation in *Boltenia*, as this is the only family of receptors thus far known to be upstream to caspase-8 and has been shown to be responsible for caspase-8 activation (Hu *et al*., 2000). Annotation searches of the Department of Energy *Ciona* genome website indicate that ascidians have TNF-like receptors and many of the signaling proteins downstream to

activated caspase-8, with the notable exception of BID (Dehall *et al*., 2002; and the present study results not shown). The absence of the BID gene in *Ciona* may be relevant to the present study as BID is immediately downstream to activated caspase-8 and functions in apoptotic signaling. BID is transported to the outer mitochondrial membrane, binds to the membrane causing the release of cytochrome c into the cytoplasm were it interacts with several other proteins to form 'apoptosomes' in mammals (Brenner and Kroemer, 2000). The absence of BID plus the interaction of caspases with endogenous inhibitors of apoptosis provide a plausible explanation why caspase-positive endoderm cells shown in the present study do not undergo PCD.

However, another protein, the transcription factor NF-κB, is known to be activated by caspase-8 and this protein can promote cell differentiation (Chaudhary *et al*., 2000; Hu *et al*., 2000). Nk-κB proteins can promote either apoptosis or cell differentiation, depending on interactions with other signaling pathways within a cell. The multiple functions of the NF-κB/Rel family proteins is well documented, including their role in ascidian notochord formation (Shimada *et al*., 2001), axis formation in *Xenopus* (Tannahill and Wardle, 1995), a wide varielty of stress-induced immune responses, differentiation of hemopoietic cells, keratinocytes and lymphoid cells (Dixit and Mak, 2002). Therefore, another possible signaling pathway, supported by the present results, is that caspase-8 may activate a NF-κB pathway in *Boltenia* endoderm cells leading to the survival of these cells during metamorphosis and their subsequent differentiation into adult cell types. A recent study has shown that caspase activation protects rat brain cells from undergoing apoptosis during ischemic conditions of cell stress (McLaughlin *et al*., 2003) supporting the present results that caspases have duel functions in both cell survival and PCD. Many additional experiments are needed to carefully dissect the complex web of interacting signaling pathways that determine life and death cell fates. In conclusion, the present results together with the results obtained from many other recent studies demonstrate, once again, that ascidian metamorphosis is an excellent whole organism model system to study the diversity and evolution of chordate mechanisms that are responsible for PCD and how these mechanisms are regulated by environmental factors.

ACKNOWLEDGMENTS

This project is funded by a "Discovery Grant" from the Natural Sciences and Engineering Research Council (NSERC) of Canada. Westwind Sealab Supplies in Victoria, British Columbia, Canada is thanked for providing me with ascidians. Reviewers' comments were helpful in the preparation of the revised manuscript. Discussions with past and present undergraduate students working in my lab helped in the development of some of the ideas and experiments presented in this paper.

REFERENCES

Abrams JM (2002) Competition and compensation: coupled to death in development and cancer. Cell 110: 403–406

Baines CP, Goto M, Downey JM (1997) Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. J Mol Cell Cardiol 29: 207–216

Bates WR, Bishop C (1996) Localization of constitutive heat shock proteins in developing ascidians. Dev Growth Differ 38: 307–314

Bates WR, Jeffery WR (1987) Alkaline phosphatase expression in ascidian egg fragments and merogons. Dev Biol 119: 382–389

Brenner C, Kroemer G (2000) Mitochondria – the death signal integrators. Science 289: 1150–1151

Chaudhary PM, Eby MT, Jasmin A, Kumar A, Liu L, Hood L (2000) Activation of the NF-κB pathway by caspase 8 and its homologs. Oncogene 19: 4451–4460

Chambon J-P, Soule J, Pomies P, Fort P, Sahuquet A, Alexandre D, Mangeat P-H , Baghdiguian S (2002) Tail regression in *Ciona intestinalis* (Prochordate) involves a capasase-dependent apoptosis event associated with ERK activation. Development 129: 3105–3114

Cloney RA (1982) Ascidian larvae and the events of metamorphosis. Am Zool 22: 817–826

Dehall P *et al*. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298: 2157–2167

Dikalov S, Landmesser U, DG Harrison DG (2002) Geldanamycin leads to superoxide formation by enzymatic and non-enzymatic redox cycling. J Biol Chem 277: 25480–25485

Dixit V, Mak, TW (2002) NF-κB signaling: many roads lead to Madrid. Cell 111: 615–619

Gummienny TL, Lambie E, Hartwieg Horvitz EHR, Hengartner MO (1999) Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. Development 126: 1011–1022

Hoffman KL, Weeks JC (2001) Role of caspases and mitochondria in the steroid-induced programmed cell death of a motoneuron during metamorphosis. Dev Biol 229: 517–536

Holland LZ, Gibson-Brown JJ (2003) The *Ciona intestinalis* genome: when the constraints are off. BioEssays 25: 529–532

Hu W-H, Johnson H, Shu H-B (2000) Activation of NF-κB by FADD, Casper, and Caspase-8. J Biol Chem 275: 10838–10844

Jacobson MD, Wiel M, Raff MC (1997) Programmed cell death in animal development. Cell 88: 347–354

Jeffery WR (2002) Programmed cell death in the ascidian embryo:modulation by FoxA5 and Manx and roles in the evolution of larval development. Mech Dev 118: 111–124

Kowalevsky A (1868) Beitrage zur Entwicklungsgeschichte der Tunicaten (Vorlaufige Mitteilung). Nachrichten Gesellschaft Wissenschaften Gottingen 19: 401–415

Matova N, Cooley L (2001) Comparative aspects of animal oogenesis. Dev Biol 231: 291–320

McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E (2003) Caspase 3 activation is essential for neuroprotection in preconditioning. PNAS 100: 715–720

Newmeyer DD, Ferguson-Miller S (2003) Mitochondria: releasing power for life and unleashing the machineries of death. Cell 112: 481–490

Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P, Di Fiore PP (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature 416: 451–455

Shimada M, Satoh N, Yokosawa Y (2001) Involvement of Rel/NF-κB in regulation of ascidian notochord formation. Dev Growth Differ 43: 145–154

Sperandio S, De Belle I, Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. PNAS 97: 14376–14381

Swalla BJ, Badgett MR, Jeffery WR (1991) Identification of a cytoskeletal protein localized in the myoplasm of ascidian eggs: localization is modified during anural development. Development 111: 425–436

Tannahill D, Wardle FC (1995) Control of axis formation in *Xenopus* by the NF-κ B-I κ B system. Int J Dev Biol 39: 549–558

(Received December 8, 2003 / Accepted February 25, 2004)