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Caspase-like Activity is Required for Programmed Nuclear Elimination during Conjugation in *Tetrahymena*

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ABSTRACT. During conjugation in the binucleate ciliate, *Tetrahymena thermophila*, the old macronucleus is eliminated as new macronuclei and micronuclei are ontogenetically derived from the zygote nucleus. The mechanism of programmed nuclear elimination in ciliates may be related to the mechanism of apoptosis in higher organisms since its chromatin undergoes major condensation, its DNA is digested into nucleosome-sized fragments, and it stains positively for TUNEL. The present study explores whether caspases are involved in programmed macronuclear degradation in *Tetrahymena*. We show here that caspase-like activity is detectable using two specific colorimetric substrates, and that the activity is reduced with specific caspase inhibitors. In addition, using the fluorigenic substrate PhiPhiLux, active caspase-like activity is detected in living cells, localized to cytoplasmic vesicles; activity is not detected in pre- or post-condensed macronuclei. Finally, three different inhibitors of caspase activity cause a block to macronuclear chromatin condensation and elimination. Therefore, a caspase-like enzyme activity is necessary for regulating macronuclear elimination in *Tetrahymena*. These data support the possibility that macronuclear elimination is related, evolutionarily, to regulated cell death in multicellular organisms.

Key Words. Apoptosis, autophagosome, autophagy, caspase, chromatin, ciliates, conjugation, macronucleus, nucleus, *Tetrahymena*.

DURING conjugation in *Tetrahymena*, and other ciliates, the macronucleus of the cell is degraded as a new macronucleus and micronucleus develop from division products of the zygote nucleus. The regulation and manner of macronuclear degradation is of interest since it is developmentally programmed, it leaves the rest of the cell intact, and it is highly specific in that other nuclei in the same cytoplasm are not targeted. Previous studies showed that macronuclear elimination in *Tetrahymena* resembles some aspects of apoptotic cell death in multicellular organisms.

In apoptosis the nucleus shrinks in size as chromatin condenses, it stains positive for TUNEL (Terminal deoxyribosyltransferase mediated dUTP nick end labeling), and its DNA is cleaved into nucleosome-sized fragments; these characteristics also pertain to *Tetrahymena* macronuclei during their elimination (Davis et al. 1992; Mpoke and Wolfe 1996; Mpoke and Wolfe 1997).

Another critical feature of apoptosis is its dependence on activation of caspases, a family of cysteine proteases (Earnshaw et al. 1999; Nicholson 1999). Here we inquired whether caspase activity is detectable in *Tetrahymena*, and whether caspase activity might be required for programmed macronuclear elimination.

MATERIALS AND METHODS

Cell culture. The cells of *Tetrahymena thermophila* strains CU428 and B2086, developed by Peter Bruns were grown and prepared for conjugation as previously described (Wolfe et al. 1999). Typically, cells begin to pair at about one hour after mixing and reach 70% to 80% paired by four hours.

Cytological analyses. Nuclei were assayed in fixed cells (with an equal vol. of 20% formaldehyde in 10 mM phosphate buffer at pH 7) by staining with 0.1 μg/ml DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride). NPG (N-propyl gallate) was used to retard photo-bleaching (Giloh and Sedat 1982).

Simultaneous visualization of lysosomal bodies and nuclei in living cells was achieved by co-staining cells with AO (Acridine Orange) and Hoechst 33342 (Mpoke and Wolfe 1997), which selectively stains degrading nuclei in apoptotic cells yellow, rather than blue, an indication that the nucleus has become acidic.

The in situ assay for active caspase-like activity in living cells was performed using PhiPhiLux according to the manufacturer's protocol.

Fluorescence microscopy. A Zeiss Axioplan epifluorescence microscope was used. Micrographs were taken manually with 4- or 8-sec exposures using a Zeiss MC100 automatic camera loaded with Fujichrome Sensia II 200 film. DAPI, Hoechst and Apofluor were excited at about 350 nm and emitted light was observed with filters for blue emission. PhiPhiLux and AO were excited at 450–490 nm and emitted light was observed with filters for green emission.

Reagents. Caspase inhibitors zVAD-fmk (Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-aldehyde), and YVAD-CHO (N-acetyl-Tyr-Val-Ala-Asp-aldehyde) were obtained from Bachem, PhiPhiLux from OncoImmunin, NPG from Sigma, AO from Chroma-Gesellschaft, and Hoechst 33342 and DAPI from Molecular Probes.

RESULTS

Chromatin condensation and macronuclear compaction is the first visible sign of macronuclear elimination, and is therefore a good cytological indicator for whether or not a given macronucleus is in the process of being degraded. We determined that the commencement of chromatin condensation occurs in the population at about 6.5 h after mixing mating types; at 6 h no macronuclear compaction has occurred. At 9 and 12 h a substantial proportion of paired cells exhibit macronuclear compaction, indicating that at these times there is considerable ongoing macronuclear degradation in the population.

We tested the effects of different concentrations of the general caspase inhibitor zVAD-fmk on macronuclear compaction. zVAD-fmk was added at at 6 h, and the number of compact macronuclei among paired cells was assayed at 12 h. Compact macronuclei were seen in 87% of control pairs (Fig. 1a), whereas that number decreased with increasing concentrations of zVAD fmk. This shows that that macronuclear condensation is sensitive to caspase inhibitor in a concentration-dependent manner.

Two inhibitors with similar solubility but different specificities were compared, both at 0.5 mM. YVAD-CHO, specific for caspase 1, and DEVD-CHO, specific for caspase-3, were added to separate samples at 6 h and macronuclear condensation was assayed at 9 h. DEVD-CHO had a slight inhibitory effect but YVAD-CHO was effective in blocking about 50% of macronuclear condensation (Fig. 1b). Therefore, three different caspase inhibitors are effective in blocking macronuclear elimination.

To determine whether caspase-like activity is measurable in *Tetrahymena*, we assayed the hydrolysis of two different caspase substrates (0.5 mM YVAD-pNA [N-acetyl-Tyr-Val-Ala-Asp-pNA (pNA = p-Nitroaniline)]) and 0.5 mM DEVD-pNA (N-

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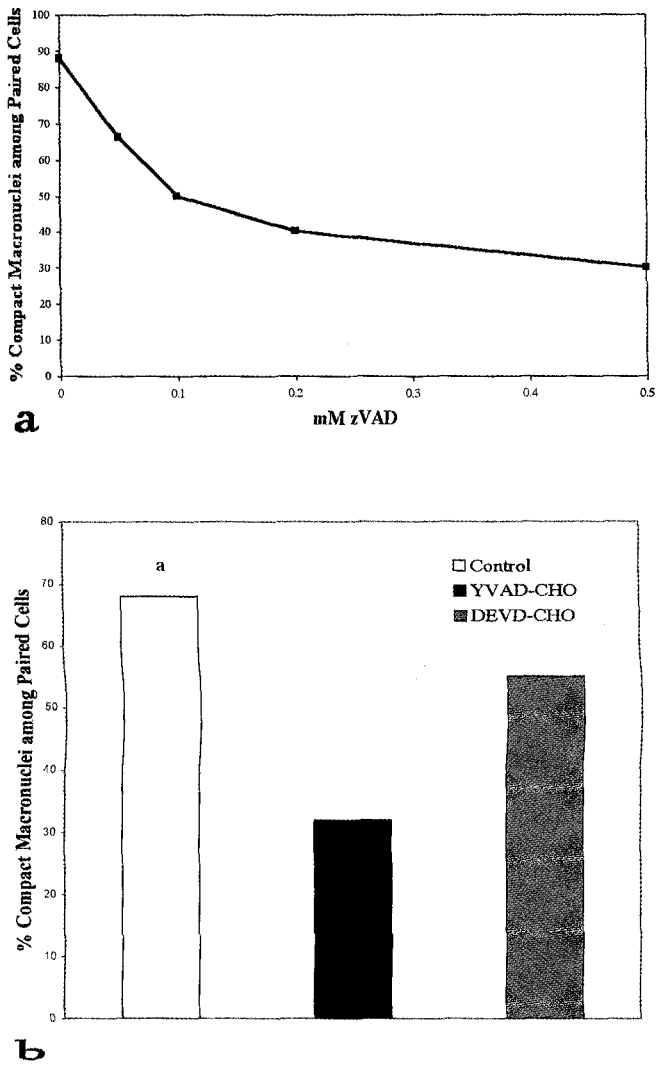

Fig. 1. Caspase inhibitors block macronuclear elimination. **1a.** Compact macronuclei in paired cells are reduced in number by zVAD, a pan-caspase inhibitor, in a concentration-dependent manner. **1b.** Compact macronuclei in paired cells are reduced in number by exposure to 0.5 mM YVAD, and inhibitor of caspase-1, and DEVD, an inhibitor of capsase-3.

Acetyl-Asp-Glu-Val-Asp-pNA (pNA = p-Nitroaniline)) by spectrophotometry in cell extracts of conjugating *Tetrahymena*, alone or together with selective inhibitors (0.5mM YVAD or 0.5 mM DEVD). Both substrates produced measurable product at 9 h after mixing cells, a time when macronuclear degradation is taking place, indicating that caspase-like activity exists in *Tetrahymena*. Moreover, caspase-like activity for each substrate was reduced 40% to 50% by both inhibitors, indicating that activity was sensitive to specific caspase inhibitors (data not shown).

We next measured caspase-like activity at different times during conjugation using the same colorimetric assay. Table 1 shows that caspase-like activity can be detected at any time during conjugation, and that product development occurs with both substrates. Whether the apparent rise in activity after 6.5 h is related to increased macronuclear degradation during that time period, or is simply statistical error, will require further analysis.

We also sought to determine the sub-cellular localization of caspase-like activity by examining live cells exposed to a fluorigenic substrate, PhiPhiLux. Fluorescence was observed amongst a 9-h group of cells, but only a small fraction of pairs was found with visible activity. Staining in those cells was localized to vesicles in the cytoplasm (Fig. 2a). In control cells without PhiPhiLux no such staining was observed. The dark center of the cells in Fig. 2a corresponds to the uncondensed macronucleus.

Table 1. Caspase-like activity in cell extracts of *Tetrahymena*. This assay was performed using a kit from BioMol. Cell lysates were made of pelleted cells from 10 mL of mixed mating types (~5 × 10^5 cells/ml) using 1 mL of (proprietary) cell lysis buffer. The tube was kept on ice for 10 min and centrifuged at 3,000 g at 4 °C for 10 min. the supernatant was transferred to a 1.5-mL Eppendorf tube and stored at −20 °C. Ninety μL of cell lysate (corresponding to ~0.45 × 10^5 cells) and 10 μL caspase substrate were placed in 96-well plates. The substrates used were DEVD-pNA for caspase-3 activity and YVAD-pNA for caspase-1 activity. The cell lysates were assayed with a microplate reader at 405 nm. The microplate reader was zeroed with 90 μL cell lysis buffer and 10 μL caspase substrate.

	μM product from substrate:	
Time in hours	YVAD-pNA	DEVD-pNA
1.5	51.0	44.2
4.0	54.4	45.9
6.5	47.6	42.5
9.0	79.9	68.0
12.0	74.8	64.6

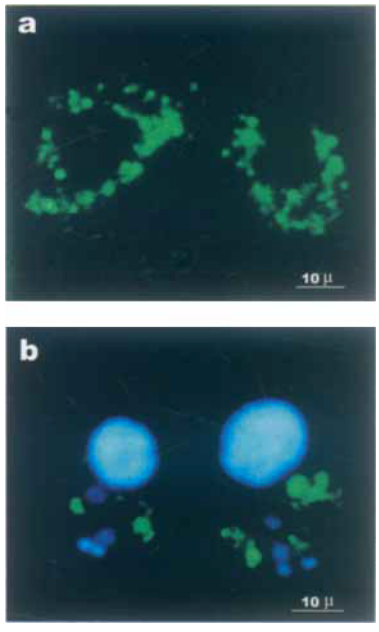

Fig. 2. Caspase activity is localized to cytoplasmic vesicles. Nine hours after conjugation, 1 mL of cells was aliquoted into microfuge tubes, centrifuged, and the pellets were resuspended in 75 μL of 10 mM PhiPhiLux (DEVD-GID) substrate solution. After one-hour incubation the samples were diluted with 1.0 mL ice-cold Tris buffer at pH 7.4 and washed once. **2a.** Activity is localized in living cells to vesicles in the cytoplasm. The large dark area is the macronucleus. **2b.** Stained also with Hoechst 33342, two separate images were superimposed using Adobe Photoshop. Caspase-like activity is associated with cytoplasmic vesicles, not nuclei. In this pair the existence of two slightly enlarged developing macronuclei is indicative of imminent macronuclear condensation.

Figure 2b is a computer-mediated overlay of two separate micrographs of the same living pair of cells double stained with PhiPhiLux and Hoechst 33342. This permits observation of any relationship between vesicles and nuclear structures. This conjugant pair contains two small developing macronuclei and two micronuclei, indicating that compaction of the old macronucleus is imminent. Here, and in repeated experiments, no activity was localized to nuclei, including degrading macronuclei. This indicates that caspase-like activity does not play a direct role in the degradation of the old macronucleus.

DISCUSSION

These experiments demonstrate that cell extracts of *Tetrahymena* possess caspase-like enzymatic activity, that activated caspase-like activity is detectable in living cells localized to cytoplasmic vesicles, and that caspase inhibitors block programmed nuclear degradation. These findings support the possibility that caspases play a role in the regulation of nuclear elimination in unicellular eukaryotes.

Our data do not indicate that caspase-like activity acts directly on the old macronucleus. Instead, it may trigger a process that leads to macronuclear degradation. The data show that new nuclear differentiation is also blocked by caspase inhibitors. Either nuclear differentiation and macronuclear degradation are separately dependent on caspase-like activity, or both are linked to a common process that is blocked by caspase inhibitors.

Recent studies in higher organisms suggest that caspases are not only involved in apoptotic cell death, but also in certain types of cell differentiation where nuclear degradation occurs. Specifically, differentiation of anucleate, but living, lens cells (Ishizaki et al. 1998) and keratinocytes (Gandarillas 2000; Gandarillas et al. 1999; Weil et al. 1999) depends upon caspase activation. In addition, nuclei are eliminated in atrophying muscle cells, and stain positively for TUNEL (Adams et al. 2001; Allen et al. 1997). Finally, in *Drosophila*, the transfer of cytoplasm from nurse cells to the developing oocyte, followed by degradation of nurse cell nuclei, is interrupted in a caspase mutant, leading to the conclusion that caspases in *Drosophila* also participate in the differentiation of oocytes (McCall and Steller 1998).

In *Tetrahymena*, macronuclear elimination shares properties with apoptosis, and the process also depends on caspase-like activity. Perhaps the more limited process of nuclear elimination, first associated with certain forms of cell differentiation in protistans, like macronuclear elimination in *Tetrahymena*, preceded apoptosis evolutionarily. Indeed, it has been proposed that the pre-existence of cell death mechanisms in protistans may have been a prerequisite for the evolution of Metazoa (Huettenbrenner et al. 2003). However, others argue that caspase-mediated apoptosis arose in multicellular organisms (Böttger and David, in press).

In situ, apoptotic cells are engulfed by neighboring cells and eliminated by phagocytosis. But how is a nucleus degraded within a living cell? It is likely that macronuclei are digested by autophagy, a process involving lysosomal fusion to a double-membraned vesicle containing the targeted material. Degrading nuclei do become acidic (Mpoke and Wolfe 1997; Santos et al. 2000), and they do become filled with lysosomal enzymes (Lu and Wolfe 2001). In addition, macronuclear elimination is blocked by 3-methyl adenine, an inhibitor of autophagy (Talchai and Wolfe 2001). Our studies of *Terahymena* macronucleus elimination suggest that intracellular elimination of nuclei may depend both on apoptotic regulation (caspase activation) and on autophagic degradation.

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