The Mechanism of caspase-3/9 activation by sfApaf-1 in unfertilized starfish eggs

Ritsuko Tamura

Apoptosis plays important roles in metazoan development and tissue homeostasis. It is executed by the activation of a family of aspartate specific cysteine proteases known as caspases. Generally, inactive zymogens of caspases, known as procaspases, are synthesized constitutively and activated by specific proteolytic cleavage. In mammals, apoptosis pathway is initiated by cellular stress, which releases cytochrome *c* from mitochondria into the cytoplasm. Cytochrome *c* binds to the WD40 repeat regions of cytosolic apoptotic-protease-activating factor 1 (Apaf-1) to form a large complex known as the apoptosome. Caspase-9, an initiator caspase, is recruited and activated by the apoptosome, and subsequently cleaves either procaspase-3 or -7 to make active effector/executioner caspase-3 or -7, respectively (Li et al., 1997 ; Yuanming et al., 1999). Association of procaspase-9 with Apaf-1 is mediated by their caspase recruitment domains (CARD) located at the amino terminal.

Meiosis reinitiation of oocytes in starfish (*Asterina pectinifera*) is stimulated by the hormone 1-methyladenine (1-MA), which is a prerequisite for fertilization. Without insemination or fertilization, endogenous caspase-3-like activity increases in aged eggs ~10 h after 1-MA stimulation, followed by blebbing and apoptotic body formation（Sasaki and Chiba, 2001）. If starfish oocytes are not treated with 1-MA, they are alive over several days in seawater. Thus, hormonal stimulation leads to apoptosis, whereas fertilization blocks the apoptotic program. As starfish is one of the species located close to the evolutionary branching point between vertebrates and nematodes, our research should help to provide clues to elucidate the relationship of apoptosis between vertebrates and nematodes which are well-characterized as model organisms. We therefore report here on the molecular mechanisms of starfish apoptosis, including the identification of caspase and its activation.

1. The mechanism of caspase-3/9 activation by sfApaf-1

We cloned a starfish caspase corresponding to mammalian effector caspase containing a CARD that is similar to the amino terminal CARD of mammalian capsase-9, and we named it procaspase-3/9. Our previous studies indicate that starfish caspase is an effector caspase. To verify the mechanism of caspase-3/9 activation, we cloned starfish Apaf-1 containing a CARD, a NOD, and 11 WD40 repeat regions, and we named it sfApaf-1. Recombinant sfApaf-1 CARD interacts with recombinant caspase-3/9 CARD and with endogenous procaspase-3/9 in cell-free preparations made from starfish oocytes, causing the formation of active caspase-3/9.

To detect endogenous sfApaf-1, we raised a specific antibody against sfApaf-1. We immunized rabbits

with sfApaf-1 CARD (1–134aa) to obtain an anti-sfApaf-1 antibody because full-length sfApaf-1 was insoluble in *Escherichia coli*. To determine whether endogenous caspase-3/9 interacts with endogenous sfApaf-1 during apoptosis, we used a cell-free preparation to perform immunoprecipitation assays using anti-caspase-3/9 antibody. As expected, sfApaf-1 was efficiently co-immunoprecipitated with caspase-3/9 from the apoptotic cell-free preparation, but not from the non-apoptotic cell-free preparation. These results indicate that the interaction between caspase-3/9 and sfApaf-1 occurred upon apoptosis.

In mammalian cells, it is reported that inhibitor proteins bind procaspase-9, preventing procaspase-9 activation. If procaspase-3/9 in starfish oocytes is blocked by endogenous inhibitor proteins, recombinant procaspase-3/9-His_6 may absorb putative inhibitors suppressing activation of endogenous procaspase-3/9. As expected, when the cell-free preparation without mitochondria was incubated with inactive recombinant procaspase-3/9-His_6, DEVDase activity increased and apoptosome-like complexes were formed in the high molecular weight fractions containing both sfApaf-1 and cleaved caspase-3/9. These results suggest that sfApaf-1 activation is not dependent on cytochrome *c*.

2. Retained eggs died by apoptosis in ovaries after spawning

Generally, apoptosis is used removing unnecessary cells, as shown in retained eggs in the frog genital tract (Iwasaki et al., 2013). To determine whether retained eggs in the starfish ovaries undergo apoptosis, we injected 1-MA into the body cavities. Around 24-36 hours after the hormonal stimulation, we detected matured eggs stained by Annexin V, indicating that the retained eggs undergo apoptosis in starfish ovaries.

In the previous studies, we reported that ovarian eggs arrest at metaphase of meiotic division I (MI) for 1-2 hours without mitogen-activated protein kinase (MAPK) activation after the hormonal stimulation (Usui et al., 2008). MI arrest is released by the activation of MAPK immediately after spawning of the maturing eggs. Then, spawned eggs develop the competence to die when high MAPK activity is maintained for several hours (MAPK-dependent period). Apoptosis occurs after this MAPK-dependent period (Sasaki and Chiba, 2004). To determine whether MAPK in the apoptotic eggs in the ovaries is activated or not, we monitored MAPK activities in retained eggs after spawning. We found that MAPK was activated around 3 hours after hormonal stimulation, and this activity was maintained for ~10 hours. Moreover, we observed female pronuclei in retained eggs 12 hours after hormonal stimulation. These results indicate that MI arrest of maturing eggs is released at ~3 hours after the hormonal stimulation, followed by apoptosis. Thus, MAPK-dependent period is essential for apoptosis occurring both inside and outside of the ovaries.