Induction of Apoptosis in Starfish Eggs Requires Spontaneous Inactivation of MAPK (Extracellular Signal-regulated Kinase) Followed by Activation of p38MAPK

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Mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase) prevents DNA replication and parthenogenesis in maturing oocytes. After the meiotic cell cycle in starfish eggs, MAPK activity is maintained until fertilization. When eggs are fertilized, inactivation of MAPK occurs, allowing development to proceed. Without fertilization, highly synchronous apoptosis of starfish eggs starts 10 h after germinal vesicle breakdown, which varies according to season and individual animals. For induction of the apoptosis, MAPK should be activated for a definite period, called the MAPK-dependent period, during which eggs develop competence to die, although the exact duration of the period was unclear. In this study, we show that the duration of the MAPK-dependent period was ~8 h. Membrane blebbing occurred ~2 h after the MAPK-dependent period. Surprisingly, when MAPK was inhibited by U0126 after the MAPK-dependent period, activation of caspase-3 occurred earlier than in the control eggs. Thus, inactivation of MAPK is a prerequisite for apoptosis. Also, even in the absence of the inhibitor, MAPK was inactivated spontaneously when eggs began to bleb, indicating that inactivation of MAPK after the MAPK-dependent period acts upstream of caspase-3. Inactivation of MAPK also resulted in the activation of p38MAPK, which may contribute to apoptotic body formation.

INTRODUCTION

Apoptosis plays critical roles in development and in the maintenance of homeostasis. Once triggered, the apoptotic program induces activation of a series of biochemical events. The best characterized pathway of apoptosis involves the release of cytochrome *c* from mitochondria, leading to the activation of caspase-9. The caspase-9 cleaves and activates caspase-3, which is the key enzyme to execute apoptosis (reviewed by Chang and Yang, 2000). Caspase-3 cleaves a large number of proteins within the cell, leading to the orderly dismantling of the apoptotic cell (reviewed by Porter and Janicke, 1999).

A mitogen-activated protein kinase kinase kinase phosphorylates and activates a MAP kinase kinase (MAPKK), which phosphorylates and activates mitogen-activated protein kinase (MAPK). In mammals, there are at least three genetically distinct groups of MAPK pathways, including extracellular signal-regulated kinase (ERK: MAPK), the c-Jun NH₂-terminal kinase (JNK), and the p38MAPK (reviewed by Widmann *et al.*, 1999; Davis, 2000). The JNK and the p38MAPK cascades are activated by many agents that induce apoptosis such as oxidative stress, UV radiation, transforming growth factor- β treatment, and anticancer drugs (Zanke *et al.*, 1996; Huot *et al.*, 1998; Tournier *et al.*, 2000; Edlund *et al.*, 2003). Inhibition of JNK and p38MAPK suppresses apoptosis induced by these agents. Although JNK and p38MAPK seem to be

involved in modulating apoptosis, ERK is generally considered as a survival factor. In *Drosophila*, the Ras-MAPK signaling pathway promotes cell survival by inhibiting the expression and activity of the proapoptotic protein Hid (Kurada and White, 1998). In rat cerebellar granule cells, MAP kinase-activated kinase, Rsk, phosphorylates the proapoptotic protein BAD. Phosphorylated BAD is inactivated and thus active Rsk prevents apoptosis by inhibiting BAD (Bonni *et al.*, 1999). Growth factor withdrawal down-regulates ERK and induces apoptosis in several cell systems, whereas activation of ERK during stress can be protective (Xia *et al.*, 1995; Guyton *et al.*, 1996). In rat PC-12 cells, apoptosis is regulated by a balance between activation of JNK, p38MAPK, and ERK (Xia *et al.*, 1995).

Fully grown starfish oocytes are arrested at prophase of meiosis I. Meiosis is reinitiated by 1-methyladenine (1-MA), which is released from follicle cells, causing germinal vesicle breakdown (GVBD) (Kanatani *et al.*, 1969). Just after GVBD, MAPK (ERK) is activated (Pelech *et al.*, 1988) by a newly synthesized Mos functioning as a mitogen-activated protein kinase kinase (Tachibana *et al.*, 2000). When fertilization occurs during meiosis, a decrease of MAPK activity by a disappearance of Mos occurs during or after the second polar body formation, which initiates DNA synthesis and embryonic development (Picard *et al.*, 1996; Tachibana *et al.*, 2000). Without fertilization, MAPK activity is maintained even after meiotic divisions (Tachibana *et al.*, 1997; Fisher *et al.*, 1998; Sadler and Ruderman, 1998), which is necessarily for induction of apoptosis (see below).

Although starfish immature oocytes can live >1 wk in seawater, postmeiotic eggs synchronously and rapidly un-

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dergo apoptosis in <24 h after 1-MA treatment (Sasaki and Chiba, 2001; Yüce and Sadler, 2001). Usually, in starfish *Asterina pectinifera*, caspase-3 activation occurs 8–12 h after 1-MA treatment, followed by membrane blebbing and apoptotic body formation. The timing of apoptosis depends on the animals and seasons, but apoptotic processes are highly synchronous in the same animal. When MAPK is blocked by an MAPK kinase inhibitor, apoptosis does not occur. Similar results are obtained when Mos synthesis is blocked by emetine to inhibit MAPK, whereas injection of recombinant Mos into emetine-treated eggs causes apoptosis several hours after injection. These results support the hypothesis that MAPK should be activated during a definite period to induce apoptosis, called the MAPK-dependent period (Sasaki and Chiba, 2001). The MAPK-dependent period starts immediately after GVBD, and inhibition of MAPK before the end of the MAPK-dependent period blocks apoptosis. To study the role of MAPK in the induction of apoptosis, it is crucial to define the MAPK-dependent period.

MATERIALS AND METHODS

Materials

U0126 (Promega, Madison, WI) and U0124 and SB203580 (Calbiochem, La Jolla, CA) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. 1-MA (1 mM), purchased from Kanto Kagaku Reagent Division (Tokyo, Japan), was dissolved in distilled water. These solutions were stored at -20°C .

Animal Maintenance, Gamete Collection, and Culture

Starfish *A. pectinifera* were collected on the Pacific coast of Honshu Island, Japan, and kept in laboratory aquaria supplied with circulating seawater at $10\text{--}17^{\circ}\text{C}$. To remove follicle cells, isolated ovaries were incubated in ice-cold Ca^{2+} -free artificial seawater (480 mM NaCl, 10 mM KCl, 27 mM MgCl_2 , 29 mM MgSO_4 , 2 mM NaHCO_3 , or Ca^{2+} -free Jamarin; Jamarin Laboratory, Osaka, Japan), and released oocytes were washed twice with Ca^{2+} -free artificial seawater. Defolliculated oocytes were stored in artificial seawater (Ca^{2+} -free Jamarin plus 9.2 mM CaCl_2) at 20°C . Oocyte maturation was induced by the addition of 1 μM 1-MA. GVBD occurred around 20 min after 1-MA treatment. 1-MA was washed out of the culture 40–60 min after 1-MA treatment.

Transmission Electron Microscopy

Small portions of the egg suspension were sampled and fixed with OsO_4 for 30 min on ice. The fixative contained 1% OsO_4 , 0.05% sodium cacodylate, and 1.5% potassium ferrocyanide in Ca^{2+} -free artificial seawater. After dehydration in an ethanol series, the oocytes were embedded in Epon resin containing 14% Quetol653 (Okenshoji, Osaka, Japan), 23% ERL4206, 63% nonenyl succinic anhydride, and 0.5% S-1 (TAAB Laboratories Equipment, Berkshire, England). Ultrathin sections were mounted on a grid coated with Bioten mesh cement (Okenshoji) and then stained with uranyl acetate and lead citrate. Grids were examined using a JEOL-1230 electron microscope (JEOL, Tokyo, Japan).

Measurement of DEVD-MCA Cleavage Activity

Eggs were collected by centrifugation at $1800 \times g$ for 2 min at 4°C . The eggs were resuspended in ice-cold buffer A (100 mM HEPES-NaOH, pH 7.5, 10 mM dithiothreitol; 0.05 μl /egg) and homogenized on ice. Egg homogenates were spun at $14,000 \times g$ for 15 min at 4°C to obtain cytosolic extracts. Proteolytic reactions were carried out in 1 ml of buffer A, containing 50 μl of cytosolic extracts and 10 μM acetyl-Asp-Glu-Val-Asp- (4-methyl-coumarinyl-7-amide) (Ac-DEVD-MCA; Peptide Institute, Osaka, Japan) at 20°C . The fluorogenic product substrate 7-amino-4-methylcoumarin was detected by excitation at 380 nm and emission at 460 nm with a fluorescence spectrophotometer (650-10 SL; Hitachi, Tokyo, Japan), and the initial velocity of hydrolysis of the substrate by DEVDase was measured.

SDS-PAGE and Western Blotting

Eggs were pelleted by brief centrifugation to remove seawater. The egg pellet was resuspended in SDS-sample buffer at 0.33 μl /egg, heated to $95\text{--}100^{\circ}\text{C}$ for 5 min, and subjected to gel electrophoresis. Typically, 10 μl of each sample (containing 30 eggs) was run on a 12.5% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride transfer membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was blocked with phosphate-buff-

ered saline (PBS) containing 5% skim milk and incubated with an anti-rat ERK1 antibody (Seikagaku, Tokyo, Japan) for 45 min or an anti-active p38MAPK antibody (Promega) for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20 (vol/vol), the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody for 45 min at room temperature and then washed again. Bound antibody was detected using an ECL Western blotting analysis system (Amersham Biosciences, Piscataway, NJ) and an LAS-1000 lumino image analyzer (Fuji Photo Film, Tokyo, Japan). Western blots were sometimes stripped by using Western blot stripping buffer (Pierce Chemical, Rockford, IL) and reprobed with another antibody. When we use highly sensitive detection reagents (ECL+; Amersham Biosciences), the membrane was incubated overnight with an anti-active p38MAPK antibody at 4°C and then incubated for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20 (vol/vol), the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at room temperature.

Preparation of Recombinant Protein

To prepare glutathione S-transferase (GST), the pGEX-6P-3 vector was purchased from Amersham Biosciences. The GST-starfish Mos (GST-Mos) construct in the pGEX-4T-2 vector was kindly provided by Dr. Kazunori Tachibana (Tokyo Institute of Technology, Tokyo, Japan). The plasmids were transformed into the BL21 bacterial strain, followed by growth at 37°C for 1 h, chilled in ice water for 15 min, and then induced with 0.5 mM isopropyl β -D-thiogalactoside at 37°C for 3 h. Bacteria were then pelleted by centrifugation at $8000 \times g$ for 5 min, resuspended in 50 ml of PBS containing 0.05% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) per liter culture, and disrupted by sonication. The GST-Mos and GST were purified with glutathione-Sepharose 4B (Amersham Biosciences) and concentrated to 1.6 mg/ml in buffer B (20 mM HEPES, pH 6.8, 88 mM NaCl, 7.5 mM MgCl_2) with Centricon YM-50 and Microcon YM-50 (Millipore). Aliquoted proteins were frozen in liquid nitrogen and stored at -80°C .

Microinjection

Microinjection into an egg and quantitation of injection volumes were performed according to the methods of Hiramoto (1974) and Kishimoto (1986). Ac-DEVD-CHO (Peptide Institute) dissolved in DMSO at a concentration of 10 mM was diluted with aspartate buffer (100 mM potassium aspartate, 20 mM HEPES, pH 7.2) to make 2.5 mM Ac-DEVD-CHO solution for injection. GST-Mos and GST was dissolved as described above. The injection volume of Ac-DEVD-CHO solution was $\sim 3\%$ of the total egg volume, and GST-Mos or GST was 10%. Oocytes were held between two coverslips separated by one piece of double-stick tape during microinjection and observation (Chiba *et al.*, 1992).

RESULTS

Morphological Changes during Starfish Egg Apoptosis

Immature oocytes of the starfish *A. pectinifera* were treated with 1-MA to reinitiate meiotic maturation. Oocytes completed both meiotic divisions to yield haploid interphase-arrested eggs (Figure 1A, a). Although the timing of the occurrence of blebbing depends on the animals, synchronous blebbing always starts between 8 and 12 h after 1-MA treatment (Sasaki and Chiba, 2001). In this experiment, membrane blebbing started 10 h after 1-MA treatment and continued for ~ 1 h (Figure 1A, b and c). Blebbing then stopped, and fragmentation of eggs forming spherical vesicles occurred (Figure 1A, d and e). Electron microscopy showed that cytoplasmic elements such as mitochondria, yolk granules, and cortical granules remained intact in the vesicles as well as in the blebbing egg (Figure 1B). Generally in apoptosis, cessation of blebbing is followed by the formation of membrane-bound, roughly spherical cytoplasmic fragments containing intact organelles, called “apoptotic bodies”, or condensation into a single, shrunken ball (Kerr *et al.*, 1972; reviewed by Mills *et al.*, 1999). Thus, it is concluded that the vesicles or fragments formed after blebbing are apoptotic bodies in starfish egg apoptosis.

Membrane blebbing and apoptotic body formation are hallmark morphological features of apoptosis. In addition, the key enzyme to execute apoptosis, caspase-3, is activated when starfish eggs initiate blebbing (Sasaki and Chiba,

Figure 1. Time course of starfish egg death after 1-MA treatment. (A) To reinitiate meiosis, oocytes were stimulated by the addition of 1 μ M 1-MA. Morphological changes were observed with a light microscope equipped with Nomarski differential interference contrast optics. Photographs of the same egg were taken at the indicated times after 1-MA treatment. (a) Mature egg with female pronucleus, 8 h 10 min. (b and c) Blebbing eggs, 10 h 10 min and 10 h 50 min, respectively. (d and e) Apoptotic body formation, 11 h 10 min and 13 h, respectively. Arrows indicate apoptotic bodies. Bar, 50 μ m. (B) Transmission electron micrographs of apoptotic eggs. (a) Representative cross section through a blebbing egg at 10 h after 1-MA treatment. Bar, 1 μ m. (b) Representative cross-section through an apoptotic body at 12 h after 1-MA treatment. Bar, 1 μ m. cg, cortical granule; yg, yolk granule; m, mitochondrion.

2001). Thus, membrane blebbing and apoptotic body formation observable with light microscopy of live samples provide an easy and reproducible assay for distinguishing viable from apoptotic cells. We used this morphological assay in the following experiments to characterize the apoptotic process in postmeiotic starfish eggs.

The MAPK-dependent Period for Induction of Apoptosis

For induction of starfish egg apoptosis, MAPK should be activated for a definite period, called the MAPK-dependent period (Sasaki and Chiba, 2001), although the exact duration of the MAPK-dependent period was still unclear. If active MAPK is detected in the eggs at time T1 and if artificial inactivation of MAPK from time T1 causes blocking of apoptosis, time T1 is determined to be within the MAPK-dependent period. If artificial inactivation of MAPK after time T2 does not inhibit induction of apoptosis, time T2 is not within the MAPK-dependent period (the MAPK-dependent period has already finished).

Thus, to measure the length of the MAPK-dependent period, we blocked MAPK by using mitogen-activated protein kinase kinase (MEK) inhibitor U0126 at various times after 1-MA treatment. Because 50% inhibitory concentration of MAPK was ~ 0.1 μ M in starfish eggs (Figure 2A), which is comparable with that of in COS-7 cells (Favata *et al.*, 1998), we treated eggs with 1 μ M U0126. The percentage of apoptotic eggs was counted 11 h after 1-MA treatment. U0126 was suitable for our purpose, because MAPK was inactivated by 1 μ M U0126 treatment within 45 min (Figure 7A). In this study, the end of the MAPK-dependent period is defined as the time of U0126 treatment after which >50% of the eggs will proceed into apoptosis even in the presence of the drugs.

Using the results in Figure 2B, the MAPK-dependent period was estimated to continue until 7 h 18 min after 1-MA

treatment (Figure 2C, a). Because GVBD occurred at 20 min after 1-MA treatment, the MAPK-dependent period should start from then. In this animal, the duration of MAPK-dependent period was ~ 7 h.

The MAPK-dependent period depends on the animal and season. The duration of the period varied from 5.5 to 8.5 h. Frequently, the end of the MAPK-dependent period was 8 h after 1-MA treatment, and 50% blebbing occurred ~ 2 h after the end of MAPK-dependent period.

When U0126 was added to the egg culture from 7 h after 1-MA treatment, 15% of eggs underwent apoptosis (Figure 2B). In these 15% of eggs, the MAPK-dependent period had probably finished. Interestingly, these eggs initiated membrane blebbing earlier than the control eggs treated with inactive analogue U0124. Similar acceleration of blebbing occurred when U0126 was administered immediately after MAPK-dependent period as shown in Figure 3A, a. U0126-treated and U0124-treated eggs revealed 50% blebbing at 8 h 05 min and 8 h 34 min after 1-MA treatment, respectively. Similar results were obtained using oocytes from different females (Figure 3, b and c). Also, U0126-treated eggs proceed from blebbing to apoptotic body formation within 1–1.5 h; essentially the same kinetics as eggs cultured in the absence of U0126 (Figure 3B). These results indicate that inactivation of MAPK after the MAPK-dependent period accelerates the initiation of blebbing but does not prolong or shorten the execution phase. These results also suggest that inactivation of MAPK after the MAPK-dependent period is prerequisite to blebbing initiation because spontaneous inactivation of MAPK occurs at about the same time as the eggs initiated membrane blebbing (Sasaki and Chiba, 2001; Figure 5A). Thus, after the MAPK-dependent period, MAPK activity seems to act to inhibit blebbing initiation.

Figure 2. The MAPK-dependent period for induction of apoptosis. (A) From 39 min after 1-MA treatment, eggs were treated with the specific MEK inhibitor U0126 or its inactive analog U0124 (100, 25, 0.4, and 0.1 μM). After incubation for 1 h (1 h 39 min after 1-MA treatment), samples from eggs were taken and analyzed by Western blots probed with an antibody against MAPK (ERK1). Activation of MAPK is evident by the shift in mobility of the bands, because active MAPK is dually phosphorylated. Arrows and arrowheads indicate active and inactive forms of MAPK, respectively. U0126 was able to inhibit MEK-dependent intracellular ERK phosphorylation. The 50% inhibitory concentration of starfish MAPK was $\sim 0.1 \mu\text{M}$. U0124 did not inhibit MAPK at concentrations up to 100 μM . (B) Eggs were treated with 1 μM U0126 or U0124 at 2 h (a), 6.5 h (b), 7 h (c), 7.5 h (d), 8 h (e), and 8.5 h (f) after 1-MA treatment. The number of apoptotic cells as determined by blebbing or apoptotic body formation was scored ($n > 100$) at 11 h after 1-MA treatment, when membrane blebbing and apoptotic body formation had occurred in the control experiments. U0126 had inhibitory effects on apoptosis, whereas U0124 had no effects. The timing of U0126 treatment required for 50% apoptosis should be present between 7 h (c; 15%) and 7.5 h (d; 72%). Thus, in this animal (female A), the estimated MAPK-dependent period is 7 h 18 min ($= 7 \text{ h} + (50 - 15)/(72 - 15) \times 30 \text{ min}$). (C) Schematic illustration of the MAPK-dependent period and initiation of blebbing. The results of three independent experiments using different females (female A [a], female B [b], female C [c]) were shown. Arrows of 1 and 50% blebbing indicate the time when 1 and 50% of the control eggs without U0126 treatment initiated blebbing. The results are representative of at least five similar experiments by using oocytes from different females.

Inactivation of MAPK after the MAPK-dependent Period Is Required for Caspase-3 Activation

In starfish eggs, execution of apoptosis (membrane blebbing and apoptotic body formation) is regulated by the activity of caspase-3 (Sasaki and Chiba, 2001). To determine whether the timing of caspase-3 activation is accelerated in U0126-treated eggs, we measured the activity of caspase-3 by the cleavage of the peptide substrate Ac-DEVD-MCA. As shown in Figure 4, A and B, both the onset of apoptosis and caspase-3 activation were accelerated by U0126 treatment. Also, initiation of $>50\%$ blebbing as well as caspase-3 activation occurred within 1 h after U0126 treatment. Thus, inactivation of MAPK after the MAPK-dependent period is required for apoptosis.

Dynamics of MAPK and p38MAPK during Starfish Egg Apoptosis

Next, we examined the timing of blebbing and dynamics of MAPK of starfish eggs without U0126 treatment. Exposure of oocytes to 1-MA led to the activation of MAPK at ~ 30 min. The active form of MAPK persists for almost 10 h after 1-MA treatment. Immediately before the onset of blebbing, a small proportion of the MAPK became inactive even in the absence of U0126 (Figure 5A). At 11 h, a large quantity of

MAPK was spontaneously inactivated, followed by initiation of blebbing. Thus, it is likely that MAPK inactivation causes execution of apoptosis. Also, artificial inactivation of MAPK after the MAPK-dependent period resulted in caspase-3 activation (Figure 4). Thus, cell death probably occurs in the following order: persistent activation of MAPK during the MAPK-dependent period, MAPK inactivation, caspase-3 activation, and execution of apoptosis. Inactivation of MAPK does not act downstream of caspase-3 as shown in Figure 7B (see below).

Another MAPK family protein, p38MAPK, plays critical roles in stress responses and apoptosis in many cell lines (reviewed by Widmann *et al.*, 1999; Harper and LoGrasso, 2001). To determine whether p38MAPK was activated in apoptotic starfish eggs, oocytes and eggs were collected and then subjected to SDS-PAGE and immunoblotting with an active p38MAPK-specific polyclonal antibody. This antibody has been shown to specifically detect dually phosphorylated p38MAPK from human, mouse, and *Drosophila* cells. As expected, we could detect a putative active starfish p38MAPK band (apparent molecular mass of 38.5 kDa) when apoptotic eggs formed (Figure 5A). Although we did not detect the strong active starfish p38MAPK band in GV oocytes (Figure 5A, 0 h), Morrison *et al.* (2000) reported that

Figure 3. Effects of MAPK inactivation on the execution of apoptosis. The initiation of membrane blebbing and the initiation of apoptotic body formation were accelerated by U0126 treatment. (A) The percentage of apoptotic cells (as determined by blebbing or apoptotic body formation) was counted at the indicated times after 1-MA treatment. The results of three independent experiments by using different females (female D [a], female E [b], female F [c]) were shown. Eggs were treated with 1 μ M U0126 or U0124 (inactive analog of U0126) at 7.5 h (a) and 8 h (b and c) after 1-MA treatment. In U0126-treated eggs, 50% apoptosis occurred at 8 h 05 min (a), 8 h 25 min (b), and 8 h 58 min (c) after 1-MA treatment. And in U0124-treated eggs, 50% apoptosis occurred at 8 h 34 min (a), 9 h 29 min (b), and 9 h 56 min (c) after 1-MA treatment, respectively. Filled circles, U0126-treated eggs. Open circles, U0124-treated eggs. The results are representative of at least eight similar experiments with oocytes from different females. (B) Eggs from female D were treated with 1 μ M U0126 or U0124 at 7.5 h after 1-MA treatment. Photographs of the same egg (a–f) treated with U0126 or the same egg (g–l) treated with U0124 were taken at 8:15 (a and g), 8:30 (b and h), 8:50 (c and i), 9:00 (d and j), 9:30 (e and k), and 10:00 (f and l) after 1-MA treatment. Representative fields were shown. Bar, 50 μ m.

p38MAPK of the starfish *Pisaster ochraceus* is activated in immature oocytes and inactivated just before or during GVBD. When we used highly sensitive reagents, we could confirm that a weak p38.5 kDa-band on immunoblot of GV oocytes (Figure 5B, 0 h) disappeared after GVBD (Figure 5B, 0.5 h).

SB203580, an inhibitor of p38MAPK but not of p38MAPK-kinase MKK3 or MKK6, prevents phosphorylation of p38MAPK in many experimental systems, presumably via inhibiting its autophosphorylation (Ge *et al.*, 2002). In addition,

p38MAPK of the starfish *P. ochraceus* is suggested to be an autophosphorylating kinase (Morrison *et al.*, 2000). If this is the case, SB203580 inhibits phosphorylation of p38MAPK in apoptotic eggs. Indeed, as shown in Figure 5C, the 38.5-kDa band was not detected by the antibody, when we treated eggs with SB203580. This finding supports the identification of p38.5 as authentic active p38MAPK.

Just around GVBD, MAPK was activated and p38MAPK was inactivated (Figure 5, A and B). Conversely, when eggs began to bleb, MAPK is inactivated and p38MAPK was

Figure 4. Inhibition of MAPK after the MAPK-dependent period is required for caspase-3 activation. (A) Eggs were treated with 1 μ M U0126 or U0124 (control inactive analog) at 7 h (a), 7.5 h (b), and 6.5 h (c) after 1-MA treatment. The percentage of apoptotic cells was counted at the indicated times after 1-MA treatment. At the same time, eggs were collected and assessed for the DEVDase activity (Figure 4B, a–c). Each symbol represents the results from 100 eggs. The results of three independent experiments by using oocytes from different females (female G [a], female H [b], female I [c]) were shown. The results are representative of six similar experiments by using oocytes from different females. Filled circles, U0126-treated eggs. Open circles, U0124-treated eggs. (B) The activity of caspase-3 was measured by the cleavage of Ac-DEVD-MCA. Before the morphological changes, DEVDase activity was not detectable; 100% corresponds to the maximum DEVDase activity (a) 2.26, (b) 0.64, and (c) 2.01 nM/min in U0126-treated eggs, and (a) 1.09, (b) 0.57, (c) 2.08 nM/min in U0124-treated eggs, respectively. Filled circles, U0126-treated eggs. Open circles, U0124-treated eggs.

Figure 5. Dynamics of MAPK and p38MAPK through starfish egg apoptosis. (A) The percentage of apoptotic cells was counted under a light microscope at the indicated timings after 1-MA treatment. Each symbol represents the results from 100 eggs. At the same time, samples from eggs were taken and analyzed by Western blots probed with an antibody against MAPK. Bound antibody was detected using an ECL Western blotting analysis system. Arrows and arrowheads indicate active and inactive forms of MAPK, respectively. After chemiluminescent detection, Western blots were stripped and the membrane was reprobed with an antibody against active p38MAPK. The result is representative of 10 or more similar experiments by using oocytes from different females. (B) Samples from eggs were taken 0, 0.5, and 10.5 h after 1-MA treatment. All of the eggs from this animal had initiated apoptotic body formation 10.5 h after 1-MA treatment. Proteins from each sample containing 30 oocytes or eggs were resolved using 12.5% SDS-PAGE and analyzed by Western blots probed with an antibody against active p38MAPK. Bound antibody was detected using an ECL+ Western blotting analysis system, which is more sensitive than ECL. Weak staining at 0 h disappeared at 0.5 h, whereas strong staining was detected at 10.5 h. (C) SB203580 prevents activation of p38MAPK. Eggs were treated with the specific p38MAPK inhibitor SB203580 (100 μ M) or DMSO (1%) 12 h after 1-MA treatment (3% of eggs had just begun to bleb). Samples were taken 16 h after 1-MA treatment. All of the DMSO-treated eggs had initiated apoptotic body formation 16 h after 1-MA treatment. Proteins from each sample were resolved using 12.5% SDS-PAGE and analyzed by Western blots probed with an antibody against active p38MAPK.

activated, suggesting that there may be some connection between the two pathways. Also, because p38MAPK was highly activated when eggs initiated blebbing (Figure 5A), starfish p38MAPK is likely to participate to the execution of apoptosis. The role of weakly activated p38MAPK in GV oocytes is unknown.

Microinjection of the GST-Starfish Mos Fusion Protein into Eggs Delayed Execution of Apoptosis

If execution of apoptosis depends on the inactivation of MAPK, apoptosis should be inhibited by the microinjection

of exogenous Mos, which activates the Mos/MEK/MAPK pathway. Indeed, when we injected recombinant GST-starfish Mos fusion protein (GST-Mos, 160 μ g/ml final concentration) into the eggs 8–9 h after 1-MA treatment (before the initiation of blebbing), initiation of blebbing of injected eggs was delayed $\sim$ 2 h (Figure 6, a–c). As a control, injection of GST alone had no effect on execution of apoptosis (Figure 7, g–i). These results indicate that continuous activation of the Mos/MEK/MAP kinase cascade after the MAPK-dependent period had inhibitory effects on execution of apoptosis. Because occurrence of blebbing was delayed but not completely blocked in GST-Mos-injected eggs, these eggs might have strong activity to inactivate the MAPK pathway, or there may be some another component to activate caspase-3 other than MAPK inactivation.

Inactivation of MAPK after the MAPK-dependent Period Is Followed by Activation of p38MAPK

To determine whether activation of p38MAPK occurred after inactivation of MAPK, we treated the eggs with U0126 at 8 h 15 min after 1-MA treatment. As shown in Figure 7A, the timing of p38MAPK activation as well as blebbing initiation was accelerated by U0126 treatment. These results strongly suggested that inactivation of MAPK acts upstream of p38MAPK.

Eggs injected with Ac-DEVD-CHO fail to undergo membrane blebbing and apoptotic body formation, because Ac-DEVD-CHO blocks caspase-3-dependent blebbing (Sasaki and Chiba, 2001). To investigate whether p38MAPK was activated downstream of caspase-3, Ac-DEVD-CHO was microinjected into the cytoplasm of mature eggs at 5 h after 1-MA treatment. As shown in Figure 7B, at 14 h after 1-MA treatment, both inactivation of MAPK and activation of p38MAPK occurred normally in the injected eggs, whereas blebbing was completely blocked. In the control, those without injection initiated blebbing, and both inactivation of MAPK and activation of p38MAPK occurred. These results indicate that p38MAPK activation is not dependent on caspase-3 activation. Thus, it is concluded that the p38MAPK activation is not a consequence of stressful conditions generated by apoptosis but is likely to occur before or in parallel with activation of caspase-3.

Involvement of p38MAPK in Apoptotic Body Formation

To determine whether p38MAPK contributed some feature of apoptosis, we treated eggs with the specific p38MAPK inhibitor SB203580 just before the onset of blebbing (10.5 h after 1-MA treatment). As shown in Figure 8A, SB203580 did not block the blebbing. Apoptotic body formation, however, was severely inhibited in the SB203580-treated eggs, exhibiting an almost rounded morphology (Figure 8, b and c, arrows) with remaining small protrusions (Figure 8, b and c, arrowheads). The small protrusions were separated spontaneously from the rounded egg, and finally they degraded and released apoptotic body-like particles (Figure 8d, arrowhead). The egg still exhibited a rounded morphology even at 20 h after 1-MA treatment (Figure 8d, arrow). These results indicate that p38MAPK may contribute to apoptotic body formation.

Discussion

In this study, we found that MAPK has both positive and negative functions in the induction of starfish egg apoptosis. During the MAPK-dependent period ($\sim$ 8 h after 1-MA treatment), inactivation of MAPK blocked apoptosis, indicating that it gives the death-activating signal. Conversely, after the

Figure 6. Microinjection of the GST-starfish Mos fusion protein into eggs. Microinjection of the GST-starfish Mos fusion protein (GST-Mos) into eggs delayed execution of apoptosis. Eggs were microinjected with GST-Mos (a–f) or GST (g–l) 8–9 h after 1-MA treatment, at a final concentration of 160 $\mu\text{g}/\text{ml}$. Photographs were taken at 11 h (a and g), 12 h (b and h), 13 h (c and i), 14.5 h (d and j), 17 h (e and k), and 19 h (f and l) after 1-MA treatment. Without injection, 50% apoptosis occurred at ~ 10.5 h. The photographs of the same egg were shown. Representative figures from three experiments by using 38 eggs were shown. Bar, 50 μm .

MAPK-dependent period but before blebbing (~ 8 – 10 h after 1-MA treatment), inactivation of MAPK resulted in caspase-3 activation, causing apoptosis. Moreover, p38MAPK, which is generally considered as a death factor (Xia *et al.*, 1995; Kummer *et al.*, 1997) was activated immediately after the inactivation of MAPK. Thus, starfish MAPK gives the death-suppressing signal after the MAPK-dependent period.

During the MAPK-dependent period, starfish eggs are likely to develop competence to die, as reported in mammalian sympathetic neurons. After nerve growth factor deprivation, apoptosis of sympathetic neurons requires the activation of two events: a protein synthesis dependent, Bax-dependent release of mitochondrial cytochrome *c* and protein synthesis-independent, Bax-independent development of competence. Unlike most cells, cytosolic cytochrome *c* is not sufficient to induce cell death in nerve growth factor-maintained sympathetic neurons but can do so in the neurons that have developed competence (Deshmukh and Johnson, 1998). It is suggested that development of competence may be the result of the loss of function of one or more members of the inhibitor of apoptosis family blocking caspases (Deshmukh *et al.*, 2002). Because cytoplasmic microinjection of cytochrome *c* into the starfish eggs during the MAPK-dependent period did not accelerate apoptosis (our unpublished data), continuous MAPK activity during the

MAPK-dependent period may contribute to development of competence for cytochrome *c* to induce cell death.

Usually in mammalian apoptosis, the MAPK signaling pathway promotes cell survival by a dual mechanism comprising the posttranslational modification to inhibit a component of the cell death machinery and the increased transcription of prosurvival genes. Because inactivation of MAPK after the MAPK-dependent period resulted in caspase-3 activation in starfish eggs (within 1 h after U0126 treatment; Figure 4B), MAPK may regulate the apoptotic machinery directly or indirectly, presumably via phosphorylation. It is reported that egg extracts prepared from the frog *Xenopus laevis* initiate and execute a full apoptotic program in vitro when egg extracts are “aged” on the bench (Newmeyer *et al.*, 1994). Interestingly, activation of the Mos/MEK/MAPK pathway inhibits postcytochrome *c* release apoptotic events in *Xenopus* extracts in the absence of new mRNA/protein synthesis (Tashker *et al.*, 2002). Because cytoplasmic microinjection of cytochrome *c* into the starfish eggs did not accelerate apoptosis as described above, starfish MAPK may also inhibit the postcytochrome *c* release event in starfish eggs.

In mammals, two proapoptotic Bcl-2-family proteins, Bad and Bim, are involved in apoptosis after withdrawal of survival factors. MAP kinase-activated kinase Rsk phosphorylates the proapoptotic protein BAD. Phosphorylated

Figure 7. Effects of U0126 and Ac-DEVD-CHO on p38MAPK activation. (A) Inactivation of MAPK was followed by activation of p38MAPK. U0126 or U0124 (1 μM) was added to the egg culture 8 h 15 min after 1-MA treatment. Samples from eggs were taken at the indicated times after 1-MA treatment. Also, the percentage of apoptotic cells was counted. Proteins from each sample were resolved using 12.5% SDS-PAGE and analyzed by Western blots probed with an antibody against active p38MAPK. After chemiluminescent detection, Western blots were stripped and membrane was reprobed with an antibody against MAPK. Arrows and arrowheads indicate active and inactive forms of MAPK, respectively. The result is representative of four similar experiments by using oocytes from different females. (B) Inhibition of caspase-3 had no effect on MAPK inactivation or p38MAPK activation. The specific caspase-3 inhibitor Ac-DEVD-CHO was microinjected into the cytoplasm of mature eggs 4 h 30 min to 5 h 15 min after 1-MA treatment. Eggs that were not injected (–) were blebbing 12 h after 1-MA treatment. Samples were taken at the indicated times after 1-MA treatment. Proteins from each sample were resolved using 12.5% SDS-PAGE and analyzed by Western blots probed with an antibody against MAPK. Arrows and arrowheads indicate active and inactive forms of MAPK, respectively. After chemiluminescent detection, Western blots were stripped and the membrane was reprobed with an antibody against active p38MAPK. The result is representative of three similar experiments with oocytes from different females.

Figure 8. Effects of p38MAPK inactivation on apoptotic body formation. Eggs were treated with 100 μ M SB203580 (a–d) or 1% DMSO (e–h) 10.5 h after 1-MA treatment. 8% of eggs had just begun to bleb 10.5 h after 1-MA treatment. Photographs were taken 11 h (a and e), 12.5 h (b and f), 14.5 h (c and g), and 20.5 h (d and h) after 1-MA treatment. The representative fields of the different eggs were shown. The result is representative of 10 or more similar experiments with eggs from different females. Arrows, a large part of the egg exhibiting a rounded morphology. Arrowheads, small protrusions of the egg. Bar, 50 μ m.

BAD is inactivated, and thus active Rsk prevents apoptosis by inhibiting BAD (Bonni *et al.*, 1999; Shimamura *et al.*, 2000). The MAPK pathway-dependent phosphorylation of Bim targets Bim for degradation by the proteasome pathway (Ley *et al.*, 2003). In the absence of survival factors, dephosphorylated Bim inhibits antiapoptotic proteins such as Bcl-2 and render the cells more susceptible to apoptogenic stimuli (Terradillos *et al.*, 2002). It is possible that starfish MAPK inactivates starfish Bad and Bim and suppresses apoptosis after the MAPK-dependent period. Spontaneous inactivation of MAPK then occurs, causing caspase-3 activation. In *Drosophila*, proapoptotic protein Hid induces apoptosis by blocking a caspase inhibitor, Diap1 (Bergmann *et al.*, 2002). It is known that activation of the Ras/MAPK pathway inhibits Hid-induced apoptosis (Kurada and White, 1998), and phosphorylation of Hid by MAPK is thought to inactivate Hid (Bergmann *et al.*, 1998). Starfish MAPK may phosphorylate and inhibit a Hid-like protein in eggs.

We also demonstrated in this study that U0126 treatment resulted in the activation of p38MAPK. This result strongly suggested that inactivation of MAPK acts upstream of p38MAPK activation. In addition, just after GVBD, MAPK is activated (Pelech *et al.*, 1988) by a newly synthesized Mos (Tachibana *et al.*, 2000), and p38MAPK was inactivated just about the same time (Figure 5B; Morrison *et al.*, 2000). Activation of MAPK may inhibit p38MAPK activation in starfish eggs.

Because activation of p38MAPK occurred spontaneously even in the eggs injected with caspase-3 inhibitor Ac-DEVD-CHO (Figure 7B), p38MAPK does not act downstream of caspase-3. Further studies are required to determine whether p38MAPK acts upstream of caspase-3.

Regulation of actin dynamics is one of the functions of the p38MAPK pathway. After activation by p38MAPK, MAP kinase-activated protein kinase-2 phosphorylates HSP27, a protein that can modulate actin polymerization (Huot *et al.*, 1998; Landry and Huot, 1999). Interestingly, apoptotic body formation is regulated by actin polymerization in starfish eggs (Sasaki and Chiba, 2001), and the p38MAPK-specific inhibitor SB203580 antagonized apoptotic body formation. Thus, it is likely that p38MAPK in starfish also contributes to actin polymerization.

We and others had demonstrated that postmeiotic starfish eggs undergo apoptosis, if they were not fertilized (Sasaki and Chiba, 2001; Yüce and Sadler, 2001). It was also reported that unfertilized ovulated murine oocytes cultured in vitro

spontaneously undergo apoptosis (Takase *et al.*, 1995; Fujino *et al.*, 1996; Perez *et al.*, 1999). Thus, in some species of animal, the default fate of the ovulated eggs is death by apoptosis. To understand normal development, it is important to know how eggs undergo apoptosis and how apoptosis is suppressed after fertilization. Starfish is a good model for studying postmeiotic egg apoptosis, because it is easy to obtain a large quantity of homogenous eggs that synchronously undergo apoptosis in vitro.

Moreover, apoptosis is a widespread event in oogenesis (reviewed by Matova and Cooley, 2001). The role of apoptosis in the female gamete life cycle has been most extensively studied in mammals, despite the difficulty in obtaining and culturing sufficient quantities of the oocytes and eggs. In mammals, more than two-thirds of the potential germ cell pool (oogonia and oocytes) is lost through apoptosis by the time of birth (reviewed by Morita and Tilly, 1999), and >99% of the postnatal oocytes, which are not ovulated, also undergo apoptosis. Although morphological changes during mammalian oocyte death are well studied, little is known about the molecular mechanisms responsible for initiating or executing oocyte apoptosis. It may be possible that studies of starfish egg apoptosis may lend insights into apoptotic events in mammals. Also, in the nematode *Caenorhabditis elegans*, more than one-half of the hermaphrodite germ cells in adult gonad are eliminated through apoptosis, when they are about to exit the pachytene stage of meiotic prophase I. Because the oocytes of mutants in the ras/MAP kinase pathway fail to exit the pachytene stage of meiosis I and fail to die, the ras/MAP kinase pathway might directly regulate the cell death machinery, or might indirectly affect germ cell apoptosis by promoting the progression of pachytene stage cells, which are resistant to apoptosis, to a later differentiation stage that is more sensitive to proapoptotic signals (Gumienny *et al.*, 1999). Although the molecular mechanisms of pro- and antiapoptotic effects of MAPK in nematode as well as starfish are still unclear, similar apoptotic pathways may be shared.

The time after ovulation during which mammalian eggs can give rise to developmentally competent embryo is short. Under in vivo conditions, ovulated mouse eggs exhibit maximum ability to fertilize for only 4–6 h (Lewis and Wright, 1935). Oocytes that are fertilized after this optimal period exhibit severely compromised developmental success that often culminates in fragmentation of blastomeres and embryonic death (Marston and Chang, 1964). Also, in starfish,

optimal development occurs when maturing oocytes are fertilized between GVBD and first polar body emission. Fertilization of eggs after completion of meiosis usually results in polyspermy (Fujimori and Hirai, 1979). Therefore, it is apparent that postovulatory processes occurring in the egg have a significant effect on development. It is tempting to speculate that with increasing time of postovulation, the ability of fertilization to inhibit apoptotic process is reduced, resulting in abnormal development.

The target molecules of MAPK and p38MAPK for apoptotic cell death as well as cell survival have to be identified to further understand the molecular mechanism of determining the egg fate, i.e., whether the cells undergo development or apoptosis.

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