Post-Cytochrome c Protection from Apoptosis Conferred by a MAPK Pathway in *Xenopus* Egg Extracts

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Submitted June 18, 2001; Revised October 31, 2001; Accepted November 14, 2001

Monitoring Editor: Tony Hunter

In response to many different apoptotic stimuli, cytochrome c is released from the intermembrane space of the mitochondria into the cytoplasm, where it serves as a cofactor in the activation of procaspase 9. Inhibition of this process can occur either by preventing cytochrome c release or by blocking caspase activation or activity. Experiments involving in vitro reconstitution of apoptosis in cell-free extracts of *Xenopus laevis* eggs have suggested that extracts arrested in interphase are susceptible to an endogenous apoptotic program leading to caspase activation, whereas extracts arrested in meiotic metaphase are not. We report here that Mos/MEK/MAPK pathways active in M phase-arrested eggs are responsible for rendering them refractory to apoptosis. Interestingly, M phase-arrested extracts are competent to release cytochrome c, yet still do not activate caspases. Concomitantly, we have also demonstrated that recombinant Mos, MEK, and ERK are sufficient to block cytochrome c-dependent caspase activation in purified *Xenopus* cytosol, which lacks both transcription and translation. These data indicate that the MAP kinase pathway can target and inhibit post-cytochrome c release apoptotic events in the absence of new mRNA/protein synthesis and that this biochemical pathway is responsible for the apoptotic inhibition observed in meiotic *X. laevis* egg extracts.

INTRODUCTION

Apoptosis, or programmed cell death, is the process by which superfluous or damaged cells are removed from the body. Apoptotic pathways are widely conserved and have been studied in organisms ranging from flies and worms to humans. The importance of apoptotic cell death to processes such as developmental body patterning, the immune response to viral infection, and the cellular response to damage cannot be underestimated—it has been estimated that >99.9% of the cells generated in the course of a human lifetime die by apoptosis (reviewed in Vaux and Korsmeyer, 1999).

Although a wide variety of stimuli can impinge upon a cell's decision to apoptose, many proapoptotic signals converge on the mitochondria, where they promote release of cytochrome c, an integral respiratory chain protein, from the mitochondrial intermembrane space into the cytoplasm (Green and Reed, 1998). Once released, cytochrome c forms a multimeric complex with Apaf-1, a 130-kDa ATP-binding

protein (Zou *et al.*, 1999). Thought to stabilize Apaf-1 in its active conformation, cytochrome c renders Apaf-1 competent to recruit the precursor form of one of the “death proteases,” caspase 9 (Li *et al.*, 1997; Hu *et al.*, 1999; Jiang and Wang, 2000). Once assembled on the Apaf-1 scaffold, caspase 9 cleaves and activates other procaspase 9 molecules within the Apaf-1/caspase 9 complex (Srinivasula *et al.*, 1998). This multimeric complex, containing Apaf-1, cytochrome c, and active caspase 9, is commonly referred to as the apoptosome (Zou *et al.*, 1999). Once activated within the apoptosome, caspase 9 may then proteolyze and activate other caspases, including caspase 3 (Li *et al.*, 1997), a protease that cleaves a large number of cellular substrates (e.g., nuclear lamins, PARP, the DNase inhibitor ICAD). These cleavage events are believed to undermine cellular structural integrity and lead to the orderly dismantling of the apoptotic cell (for review see Porter and Janicke, 1999).

Caspase activity is opposed by IAP (inhibitor of apoptosis) proteins. IAPs have been shown to bind and potently inhibit many caspases, including caspases -3, -7, and -9, that are known to act downstream of cytochrome c release (Roy *et al.*, 1997; Deveraux *et al.*, 1998; Deveraux and Reed, 1999). Because these IAPs can block cytochrome c-induced caspase activation, they are potent antagonists of cytochrome c-dependent apoptosis. In turn, IAP function can be antagonized

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01-06-0291. Article and publication date are at www.molbiol-cell.org/cgi/doi/10.1091/mbc.01-06-0291.

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by a diverse group of molecules including the *Drosophila* proteins HID, GRIM, and Reaper (Vucic *et al.*, 1997, 1998; Goyal *et al.*, 2000) and the human protein SMAC/Diablo. In human cells, SMAC/Diablo binds IAPs and potentiates cytochrome c-dependent caspase 9 processing (Du *et al.*, 2000; Verhagen *et al.*, 2000); therefore, its overexpression increases cellular sensitivity to apoptotic stimuli.

A number of signaling pathways that protect cells from apoptosis appear to block mitochondrial cytochrome c release, which is regulated in an antagonistic manner by pro- and antiapoptotic members of the Bcl-2 protein family (reviewed in Gross *et al.*, 1999a). When overexpressed, proapoptotic family members such as Bak and BID potentiate cytochrome c release, whereas their antiapoptotic counterparts, Bcl-X_L and Bcl-2, oppose this effect and promote cell survival, either by inhibiting prodeath Bcl-2 family members or by acting directly on mitochondrial components to prevent cytochrome c release (Li *et al.*, 1998; Luo *et al.*, 1998; Desagher *et al.*, 1999; Griffiths *et al.*, 1999; Gross *et al.*, 1999b). Although several reports suggest that apoptosis can also be inhibited *after* the release of cytochrome c from mitochondria (Deshmukh and Johnson, 1998; Erhardt *et al.*, 1999), the signaling pathways effecting such protection, the physiological settings in which this type of cellular protection occurs, and the precise mechanisms of protection have not been clearly defined.

Egg extracts prepared from the frog *Xenopus laevis* provide a useful tool for studying complex cellular processes in vitro. Although best known for their use in reconstituting cell cycle processes and nuclear trafficking of macromolecules, these extracts also contain a full complement of apoptotic regulators. Indeed, when egg extracts are "aged" on the bench, they spontaneously recapitulate a range of apoptotic processes, including nuclear fragmentation, DNA laddering, and caspase activation (Newmeyer *et al.*, 1994). These spontaneous apoptotic processes can be blocked by addition of exogenous antiapoptotic proteins such as Bcl-2 (Evans *et al.*, 1997b) and accelerated by proapoptotic proteins such as Bid (Kluck *et al.*, 1999) and the *Drosophila* Reaper protein (Evans *et al.*, 1997b). Although spontaneous apoptosis in egg extracts (as well as apoptosis induced by Bid or Reaper) will not occur in the absence of mitochondria (Newmeyer *et al.*, 1994), addition of exogenous cytochrome c to fractionated extracts that lack mitochondria results in robust caspase activation (Kluck *et al.*, 1997).

The body of the female frog houses a large pool of immature oocytes, which are arrested in prophase of the cell cycle. To stimulate egg production, adult female frogs are subjected to a hormonal regimen that promotes oocyte maturation. Progesterone treatment causes immature oocytes, which are arrested at the start of Meiosis I, to resume progression through the cell cycle while moving down the egg-laying tract, before arresting in metaphase of Meiosis II because of high levels of CSF (cytostatic factor) activity (reviewed in Palmer and Nebreda, 2000). Once they are laid, eggs remain arrested in Meiosis II until fertilization, which causes the release of calcium from intracellular stores and induces entry into the cell cycle. Exit from M phase requires this calcium release, which results in the destruction of both mitotic cyclins and Mos, a MEK kinase that is an integral component of CSF activity and is responsible for activation

of the MAP kinase pathway in maturing oocytes and eggs (Sagata *et al.*, 1989; Watanabe *et al.*, 1991).

Although the *Xenopus* egg is arrested in metaphase of Meiosis II, lysis of the eggs by centrifugation while preparing the extracts used in apoptotic reconstitution causes calcium release from internal stores; in the absence of calcium chelators, this release promotes degradation of cyclins and Mos and progression into interphase. The addition of cycloheximide renders the extracts unable to synthesize new cyclins and therefore unable to reenter mitosis. Interestingly, Morin and colleagues noted that when eggs are lysed in the presence of calcium chelators in order to preserve their true cell cycle state (Meiotic metaphase), the resulting extracts are markedly refractory to apoptosis (Faure *et al.*, 1997). To explore the interplay between cell cycle state, signaling pathways, and apoptotic onset, we wanted to understand the reason for the differing susceptibility of S phase (interphase) and M phase extracts to undergo apoptosis. We report here that Mos-mediated activation of the ERK MAP kinase pathway, but not Cdc2/Cyclin activity, is necessary and sufficient to render M phase extracts refractory to apoptosis. Strikingly, this MAPK-mediated protection from apoptosis is transcription independent and occurs predominantly after the release of cytochrome c from mitochondria. Moreover, recombinant Mos, MEK, or ERK proteins are sufficient to block cytochrome c-dependent caspase activation in purified *Xenopus* egg cytosol. These results demonstrate that the MAP kinase pathway biochemically targets and inhibits post-cytochrome c apoptotic events in *Xenopus* eggs.

MATERIALS AND METHODS

Preparation of Crude *Xenopus* Egg Extracts

To induce egg laying, mature female frogs were injected with 100 U pregnant mare serum gonadotropin (Calbiochem, La Jolla, CA) to induce oocyte maturation, followed by injection (3–10 d later) with human chorionic gonadotropin (hCG; Sigma, St. Louis, MO). Twenty to 24 h after hCG injection eggs were harvested for extract production. Jelly coats were removed from the eggs by incubation with 2% cysteine, pH 8.0, washed three times in modified Ringer solution (1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 25 mM CaCl₂, 5 mM HEPES, pH 7.8, 0.8 mM EDTA), and then washed in either ELB (250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM HEPES, pH 7.7) for S extract production or in ELB-CSF (ELB + 5 mM EGTA, pH 8.0) for CSF extract production. Eggs were packed by low-speed centrifugation at 400 × g. After addition of aprotinin and leupeptin (final concentration, 5 µg/ml), cytochalasin B (final concentration, 5 µg/ml), and cycloheximide (50 µg/ml), eggs were lysed by centrifugation at 10,000 × g for 15 min.

Fractionation of Crude *Xenopus* Egg Extracts

To separate mitochondrial and cytosolic components, crude extract was centrifuged at 55,000 rpm (250,000 × g) in a Beckman TLS-55 rotor for the TL-100 centrifuge (Beckman Instruments, Fullerton, CA). The cytosolic fraction was removed and recentrifuged at 55,000 rpm for an additional 25 min, then aliquotted, and frozen in liquid nitrogen for future use. The mitochondrial fraction was diluted 1:1 in MIB (10 mM HEPES, pH 7.5, 60 mM sucrose, 210 mM mannitol, 1 mM ADP, 10 mM KCl, 10 mM succinate, 5 mM EGTA) plus 0.5 mM DTT and then spun through an MIB percoll gradient (42% percoll in MIB, 37% percoll in MIB, 30% percoll in MIB, 25% percoll in MIB). The recovered mitochondrial fraction was washed in MIB and pelleted at 750 × g for 10 min. The pellet was diluted 1:1 in MIB,

then aliquotted, and frozen in liquid nitrogen for future use. All extract components were stored at -80°C .

Production of Mitochondrial Lysates

Frozen mitochondrial pellets were diluted 1:1 in MIB + 25 mM CHAPS on ice for 15 min and then spun through a $0.1\text{-}\mu\text{m}$ ultrafree-MC filter (Millipore, Bedford, MA) for 15 min at 11,000 rpm in an Eppendorf 5415 C microfuge (Fremont, CA). The filtrate was collected, and protein concentration was measured using the Bio-Rad system (Bio-Rad Protein Laboratories, Hercules, CA).

Immunodepletion Assays

For MEK depletion experiments, Protein A-Sepharose beads (Sigma) were washed in PBS and incubated with anti-MEK antibody (kindly provided by Dr. James Ferrell) for 1 h at 4°C . Bead-antibody complexes were recovered, washed in ELB, and then incubated with 100 μl crude extract/25 μl beads. After 1 h at 4°C the antibody-bead complexes were pelleted, and the supernatant was transferred to another tube containing more bead-bound antibody. After a second round of immunodepletion the supernatant was collected and supplemented with ATP-regenerating system (10 mM phosphocreatine, 2 mM ATP, and 150 mg/ml creatine phosphokinase). Extract was then incubated at room temperature and analyzed for caspase 3 activity.

Production of his-tagged Proteins

His-MEK R4F and his-MEK kinase dead constructs in the pRSET vector were all kindly provided by Dr. Tom Guadagno. The plasmids were transformed into the BL21DE3 bacterial strain, grown at 37°C for 2 h, and then induced with 0.4 mM IPTG for 4 h. Bacteria were then pelleted at $6000 \times g$ for 10 min in a Beckman JLA-10.5 rotor, washed in PBS, and then repelleted. Pellets were frozen in liquid nitrogen and stored at -80°C . For protein production, bacteria were resuspended in 12.5 ml lysis buffer (50 mM HEPES, pH 7.7, 750 mM sucrose, 150 mM NaCl, 0.1% Triton X-100) per liter culture, to which had been added 5 mM β -mercaptoethanol, 1 mM PMSF, 5 μM aprotinin and leupeptin, and 0.8 mg/ml lysozyme. Pellets were allowed to lyse on ice for 1 hour, at which point MgCl_2 (final concentration, 20 mM), sodium deoxycholate (final concentration, 0.15%), and DNase (0.1 mg total) were added, and the lysate was left to incubate on ice until no longer viscous, $\sim 10\text{--}20$ min. Lysate was centrifuged at 12,000 rpm in a Beckman JS-13.1 rotor for 30 min. Lysate was then poured three times over 300 μl Ni-NTA agarose (QIAGEN, Santa Clarita, CA) that had been washed in lysis buffer. Bead-bound protein was then washed in 15 ml lysis buffer plus 400 mM NaCl and 20 mM imidazole, then 15 ml lysis buffer alone. For protein elution, beads were eluted with 5×500 μl lysis buffer plus 200 mM imidazole, then the eluate was concentrated in a Centricon-30 (Millipore), diluted into ELB, then recentrifuged to the desired volume. Aliquotted proteins were frozen in liquid nitrogen and stored at -80°C .

Production of MBP-Mos Protein

The plasmids pMALcRI-XE and pMALcRI-XE(KM) (Yew *et al.*, 1992) encoding *Xenopus* Mos were expressed in the Topp3 bacterial strain, grown 2 h at 37°C , then induced with 0.4 mM IPTG for 2 h at 37°C . Bacteria were pelleted at $6000 \times g$ for 10 min in a Beckman JLA-10.5 rotor, washed in PBS, then repelleted. Pellets were frozen in liquid nitrogen and stored at -80°C . For protein preparation, pellet was resuspended in 25 ml MBP lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA) to which had been added 1 mg/ml lysozyme, 5 $\mu\text{g/ml}$ aprotinin and leupeptin, and 1 mM PMSF. Resuspended bacteria were then lysed by French press. The lysate was centrifuged at $9000 \times g$ for 20 min. The supernatant was removed and run over Q Sepharose resin that had been equilibrated with MBP lysis buffer. Salt was added to the flow-through to reach

a final concentration of 0.5 M NaCl. The flow-through was passed twice over an amylose resin, which was then washed with amylose column buffer (20 mM HEPES, pH 6.8, 88 mM NaCl, 7.5 mM MgCl_2) plus 410 mM NaCl, then with amylose column buffer alone. Protein was eluted with 10×1 ml fractions of amylose column buffer plus 10 mM maltose. The fractions with the highest protein concentration as measured by A_{280} were pooled and concentrated using PEG (Sigma), then dialyzed overnight in ELB. Protein was aliquotted and frozen in liquid nitrogen, then stored at -80°C .

Caspase 3 Activity Assays

To measure caspase 3 activity, 3 μl of each sample was incubated with 10 μl colorimetric substrate AC-DEVD-pNA (Biomol) in Assay Buffer (50 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, and 10 mM DTT) for 1 h at 37°C . After a 1-h incubation the reaction was stopped by the addition of 0.2 μM Ac-DEVD-CHO (Biomol, Plymouth Meeting, PA). Reaction was read at 405 nm with a Labsystems Multiscan Plus plate reader (Fisher Scientific, Pittsburgh, PA).

Cytochrome c Release Assays

For assays in crude extracts the extract was supplemented with ATP regenerating mixture. At various time points cytosolic cytochrome c content was analyzed by diluting 15 μl crude extract into 15 μl ELB and filtering diluted extract through a $0.1\text{-}\mu\text{m}$ ultrafree-MC filter (Millipore). The filtrate was run on 17.5% SDS-PAGE minigels and blotted with anticytochrome c antibody (Cat no. 556433; PharMin-gen, San Diego, CA).

ERK Thiophosphorylation

Recombinant ERK1 (Cat no. 14-188; Upstate Biotechnology, Lake Placid, NY) was thiophosphorylated by diluting 12.5 μl enzyme (stored in PBS + 50% glycerol) 1:1 with $2\times$ thiophosphorylation buffer (40 mM Tris, pH 7.5, 40 mM MgCl_2 , 0.2 mM EDTA, 30 mM β -mercaptoethanol, and 1 mM ATP- γ -S) and incubating with his-MEK immobilized on nickel beads for 4 h at 30°C . As a control, the reaction was also carried out with PBS + 50% glycerol not containing any enzyme. The beads were centrifuged to remove the MEK kinase, and the thiophosphorylated ERK was collected. To remove residual ATP- γ -S, the activated enzyme was diluted out to 500 μl in ELB and passed through a Microcon YM-10 (Cat no. 42406; Millipore, Bedford, MA) until a >10 -fold reconcentration was achieved; this step was repeated three times. ERK activity was measured using recombinant MBP as a substrate.

RESULTS

M Phase Extracts Are Resistant to Apoptosis

As described above, it has been observed that interphase egg extracts are considerably more susceptible to apoptosis than are extracts prepared so as to preserve the meiotic arrest of the egg. To verify this observation, we wanted to compare spontaneous apoptotic activity in extracts stably arrested in M phase (hereafter referred to as CSF extracts, for "cytostatic factor-arrested") and interphase (S) extracts as well as in CSF extracts that had been released into interphase by addition of exogenous calcium (CSF + Ca^{2+}). To exclude the possibility that apoptotic inhibition was due to artificial sequestration of calcium by the chelating agent used during CSF extract preparation, EGTA was also added to S extracts (S + EGTA) after Ca^{2+} -induced release into interphase; these extracts are unable to return to an M phase state because mitotic cyclins are not present.

Figure 1. CSF extracts do not undergo apoptotic caspase activation. Crude interphase (S), meiotic (CSF), and CSF extracts that had been supplemented with exogenous calcium in order to drive them into interphase (CSF + Ca^{2+}) were analyzed for caspase activity at various time points using the model caspase substrate AC-DEVD-pNA. As a control, EGTA was added to S extracts after lysis-induced entry into interphase (S + EGTA). Cleavage of the caspase substrate AC-DEVD-pNA was measured spectrophotometrically at 405 nm (see MATERIALS AND METHODS).

As an apoptotic marker, we chose to evaluate caspase activity as measured by cleavage of the model caspase substrate AC-DEVD-pNA; cleavage of the substrate results in a product that can be monitored spectrophotometrically at 405 nm. In a typical experiment, both types of interphase extract (S and S + EGTA) developed caspase activity at hour 3 of a 6-h incubation, whereas the released CSF extract (CSF + Ca^{2+}) was slightly delayed in apoptotic activation, exhibiting robust caspase activity after 4 h. Of all the extracts tested, only stably arrested CSF extracts showed no spontaneous caspase activity over the time course observed (Figure 1). This experiment confirms the observation that CSF extracts are resistant to apoptosis and also demonstrates that this resistance is due to properties of the meiotic CSF extract, rather than to nonspecific effects of the chelating agent.

CSF Extracts Are Resistant to Cytochrome *c*-induced Caspase Activation

In many cell types, mitochondria serve as a repository of proapoptotic components that are released into the cytosol upon receipt of apoptotic stimuli (reviewed in Earnshaw, 1999). As reported by Newmeyer and colleagues (Newmeyer *et al.*, 1994), apoptosis in the *Xenopus* extract system is absolutely dependent on a heavy membrane fraction containing mitochondria, implying that mitochondrial factors are required to generate spontaneous caspase activity in the extract. Given this observation, we hypothesized that CSF and interphase extracts might differ either in their propensity to release mitochondrial factors or in their susceptibility to the proapoptotic influence of such factors once released.

As a marker for release of mitochondrial contents, we elected to monitor efflux of cytochrome *c*, the only apoptotic regulator known to reside in the mitochondrial intermembrane space thus far well-characterized in the *Xenopus* system. At various time points after initiating room tempera-

Figure 2. CSF extracts release cytochrome *c* but do not activate caspases. (A) Crude CSF extract and CSF extract that had been supplemented with exogenous calcium was analyzed for cytochrome *c* release at various time points by filtering aliquots of extract through a 0.1- μm microfilter. The filtrate, which lacks mitochondria, was analyzed by SDS-PAGE and Western blotting using an anticytochrome *c* antibody. (B) Crude untreated CSF extract and CSF extract that had been supplemented with exogenous calcium was assayed for caspase activity as described for Figure 1.

ture incubation, we passed M phase (CSF) and interphase (CSF + Ca^{2+}) extracts through 0.1- μm filters in order to exclude all intact organelles, including mitochondria. The filtrate, which contains cytosolic components but lacks mitochondria, was then assayed for the presence of cytochrome *c* by SDS-PAGE and immunoblotting with anticytochrome *c* antibodies. Interestingly, both the interphase (CSF + Ca^{2+}) and M phase (CSF) extracts showed robust cytochrome *c* release, although the CSF extract lagged slightly behind the interphase extract (Figure 2A). However, by 4 h, the mitochondria within the CSF extract had released considerable quantities of cytochrome *c*, yet did not, even by hour 7 of the experiment, activate caspases (Figure 2B). From these data we concluded that CSF phase extracts are quite capable of inducing cytochrome *c* release, yet still do not activate caspases. Although the slight lag in cytochrome *c* release compared with S extracts indicates that factors within the CSF extract may retard release of mitochondrial components, our data strongly indicate that CSF extracts also contain potent factors that can prevent caspase activation downstream of mitochondrial cytochrome *c* release. These factors appear to be either lacking or less active in interphase extracts.

To demonstrate unequivocally the differential sensitivity of these extracts to mitochondrial contents as a whole (which contain not only cytochrome *c*, but presumably homologues of other apoptotic regulators such as SMAC/Diablo and AIF), we separated mitochondrial and cytosolic fractions from crude extract by centrifugation and then lysed the mitochondria in a detergent-containing buffer and recombined this lysate with purified cytosol derived from either the S or CSF extracts (US or UCSF, for ultra-centrifuged S or

Figure 3. CSF cytosol is resistant to caspase activation. Crude S and CSF extract were further fractionated into membranous and cytosolic fractions. (A) Mitochondrial fractions that had been purified away from the membranous fraction by centrifugation through a percoll gradient were then lysed in a buffer-containing detergent. The lysate was then filtered through a 0.1- μ m microfilter. The filtrate was then added to S or CSF cytosol (US or UCSF, respectively) to a final concentration of 1 mg mitochondrial protein per 60 mg extract protein. Caspase activity was measured as described for Figure 1. Cytochrome c is required for caspase activation by mitochondrial lysate. (B) Purified cytochrome c was added to US or UCSF to a final concentration of 1 μ g cytochrome c per 40 mg extract protein. Caspase activity was measured as described in Figure 1.

CSF, respectively). These reconstituted extracts were then incubated at room temperature and monitored for the development of caspase activity. As shown in Figure 3A, the CSF cytosol was markedly refractory to induction of caspase activity by total mitochondrial protein, although excess mitochondrial protein could overcome this resistance (our unpublished results). In contrast, S cytosol was fully susceptible to caspase activation even by low concentrations of mitochondrial protein. These results indicate that CSF and S phase extracts are differentially sensitive to proapoptotic factors present in the mitochondria, and that cytosolic factors present in CSF extracts can protect extracts from these proapoptotic factors.

It has been shown that exogenous cytochrome c is sufficient to activate caspases 9 and 3 in purified cytosol (Kluck *et al.*, 1997; Li *et al.*, 1997). Because we had demonstrated that cytochrome c is released from mitochondria in CSF extracts

and that CSF extracts are relatively insensitive to the proapoptotic influence of mitochondrial contents, we wanted to determine whether factors within CSF extracts could prevent caspase activation by pure cytochrome c. Therefore, we added purified cytochrome c (Sigma) to S or CSF cytosol (lacking mitochondria) and monitored caspase activity. As shown in Figure 3B, when compared with interphase cytosols, CSF cytosols were markedly resistant to cytochrome c-induced caspase activation.

Mos/MEK Kinase Pathway Activity Is Necessary and Sufficient for Apoptotic Inhibition in M Phase Extracts

The most notable difference between CSF and interphase extracts is the presence of high levels of mitotic cyclin/Cdk activity in the former. Indeed, during conversion of mitotic extracts to interphase extracts (or during lysis of eggs in the absence of calcium chelators), the mitotic cdk cdc2/cyclin B is inactivated by calcium-dependent destruction of cyclin B (Watanabe *et al.*, 1991). We therefore assumed that the difference between M and S extracts might lie in the differing levels of cdc2/cyclin B activity. In support of this notion, we found that addition of recombinant cyclin B to interphase extracts could prevent the development of caspase activity (Figure 4A). We were surprised, therefore, when the drug roscovitine, a potent inhibitor of cdc2/cyclin B activity, was unable to promote apoptosis in CSF extracts (our unpublished results). However, because the addition of cyclin B to interphase extracts also activates the MEK/MAP kinase pathway (Guadagno and Ferrell, 1998), which is also highly active in CSF extracts, we hypothesized that a MAP kinase pathway might be responsible for the observed apoptotic inhibition.

The MEK kinase Mos, together with cyclin B, is a primary target of calcium-dependent destruction during the transition from M phase to interphase in the *Xenopus* system (Watanabe *et al.*, 1991). On the basis of our previous results, we hypothesized that continued stimulation of Mos/MEK kinases might be required to block apoptosis in CSF extracts. To test this hypothesis, we immunodepleted endogenous MEK from crude CSF extracts using an anti-MEK antibody. As shown in Figure 4B, immunodepletion of CSF extracts with MEK antibodies, but not control IgG, restored apoptotic activity, indicating that MEK, and, by extension, its activator, Mos, are required to maintain apoptotic inhibition in CSF extracts.

To determine if Mos activation of MEK was sufficient to recapitulate the post-cytochrome c protection from apoptosis observed in CSF extracts, we incubated interphase cytosol with recombinant tagged wild-type Mos (WT Mos) or kinase-inactive Mos (K $\rightarrow$ M Mos) and then added recombinant cytochrome c. Mos kinase activity was sufficient to block caspase activity in the presence of cytochrome c (Figure 5A). This effect was completely reversed by UO126, a MEK inhibitor, indicating that Mos-dependent inhibition of cytochrome c-mediated caspase 3 activity is, as anticipated, mediated through MEK.

We extended these findings by incubating interphase cytosol with recombinant constitutively active (R4F) or kinase-dead (KD) MEK and then adding exogenous cytochrome c. R4F MEK alone, but not its kinase inactive variant, was

Western blotting; 95–99% depletion was achieved (our unpublished results). After immunodepletion, extracts were incubated at room temperature and caspase activity was measured at various time points as described in Figure 1. Note that immunodepletion of the extract itself delays apoptotic onset somewhat; this effect is most likely due to dilution of the extract during the immunodepletion process.

sufficient to block cytochrome *c*-induced caspase activity (Figure 5B). Collectively, these data indicate that MEK activity is sufficient to maintain apoptotic inhibition in mitotic extracts and suggest that this inhibition is, at least in part, directed at post-cytochrome *c* apoptotic events.

ERK Is Sufficient to Block Cytochrome *c*-dependent Caspase Activation

Because our experiments demonstrated that MEK was able to block cytochrome *c*-dependent caspase activation, we wanted to determine whether this antiapoptotic effect was exerted through its target kinase, the serine/threonine kinase MAP kinase, ERK. Because there are no ERK-activating stimuli in the interphase extract, it was first necessary to activate purified wild-type ERK protein using recombinant MEK. To render the activated ERK resistant to inactivating phosphatases present in the extract, we carried out this phosphorylation in a reducing buffer in the presence ATP- γ -S (Haccard *et al.*, 1993). As shown in Figure 6, interphase egg cytosol supplemented with this activated ERK preparation was resistant to cytochrome *c*-induced caspase activation. Collectively, these data demonstrate that the Mos-MEK-ERK pathway can target and inhibit post-cytochrome *c* apoptotic events. Moreover, the resistance of CSF extracts to cytochrome *c* results from the constitutive activation of this pathway in *Xenopus* eggs.

DISCUSSION

Egg extracts prepared from the frog *Xenopus laevis* can initiate and execute a full apoptotic program *in vitro*. We have used this biochemically tractable system to demonstrate that the Mos/MEK/MAP kinase pathway is necessary and sufficient to inhibit *in vitro* apoptotic processes and that this inhibition is directed at a post-cytochrome *c* release, pre-caspase activation step. Because *Xenopus* egg extracts lack pol II transcription and are prepared in the presence of cycloheximide, this surprising result indicates that the MAP kinase molecule ERK can prevent cytochrome *c*-dependent caspase activation in the absence of transcription or protein synthesis.

Figure 4. Depletion of MEK promotes apoptosis in crude CSF extracts. (A) Buffer or recombinant cyclin B was added to crude S extract and apoptosis was measured using an AC-DEVD-pNA cleavage assay. (B) MEK was immunodepleted with purified anti-MEK antibody (see MATERIALS AND METHODS) or mock-depleted with purified IgG from 100 μ l crude extract in two successive rounds of depletion at 4°C. Immunodepletion was assayed using SDS-PAGE and

Post-Cytochrome *c* Protection from Apoptosis

Our results, along with those of Morin and colleagues, demonstrate that CSF extracts, made so as to preserve the meiotic arrest of the intact egg, are refractory to the *in vitro* apoptotic program initiated in interphase egg extracts (Faure *et al.*, 1997). Interestingly, we have shown that CSF extracts release cytochrome *c*, albeit with slightly delayed kinetics when compared with interphase extracts, and yet remain resistant to caspase activation. This result suggests that some component of CSF extracts can inhibit caspase activation at a step downstream from cytochrome *c* release; we have demonstrated that the required factor(s) is an activated MAP kinase pathway.

The ability of MAP kinase pathways to promote cell survival has been well documented (Bonni *et al.*, 1999; Hetman *et al.*, 1999; Holmstrom *et al.*, 2000). However, because of the limitations imposed by tissue culture systems, it has not been feasible to separate out transcription-dependent and -independent effects of MAP kinase activation on post-cytochrome *c* events. For example, Cooper and colleagues (Erhardt *et al.*, 1999) have shown that lysates from Rat-1 cells transfected with B-Raf, an upstream activator of MAP kinases, are resistant to cytochrome *c*-induced caspase activation. They have proposed that MAP kinase pathway activation may result in increased expression of antiapoptotic molecules, such as IAPs, that can inhibit caspase activation downstream from cytochrome *c* release. However, because our system does not support transcription or translation, our data demonstrate the existence of a more direct biochemical role for the MAP kinase pathway in preventing caspase activation after release of cytochrome *c* from the mitochondria.

The existence of a MAPK-mediated mechanism to prevent apoptosis after cytochrome *c* release begs the question as to why cells would inhibit these processes when the more upstream event, release of cytochrome *c* from the mitochondria, is so thoroughly regulated. The simple answer may be that apoptosis, like any other cellular process, is regulated at multiple steps so as to prevent cells from making the “wrong” decision and that multiple negative regulatory events help to protect the cell in case of accidental damage. A more complex answer may involve examining the types of cells in which this protection can be observed. For example,

Figure 5. Mos and MEK prevent cytochrome c-dependent caspase activation. (A) Interphase cytosol (US) was treated for one hour at room temperature with recombinant tagged wild-type Mos (WT Mos) or with kinase dead Mos (K → M Mos), in the presence or absence of 50 μ M UO126, a MEK inhibitor. Purified cytochrome c was then added to US or UCSF to a final concentration of 1 μ g cytochrome c per 40 mg extract protein. Caspase activity was measured using an AC-DEVD-pNA cleavage assay. (B) Interphase cytosol was treated with recombinant tagged constitutively active MEK (R4F MEK) or kinase dead MEK (KD MEK) for one hour at room temperature. Cytochrome c was then added to 1 μ g per 40 mg extract protein. Caspase activity was measured as described for Figure 1.

Deshmukh and Johnson (1998) have demonstrated that sympathetic neurons are insensitive to microinjected cytochrome c in the presence of growth factors; they propose that a high level of resistance to cytochrome c-induced apoptosis may be necessary for cells such as postmitotic neurons, which are not easily replaced. In *Xenopus*, the apoptosis-resistant meiotic stage of the cell cycle corresponds to eggs that are en route to being laid or have already been laid. Although these cells can easily be replaced, allowing gametes to apoptose is disadvantageous for organisms such as frogs, which have a low energy investment in their offspring and hence are advantaged by producing the largest possible number of gametes available for fertilization. Alternatively, it may be that apoptotic inhibition is simply a byproduct of the high level of Mos/MEK/MAP kinase activity required to maintain the metaphase II meiotic arrest. In somatic cells, this degree of MAP kinase activation would be observed only after particular signaling events, whereas in the egg this pathway is, by necessity, constitutively active.

Because interphase egg extracts, which no longer have high levels of MAP kinase activity, do not spontaneously

Figure 6. Thiophosphorylated ERK inhibits cytochrome c-dependent caspase activation. Interphase cytosol was treated with thiophosphorylated ERK or equivalently prepared buffer (see MATERIALS AND METHODS) for 30 min at room temperature. Cytochrome c was then added to 1 μ g per 40 mg extract protein. Caspase activity was measured as described for Figure 1.

release cytochrome c until they have been incubated at room temperature for prolonged periods, we assume that there are apoptotic inhibitors operating before cytochrome c release in these extracts (and most likely, in the early fertilized embryos that they mimic). Indeed, it has been suggested that apoptosis is suppressed during the early cleavages in the *Xenopus* embryo (premid blastula transition) by maternally encoded apoptotic inhibitors (Hensley and Gautier, 1997; Stack and Newport, 1997). Although post-cytochrome c protection conferred by MAPK is likely to be lost at fertilization, other, pre-cytochrome c release mechanisms must act to prevent apoptosis during the early embryonic cleavages.

MAP Kinase and the Apoptosome

Once released into the cytosol, the primary function of cytochrome c is to nucleate the apoptosome through recruitment of Apaf-1 and caspase 9. Because our data indicate that MAP kinase targets a post-cytochrome c event, it seems likely that the MAP kinase pathway might target and modulate this initial downstream event, the formation or function of the apoptosome. In theory, apoptosomal inhibition could result from a change in the composition of the apoptosome or from the posttranslational modification (i.e., phosphorylation) of preexisting components. However, the possible targets are not limited to Apaf-1, caspase 9, and cytochrome c; a comparison of apoptosomes isolated from cell lysates with *in vitro* reconstitutions using purified recombinant components (i.e., caspase 9, cytochrome c, Apaf-1, and dATP) have suggested that apoptosomes from cell lysates may contain additional factors (Cain *et al.*, 2000). Moreover, a number of accessory proteins associated with apoptosomes have been described (e.g., Aven [Chau *et al.*, 2000] and NAC [Chu *et al.*, 2001]). The function of these or other novel molecules may be altered by MAP kinase phos-

phorylation; further investigation will concentrate on identifying the relevant MAP kinase target(s). Another possibly relevant MAP kinase target is HID, a known *Drosophila* IAP inhibitor. Because HID has been shown to be a MAP kinase substrate in flies (Bergmann *et al.*, 1998), it is attractive to speculate that apoptosomal association of IAPs might be altered in response to the activity of an HID-like protein in egg extracts.

In aggregate, our data both explain the relative resistance of meiotic extracts to apoptosis and describe the pathway responsible for this phenomenon. The inhibition of cytochrome c-dependent caspase activation by ERK, coupled with our use of a transcriptionally/translationally inert system, demonstrates unequivocally the existence of a purely posttranslational inhibition of apoptosis by MAP kinase pathways. Moreover, a good deal of this inhibition appears to occur after mitochondrial release of cytochrome c, providing a novel context in which this type of inhibition can be observed. Finally, these findings offer a starting point for future identification of possible MAP kinase-modified apoptotic regulators, including apoptosomal components, IAPs and their regulators, and other proteins acting downstream of mitochondrial cytochrome c release.

ACKNOWLEDGMENTS

The authors are grateful to Jim Ferrell and Tom Guadagno for their generous provision of MEK/MAP kinase clones and antibodies and also thank Katherine Swenson-Fields for the Mos clones. They thank Danny Lew, Jesse Smith, and Katherine Swenson-Fields for critical reading of the manuscript. This work was supported by National Institutes of Health grants GM56518 and GM61919, an American Heart Association grant, and an IDEA grant from the USARMC, to S.K. M.O. and J.S.T. are predoctoral fellows of the Breast Cancer Research Program of the USARMC. S.K. is a Scholar of the Leukemia and Lymphoma Society.

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