Hyperosmotic shock induces early calpain activation, Smac/DIABLO release from the mitochondria, and p38/JNK activation in *Xenopus* oocytes. These pathways regulate late cytochrome c release and caspase-3 activation. Here, we show that JNK1-1 and JNK1-2 are activated early by osmostress, and sustained activation of both isoforms accelerates the apoptotic program. When caspase-3 is activated, JNK1-2 is proteolyzed at Asp-385 increasing the release of cytochrome c and caspase-3 activity, thereby creating a positive feedback loop. Expression of Bcl-xL markedly reduces hyperosmotic shock-induced apoptosis. In contrast, expression of Bid induces rapid caspase-3 activation, even in the absence of osmostress, which is blocked by Bcl-xL co-expression. In these conditions a significant amount of Bid in the cytosol is mono- and bi-ubiquitinated. Caspase-3 activation by hyperosmotic shock induces proteolysis of Bid and mono-ubiquitinated Bid at Asp-52 increasing the release of cytochrome c and caspase-3 activation, and thus creating a second positive feedback loop. Revealing the JNK isoforms and the loops activated by osmostress could help to design better treatments for human diseases caused by perturbations in fluid osmolarity.

Hyperosmotic shock induces cytochrome c release and caspase-3 activation in *Xenopus* oocytes (1). Recently, we have shown that hyperosmotic shock also induces rapid calpain activation and Smac/DIABLO (second mitochondrial-derived activator of caspases/direct IAP-binding protein with low PI Smac/DIABLO) release from the mitochondria, as well as p38 and JNK (c-Jun N-terminal kinase) activation. These four pathways, induced early by osmostress, converge on the mitochondrial to trigger late cytochrome c release and caspase-3 activation (2). Moreover, we have found that caspase-3 activation induces rapid phosphorylation of p38, thus creating a positive feedback loop in osmostress-induced apoptosis (3). However, the role of Bcl-2 family members in osmostress-induced apoptosis was not addressed in previous studies. It is not clear which specific p38 and JNK isoforms are activated by osmostress and how they regulate the apoptotic program. Bid is a member of the BH3-only proteins that plays a crucial role in regulating the permeability of the outer mitochondrial membrane. The BH3 region is required for interaction with both pro-apoptotic Bax or anti-apoptotic protein Bcl-xL (4). Bid also contains a large unstructured loop (amino acids 42–79) with a variety of sites that are subjected to post-translational modifications, regulating Bid localization and apoptotic function (5). Bid is a caspase-8 substrate, and the resulting tBid translocates to mitochondria and initiates mitochondrial protein release. The cleavage site of caspase-8 in human Bid is Asp-60 (6) and in *Xenopus laevis* is Asp-52 (7). *Xenopus* Bid can also be proteolyzed by caspase-10β (7). In addition, it has been reported that caspase-3 can cleave human Bid at Asp-60 (8), and cleavage sites for non-caspase proteases have been detected, such as Gly-70 for calpain, Arg-71 for cathepsins, and Asp-75 for granzyme B (5). Finally, human Bid is cleaved at Leu-25 by an unknown protease in a JNK-dependent manner generating a large C-terminal fragment (tBid) that could accumulate at the mitochondria like tBid (9). In summary, when Bid is cleaved by proteases, the C-terminal product accumulates at the mitochondria inducing apoptosis. This implicates the N terminus of Bid as a negative regulatory sequence that prevents the mitochondrial localization of Bid, thereby preventing apoptosis (10). Bid cleavage mediated by caspase-8 can be attenuated when residues in the vicinity of the cleavage site are phosphorylated. In human Bid, phosphorylation at Thr-59 severely inhibits Bid cleavage by caspase-8 (11). Similarly, phosphorylation of murine Bid (at Ser-61 and Ser-64) also attenuates its cleavage by caspase-8 (12). In human Bid, the C-terminal fragment (tBid) is polyubiquitinated and degraded by the proteasome (13), and it has been reported that the N-terminal fragment (nBid) is also polyubiquitinated in Ser/Thr/Cys residues in an unconventional manner and degraded by the proteasome (14). In contrast, *Xenopus* Bid is mono- and bi-ubiquitinated in egg extracts but not degraded by the proteasome (15). There are at least three important sites for ubiquitination (Lys-18, Lys-21, and Lys-37) in *Xenopus* Bid, located close to the cleavage site Asp-52. However, the role of mono- and bi-ubiquitinated Bid in apoptosis is unknown (15).

JNKs regulate osmostress-induced apoptosis in *Xenopus* oocytes (2), but the isoforms activated have not been characterized. Three distinct JNK genes have been isolated in mammalians (16). The *jnk1* and *jnk2* are ubiquitously expressed. In contrast, the *jnk3* expression pattern is relatively restricted to brain, heart, and testis. The three *jnk* genes express at least 10 JNK kinase; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; Ub, ubiquitin; ANOVA, analysis of variance.
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isoforms by distinct modification in selective transcription and alternative splicing. Thus, transcripts derived from the jnk1 gene present alternative slicing at the C terminus yielding short and long JNK-1 variants (JNK1-1 and JNK1-2, respectively). JNK proteins are activated by concomitant phosphorylation on Thr and Tyr in the TPY motif in the activation loop by MKK7 and MKK4 (SEK1), which are also activated by dual phosphorylation on two residues in the activation loop by an upstream kinase. Several MAP3Ks that phosphorylate and activate MKK4 and/or MKK7 have been isolated (16). JNK may have pro- or anti-apoptotic roles in a stimulus- and tissue-dependent mechanism (16–18). It seems that a transient activation of JNK is a struggle for survival, whereas sustained activation of JNK induces apoptosis (19). JNK can regulate the pro- or anti-apoptotic proteins residing in the mitochondria, such as Bax, Bid, Bad, Bim, Bmf Bcl-2, Bcl-xL, or Mcl-1 (17, 18, 20–23). Interestingly, human JNK1 and JNK2 are proteolyzed by caspase-3, and the cleaved proteins have similar activities as the full-length pJNKs (24). However, the role of JNK proteolysis in the regulation of apoptosis is unknown.

Here, we show that hyperosmotic shock activates the JNK1-1 and JNK1-2 isoforms in Xenopus oocytes, thus accelerating osmostress-induced apoptosis. Moreover, caspase-3-dependent proteolysis of JNK1-2 regulates cytochrome c release and caspase-3 activation, creating a positive feedback loop. We also show that osmotic stress-induced apoptosis is markedly reduced by Bcl-xL overexpression. Importantly, caspase-3-dependent proteolysis of Bid engages another positive feedback loop that in combination with others, as reported previously, would complete the apoptotic program.

Experimental Procedures

Oocyte Isolation and Treatment—Oocytes were obtained from sexually mature X. laevis females (purchased from Centre d’Elevage de Xenopes, Montpellier, France, or from Xenopus Express, Vernassal, France) anesthetized in 0.02% benzocaine, and portions of ovaries were removed through a small incision on the abdomen. The incision was sutured, and the animal was returned to a separate tank until it had fully recovered from the anesthesia. It was then returned to a large tank in which all the frogs were kept for at least 4 weeks until the next surgery. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Autònoma de Barcelona (Permit Number: CEEAH 439), and all efforts were made to minimize animal suffering. The tissue was dissected into small pieces if the ovaries were healthy enough to use. Oocytes were defolliculated for 2–3 h at room temperature with collagenase/dispase (0.8 mg/ml (Sigma) and 0.48 mg/ml (Roche Applied Science)) in MBS (5 mM HEPES, 88 mM NaCl, 1 mM KCl, 1 mM MgSO4·7H2O, 2.5 mM NaHCO3, 0.7 mM CaCl2, pH 7.8) with gentle agitation. The defolliculated oocytes were then washed thoroughly with MBS and transferred to a Petri dish. Stage VI oocytes in good condition were sorted manually and incubated overnight in MBS at 18 °C. The next day, healthy survivors were selected and transferred to a Petri dish containing fresh MBS. Oocytes were exposed to hyperosmotic shock by transferring them to a new dish containing MBS with 300 mM sorbitol, collected at different times, and treated as described below. Some oocytes were incubated with drugs dissolved in MBS at the concentrations and times indicated or injected with capped RNAs (cRNAs) and exposed to hyperosmotic shock.

Inhibitors—Z-DEVD-fmk, ALLN (Calbiochem), MDL28170 (Sigma), and Z-VAD-fmk (Bachem) were dissolved in DMSO to prepare stock solutions. Oocytes were pre-incubated for 1 h with the corresponding inhibitor dissolved in MBS to a final concentration of 50 or 25 μM (ALLN) and then incubated for the indicated times with the same concentration of inhibitor dissolved in sorbitol (300 mM). We used a higher concentration of inhibitors compared with mammalian cells due to specific properties of Xenopus oocytes (presence of vitelline membrane and the yolk) that reduce the actual concentration of drugs at the cell membrane. In general, IC50 values are ~10–20-fold higher when the drugs are applied to the extracellular surface of Xenopus oocytes (25, 26). Some oocytes were injected with the inhibitors or DMSO as a solvent control. The caspase-3 inhibitor Ac-DEVD-CHO (Molecular Probes) was injected at 1 μM (final concentration in the oocyte).

Oocyte Lysis and Western Blot Analysis—Fresh oocytes were lysed by pipetting up and down in 200 μl (pools of 20 oocytes) of ice-cold extraction buffer (0.25 M sucrose, 0.1 M NaCl, 2.5 mM MgCl2, 20 mM HEPES, pH 7.2) containing 1 mM EDTA, 1 mM EGTA, protease inhibitors (10 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml aprotinin), and phosphatase inhibitors (50 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate). Samples were clarified by centrifugation at 14,500 rpm for 5 min, and supernatants were collected and processed for immunoblotting or caspase assay as described below. The whole supernatants were denatured with Sample Buffer (50 mM Tris-HCl, pH 6.8, SDS 2%, 100 mM dithiothreitol, 10% glycerol) and subjected to 10 or 15% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Uniformity of samples loading was verified by Ponceau (Sigma) staining of the blots. Membranes were blocked for 1 h with 5% dried skimmed milk in TBST (50 mM Tris, 150 mM NaCl, 100 mM KCl, pH 7.4, and 0.1% Tween 20) and then incubated with the following polyclonal antibodies from Cell Signaling: anti-AMP-activated protein kinase α (2532); anti-pp38 (Thr-180/Tyr-182) (9211); anti-pJNK (Thr-183/Tyr-185) (9251); anti-JNK (9252); and anti-cleaved caspase-3 (Asp-175) (9661). Polyclonal anti-Smac/DIABLO (2409, ProSci), monoclonal anti-Myc (M4439, clone 9E10, Sigma), monoclonal anti-β-actin (A19789, Sigma), monoclonal anti-ATP synthase α (A21350, Invitrogen), monoclonal anti-Ub (sc-8017, Santa Cruz Biotechnology), and monoclonal anti-cytochrome c (556432, Pharmingen) were also used. Antibody binding was detected with horseradish peroxidase-coupled secondary antibody and the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

Mitochondrial and Cytosolic Fractions—For subcellular fractionation, 30 oocytes were lysed in 300 μl of ice-cold extraction buffer, as described previously, and the extract obtained was centrifuged at 1000 × g for 10 min at 4 °C to remove lipids and the yolk. The supernatant was isolated and centrifuged at 16,000 × g for 15 min at 4 °C. The supernatant obtained (cytosolic fraction) was stored at −20 °C, and the pellet (mitochon-
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JNK1-1 and JNK1-2 Are Activated by Hyperosmotic Shock—It has been reported that hyperosmotic shock induces rapid activation of two JNK isoforms (p40 and p49) in Xenopus oocytes (1, 2, 28), but these isoforms have not been characterized. The wild type isoforms JNK1-1 (384 amino acids) and JNK1-2 (426 amino acids) were cloned by RT-PCR from stage VI oocytes. Both isoforms are generated by alternative splicing from a single gene (jnk1) and differ at the C-terminal sequence. These isoforms, or the corresponding catalytically inactive mutants JNK1-1-AR and JNK1-2-AR (with mutations in amino acids Thr-183 to Ala and Tyr-185 to Phe in both isoforms), with a Myc tag attached at the N terminus, were expressed in Xenopus oocytes and exposed to hyperosmotic shock. Wild type JNK1-1 (40 kDa), JNK1-2 (49 kDa), or the corresponding catalytically inactive mutants AF showed no phosphorylation in untreated oocytes (Fig. 1A). The Myc tag in JNK1-1 and JNK1-2 produced a slight increase in molecular weight compared with endogenous JNK isoforms. Hyperosmotic shock induced phosphorylation of endogenous JNK isoforms (p40 and p49) and of expressed JNK1-1 or JNK1-2, but not of the catalytically inactive mutants JNK1-1-AR or JNK1-2-AR (Fig. 1B). Interestingly, expression of JNK1-1 or JNK1-2 increased caspase-3 activity in oocytes treated with 300 mM sorbitol for 2 h compared with water-injected oocytes or oocytes expressing the mutants JNK1-1-AR or JNK1-2-AR (Fig. 1B), although the differences were not statistically significant. These data demonstrate that JNK1-1 and JNK1-2 are activated by hyperosmotic shock and suggest that they might regulate osmostress-induced apoptosis.

Co-expression of M KK 7 with JNK1-1 or JNK1-2 Accelerates Osmostress-induced Apoptosis—Previously, we have reported that sustained activation of p38 and JNK with the upstream kinase MEKK1 accelerates osmostress-induced apoptosis (2). To address more specifically the role of JNK in osmostress-induced apoptosis, we cloned Xenopus MKK7 (a MAPKK-specific activator of the JNK pathway) and mutated amino acids Ser-268, Thr-272, and Ser-274 to Asp, Glu, and Asp, respectively (MKK7-DED). This mutant has been reported previously as a constitutively active MKK7 (29). Expression of MKK7-DED induced phosphorylation of endogenous JNK1-1 and JNK1-2, as well as the co-expressed JNK1-1 or JNK1-2, but not phosphorylation of p38 (Fig. 1C). Untreated oocytes, in all conditions, showed no significant levels of caspase-3 activity (Fig. 1D). Expression of MKK7-DED in oocytes treated with sorbitol (300 mM) for 1 h showed increased caspase-3 activity compared with water-injected oocytes, although the differences were not statistically significant (Fig. 1D). Co-expression of MKK7-DED with JNK1-1 or JNK1-2 increased the caspase-3 activity induced by osmostress compared with MKK7-DED or water-injected oocytes (Fig. 1D), which was correlated with higher levels of cleaved caspase-3 detected by Western blot (Fig. 1C). Note that the oocytes expressing MKK7 plus JNK1-2 and exposed to osmostress for 1 h presented a decrease of JNK1-2 and the appearance of a new band with similar molecular weights to JNK1-1, detected with JNK or Myc antibodies (Fig. 1D).
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1C), suggesting that hyperosmotic shock induced the proteolysis of JNK1-2 (see below). The oocytes injected with MKK7-DED or with MKK7-DED plus JNK1-1 or JNK1-2 and exposed to osmostress for 2 or 3 h showed higher caspase-3 activity compared with water-injected oocytes exposed to osmostress for the same time, and the differences were statistically significant (Fig. 1D). These results indicate that sustained activation of JNK1-1 and JNK1-2 accelerates hyperosmotic shock-induced apoptosis.

Osmostress Induces the Proteolysis of JNK1-2 by Caspase-3—Incubation of the oocytes with 300 mM sorbitol during several hours induced the disappearance of endogenous JNK1-2 (p49)
and an apparent increase in the band corresponding to endogenous JNK1-1 (p40) (Fig. 2A). Several Western blots were quantified by densitometry showing that a 40% decrease in JNK1-2 (p49) was correlated with a 40% increase in the band corresponding to JNK1-1 (p40) (Fig. 2B). The proteolysis of JNK1-2 was inhibited by Z-VAD-fmk or the specific caspase-3 inhibitor Z-DEVD-fmk, but not by calpain inhibitors ALLN or MDL28170 or EGTA microinjection (Fig. 2C), demonstrating that cleavage of JNK1-2 into a JNK1-1 like fragment is caspase-3-dependent. Although there are no previous reports about the proteolysis of *Xenopus* JNKs by caspasases, it has been reported that human JNK1 and JNK2 are proteolyzed by caspase-3 (24).

To confirm that caspase-3 proteolyzes *Xenopus* JNK1-2, we microinjected oocytes with cytochrome *c*, which has been reported to activate caspase-3 very quickly (30). As depicted in Fig. 2D, cytochrome *c* microinjection induced rapid caspase-3 activation and disappearance of JNK1-2 (p49) with a corresponding increase of the JNK1-1 (p40) band. In contrast, cytochrome *c* coinjected with the broad caspase inhibitor Z-VAD-fmk or the specific caspase-3 inhibitor Ac-DEVD-CHO did not induce JNK1-2 proteolysis. In accordance with previous experiments, co-injection of calpain inhibitors (ALLN, MDL2817, or EGTA) did not reduce JNK1-2 proteolysis induced by cytochrome *c* (Fig. 2E). These data clearly indicate that JNK1-2 is a caspase-3 substrate in *vivo*.

**JNK1-2 Cleavage by Caspase-3 at Asp-385 Engages a Positive Feedback Loop Increasing the Release of Cytochrome *c* and Caspase-3 Activation**—Caspar substrate cleavage sites scanning via the online software “Cascleave” (31) indicated that there are two potential caspase-3 cleavage sites at Asp-385 and Asp-412 at the C terminus of JNK1-2 (Fig. 3A). The mutants JNK1-2D385A (Asp-385 mutated to Ala) and JNK1-2D412A (Asp-412 mutated to Ala) were expressed in *Xenopus* oocytes and exposed to hyperosmotic shock. As shown in Fig. 3B, JNK1-2D385A was not proteolyzed 3 h after hyperosmotic shock, whereas JNK1-2D412A and wild type JNK1-2 were cleaved. Furthermore, truncated JNK1-2Δ385 had the same molecular weight that cleaved wild type JNK1-2 (Fig. 3B). In contrast, truncated JNK1-2Δ412 showed a higher molecular weight than cleaved JNK1-2 and was proteolyzed after hyperosmotic shock (Fig. 3B). As shown in Fig. 3C, the proteolysis of JNK1-2 or JNK1-2D412A was protected by Z-VAD-fmk but not by the calpain inhibitor MDL28170, as expected. Importantly, expression of JNK1-2 increased the release of cytochrome *c* and caspase-3 activity induced by osmolarity at 3 h after treatment compared with JNK1-2Δ385A or water-injected oocytes (Fig. 3D). This result clearly indicates that cleavage of JNK1-2 by caspase-3 accelerates the apoptotic program via increasing the release of cytochrome *c* and caspase-3 activation, thus creating a positive feedback loop. Expression of JNK1-2Δ385, the cleaved product of JNK1-2 by caspase-3, had a similar effect to JNK1-2 in oocytes exposed to osmotic shock for 3 h (Fig. 3D), but showed a significant increase in caspase-3 activity compared with JNK1-2 at 2 h after treatment (Fig. 3E). These data indicate that proteolysed JNK1-2 accelerates osmolarity-induced apoptosis.

**Bel-x<sub>L</sub> Expression Markedly Reduces Osmostress-induced Apoptosis**—Bcl-2 family members are key proteins in regulating mitochondrial membrane permeability during apoptosis. One anti-apoptotic protein, Bcl-x<sub>L</sub>, and three pro-apoptotic proteins (Bak, Bax, and Bid) were cloned by RT-PCR from stage VI oocytes. Bcl-x<sub>L</sub>, Bak, and Bid were cloned into FTX5 plasmid containing a Myc tag at the N terminus, whereas Bak was cloned into the FTX4 plasmid, without a Myc tag. Microinjection of Bak, Bax, or Bid cRNAs induced rapid caspase-3 activity even without hyperosmotic shock treatment (data not shown). However, microinjection of Bel-x<sub>L</sub> cRNA did not induce caspase-3 activity (Fig. 4A, *time 0 h*). Furthermore, when Bcl-x<sub>L</sub> was expressed in combination with Bak, Bax, or Bid, there was no caspase-3 activation (Fig. 4A, *time 0 h*). In these conditions, Bcl-x<sub>L</sub> and most Bak protein were located in the mitochondria, whereas Bid was detected in the cytosolic fraction as two major bands with different molecular weights that we labeled in the blot as Bid and Bid* (Fig. 4B, *time 0 h*). In the next section we will show that Bid* corresponds to Bid mono-ubiquinated (Bid-UB). This Western blot cannot differentiate Bcl-x<sub>L</sub> from Bid in the mitochondrial fraction, because they have similar molecular weights (see below to clarify this point). Hyperosmotic shock treatment for 4 h decreased the amounts of cytosolic Bak and Bid. Intriguingly, a short fragment (15 kDa) was detected with Myc antibodies in the oocytes microinjected with Bcl-x<sub>L</sub> and Bid (Fig. 4B, *time 4 h*). In the next section we will show that this fragment came up as a consequence of Bid proteolysis. Importantly, Bcl-x<sub>L</sub> expression markedly reduced caspase-3 activation and cytochrome *c* release induced by hyperosmotic shock compared with water-injected oocytes or oocytes expressing Bak, but not in oocytes expressing Bid, and with partial protection in the oocytes expressing Bax (Fig. 4, *A and B, time 4 h*). The decrease in caspase-3 activity in oocytes expressing Bcl-x<sub>L</sub> compared with oocytes expressing Bcl-x<sub>L</sub> + Bid or water-injected oocytes was statistically significant (Fig. 4C). The above results demonstrate that overexpression of Bcl-x<sub>L</sub> protects oocytes from osmocytosis-induced apoptosis and suggest that Bid might be proteolysed in oocytes exposed to hyperosmotic shock.
FIGURE 2. JNK1-2 is proteolyzed into a JNK1-1-like protein by caspase-3. A, hyperosmotic shock induces the proteolysis of JNK1-2 (p49). Xenopus oocytes were treated with 300 mM sorbitol, and samples were collected at the indicated times and analyzed by Western blot. B, JNK1-2 (p49) is proteolyzed into a JNK1-1 (p40)-like protein by hyperosmotic shock. Xenopus oocytes were treated with 300 mM sorbitol, and samples were collected 3 and 4 h after treatment and analyzed by Western blot in four independent experiments (lower panel). JNK proteins were quantified by densitometry, and the values obtained for p49 (JNK1-2) and p40 (JNK1-1) at 3 h are referred to as 100 arbitrary units (AU) and compared with values obtained at 4 h (upper graph). Data are represented as mean ± S.E. of four independent experiments; *, p < 0.05 paired t test. C, JNK1-2 proteolysis induced by hyperosmotic shock is caspase-3-dependent but calpain-independent. Oocytes were treated with 300 mM sorbitol with or without the pan-caspase inhibitor Z-VAD-fmk (50 μM), the caspase-3 inhibitor Z-DEVD-fmk (50 μM), the calpain inhibitors ALLN (25 μM), MDL28170 (50 μM), or DMSO (as a solvent control) and 4 h later were collected and analyzed by Western blot. Some oocytes were injected with H2O or EGTA (0.5 mM final concentration in the oocyte) and treated with 300 mM sorbitol for 4 h. D, cytochrome c microinjection induces JNK1-2 proteolysis. Xenopus oocytes were injected with MBS or cytochrome c (0.5 μM, final concentration in the oocyte) and collected at different times. Some oocytes were co-injected with cytochrome c plus Z-VAD-fmk (50 μM) or caspase-3-specific inhibitor Ac-DEVD-CHO (1 μM) and then incubated for the indicated times in MBS. Some oocytes were non-injected and non-treated (control). Samples were collected at different times and analyzed by Western blot and caspase-3 activity assay giving value 1 to control oocytes. The results presented are representative of three independent experiments. E, JNK1-2 proteolysis induced by cytochrome c microinjection is caspase-3-dependent but calpain-independent. Oocytes were injected with cytochrome c (0.5 μM) with or without the inhibitors Z-VAD-fmk (50 μM), Ac-DEVD-CHO (1 μM), ALLN (25 μM), MDL28170 (50 μM), and EGTA (0.5 mM), collected 2 h later, and analyzed by Western blot and caspase-3 activity assay. AMPK, AMP-activated protein kinase.
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B

C

D

E
Bid and Mono-ubiquitinated Bid Are Proteolyzed during Osmostress-induced Apoptosis—To confirm which protein, Bcl-xL or Bid, is proteolyzed during osmostress-induced apoptosis, two different combinations of Bcl-xL and Bid cRNAs were injected in Xenopus oocytes as follows: FTX5-Bcl-xL/FTX4-Bid (represented as Bid (Myc-) in Fig. 5A) or FTX4-Bcl-xL/FTX5-Bid (represented as Bid (Myc+) in Fig. 5A). This allowed us to express high levels of both proteins without any apoptotic effect in untreated oocytes and to detect by Western blot Bcl-xL or Bid, when the Myc tag is attached at the N terminal position of Bak, Bid, Bcl-xL, and Bid* (Bid-Ub). The 15-kDa band, detected with Myc antibodies, corresponds to N-terminal Bid proteolytic fragment induced by hyperosmotic shock treatment. The results presented are representative of three independent experiments. C, Bcl-xL expression significantly reduces caspase-3 activity induced by osmостress, which is counteracted by Bid co-expression. Oocytes were injected with H2O, cRNA Bcl-xL, or the combination Bcl-xL + Bid and 18 h later were exposed to hyperosmotic shock (300 mM sorbitol) for 4 h. Caspase-3 activity is the average ± S.E. of four independent experiments, *, p < 0.05 when compared Bcl-xL versus Bcl-xL + Bid or H2O injected oocytes (ANOVA and Newman-Keuls multiple comparison test). AU, arbitrary units.

FIGURE 4. Bcl-xL expression protects Xenopus oocytes from osmostress-induced apoptosis and Bid co-expression counteracts this protection. A, caspase-3 activity induced by osmостress is regulated by expression of Bcl-2 family members. Xenopus oocytes were injected with H2O, cRNA Bcl-xL, or the combination Bcl-xL + Bak, Bcl-xL + Bax, Bcl-xL + Bid and 18 h later were exposed to hyperosmotic shock (300 mM sorbitol) for 4 h. Oocytes were collected at 0 and 4 h after treatment to obtain cytosolic and mitochondrial fractions. Caspase-3 activity was determined in the cytosolic fractions giving value 1 to non-treated water-injected oocytes. B, expression and subcellular distribution of Bcl-2 family member in Xenopus oocytes. Both cytosolic and mitochondrial fractions were analyzed by Western blot. Bcl-xL, Bak, and Bid were Myc-tagged. CC is the abbreviation for cytochrome c. ATP-synthase and AMP-activated protein kinase (AMPK) were measured as markers of mitochondrial and cytosolic fractions, respectively. The labels on the left indicate the position of Bak, Bid, Bcl-xL, and Bid* (Bid-Ub). The 15-kDa band, detected with Myc antibodies, corresponds to N-terminal Bid proteolytic fragment induced by hyperosmotic shock treatment. The results presented are representative of three independent experiments. C, Bcl-xL expression significantly reduces caspase-3 activity induced by osmостress, which is counteracted by Bid co-expression. Oocytes were injected with H2O, cRNA Bcl-xL, or the combination Bcl-xL + Bid and 18 h later were exposed to hyperosmotic shock (300 mM sorbitol) for 4 h. Caspase-3 activity is the average ± S.E. of four independent experiments, *, p < 0.05 when compared Bcl-xL versus Bcl-xL + Bid or H2O injected oocytes (ANOVA and Newman-Keuls multiple comparison test). AU, arbitrary units.

FIGURE 3. Osmostress-induced cleavage of JNK1-2 at Asp-385 by caspase-3 positively regulates cytochrome c release and caspase-3 activation. A, N-terminal sequences alignment of Xenopus JNK1-1 and JNK1-2. B, hyperosmotic shock induces JNK1-2 cleavage at residue Asp-385 but not at residue Asp-412. Xenopus oocytes were injected with H2O or cRNA of wild type JNK1-1, JNK1-2, mutants JNK1-2D385A, JNK1-2D412A, JNK1-2Δ358, or JNK1-2Δ412 (all Myc-tagged) and 18 h later were exposed to osmotic shock (300 mM sorbitol), collected at different times, and analyzed by Western blot with Myc antibodies. C, JNK1-2 and JNK1-2D385A proteolysis induced by osmостress is caspase-dependent but not calpain-dependent. Oocytes were injected with H2O or cRNA JNK1-2 and JNK1-2D412A and 18 h later were exposed to 300 mM sorbitol for 4 h in the presence or absence of DMSO (solvent control), pan-caspase inhibitor Z-VAD-fmk, caspase-3 inhibitor Z-DEVD-fmk, or calpain inhibitor MDL28170 (all inhibitors at 50 μM), and analyzed by Western blot. D, JNK1-2 cleavage at Asp-385 positively regulates cytochrome c release and caspase-3 activation. Oocytes were injected with H2O or cRNA JNK1-2, JNK1-2D385A, or JNK1-2Δ385 and 18 h later were exposed to osmotic shock (300 mM sorbitol) for 3 h, or non-treated and analyzed by Western blot (bottom panel) and caspase-3 activity assay (upper graph) giving value 1 to non-treated water-injected oocytes. CC in the blot is the abbreviation for cytochrome c. The data presented in the graph are the average ± S.E. of four independent experiments, *, p < 0.05 when compared JNK1-2 versus JNK1-2Δ385 (ANOVA and Newman-Keuls multiple comparison test). E, JNK1-2Δ385 accelerates osmostress-induced apoptosis. Oocytes were injected with H2O or different cRNA, as described in D, and treated with 300 mM sorbitol for 2 h. Caspase-3 activity is the average ± S.E. of eight independent experiments, *, p < 0.05 when compared JNK1-2 versus JNK1-2Δ385 (ANOVA and Newman-Keuls multiple comparison test). AMPK, AMP-activated protein kinase.
expected molecular mass of 8.5 kDa, this could explain the two extra bands obtained in our blots. Western blot analysis with ubiquitin antibodies confirmed that the two extra bands corresponded to mono- and bi-ubiquitinated. *Xenopus* oocytes were injected with H₂O (control, 1st and 4th lanes) or cRNAs in two combinations as follows: FTX5-Bcl-xL, plus FTX4-Bid (represented as Bid (Myc –), 2nd and 5th lanes) and FTX4-Bcl-xL, plus FTX5-Bid (represented as Bid (Myc +), 3rd and 6th lanes), with FTX5 constructs containing a Myc tag at the N terminus and 18 h later were exposed to hyperosmotic shock (300 mM sorbitol) for 4 h. Oocytes were collected at 0 and 4 h after treatment to obtain cytosolic fractions and analyzed by Western blot to measure levels of Bid, Bid-Ub, Bid-2Ub, nBid-Ub, nBid-2Ub, and cleaved-caspase-3, using antibodies against Myc, Ub, or cleaved caspase-3. *Left diagram* indicates Bid proteins corresponding to bands obtained in the Western blot, except the band marked with an asterisk, which represents a non-specific band detected with Myc antibodies. The *top left panel*, in which the background for the non-specific band (*) is lighter, comes from another Western blot. *B*, sequence alignment of *X. laevis* Bid with caspase-3 proteins from *X. laevis* and *Homo sapiens*. Proteolysis of the three proteins by caspsases exposes identical three amino acids (ETD) at the C terminus, which can be recognized with cleaved caspase-3 antibodies from Cell Signaling.

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**FIGURE 5.** *Bid and mono-ubiquitinated Bid are proteolyzed during hyperosmotic shock-induced apoptosis.* *A*, Bid expressed in *Xenopus* oocytes is mono- and bi-ubiquitinated. *Xenopus* oocytes were injected with H₂O (control, 1st and 4th lanes) or cRNAs in two combinations as follows: FTX5-Bcl-xL, plus FTX4-Bid (represented as Bid (Myc –), 2nd and 5th lanes) and FTX4-Bcl-xL, plus FTX5-Bid (represented as Bid (Myc +), 3rd and 6th lanes), with FTX5 constructs containing a Myc tag at the N terminus and 18 h later were exposed to hyperosmotic shock (300 mM sorbitol) for 4 h. Oocytes were collected at 0 and 4 h after treatment to obtain cytosolic fractions and analyzed by Western blot to measure levels of Bid, Bid-Ub, Bid-2Ub, nBid-Ub, nBid-2Ub, and cleaved-caspase-3, using antibodies against Myc, Ub, or cleaved caspase-3. *Left diagram* indicates Bid proteins corresponding to bands obtained in the Western blot, except the band marked with an asterisk, which represents a non-specific band detected with Myc antibodies. The *top left panel*, in which the background for the non-specific band (*) is lighter, comes from another Western blot. *B*, sequence alignment of *X. laevis* Bid with caspase-3 proteins from *X. laevis* and *Homo sapiens*. Proteolysis of the three proteins by caspsases exposes identical three amino acids (ETD) at the C terminus, which can be recognized with cleaved caspase-3 antibodies from Cell Signaling.
osmotic shock treatment, the amount of cytosolic Bid-2Ub was unchanged (Fig. 5A). Because nBid-Ub and nBid-2Ub accumulated in the cytosol, this suggests that nBid-2Ub came from the ubiquitination of nBid and/or nBid-Ub instead of direct cleavage of Bid-2Ub.

In traditional signaling pathways, Bid is cleaved by caspase-8 generating the so-called tBid fragment to regulate the mitochondrial outer membrane permeability. For Xenopus Bid, this cleavage site has been reported at Asp-52 (7). As shown in Fig. 5B, Bid proteolysis at Asp-52 exposes three amino acids (−ETD−), similar to activated caspase-3. It has been reported that cleaved caspase-3 antibody from Cell Signaling Technology (Ref. #9661) recognizes the epitope ETD, and therefore it can be used for detection of other caspase-9 substrates (32). Because the epitope ETD at the N-terminal fragment of Bid (nBid) is identical to cleaved caspase-3, it should be detected by the Cell Signaling antibody. As shown in Fig. 5A, lower panel, in addition to activated caspase-3 (18 kDa), two extra bands corresponding to nBid-Ub and nBid-2Ub were recognized by caspase-3 antibody in the cytosolic pool, thus confirming the identity of the Bid fragments. Moreover, nBid-Ub and nBid-2Ub were not detected with caspase-3 antibodies in oocytes expressing the mutants Bid-D52N (non-proteolyzed by caspases) or Bid-nonUb (non-ubiquitinated) and exposed to hyperosmotic shock, thus corroborating the identity of these bands (see Fig. 7A). In conclusion, Xenopus Bid is present in the oocytes as non-ubiquitinated, mono-, and bi-ubiquitinated forms, and hyperosmotic shock induces cleavage of non-ubiquitinated and mono-ubiquitinated Bid producing nBid-Ub and nBid-2Ub in the cytosol.

**Hypersosmotic Shock Induces Marked Proteolysis of Bid by Caspase-3**—Next, we analyzed the proteolysis of Bid induced by osmstress in a time course experiment. Hypersosmotic shock induced weak proteolysis of Bid at 1 h, detected by appearance of a 15-kDa band, which was synchronized with Smac/DIABLO and cytochrome c release (Fig. 6A). At this time, a slight increase of caspase-3 activity was detected in the enzymatic assay, but not by Western blot (Fig. 6A). Indeed, nBid-Ub and nBid-2Ub were not detected with the cleaved caspase-3 antibody (Fig. 6A). Importantly, caspase-3 activity was detected by Western blot or by enzymatic assay at 3 and 4 h after osmstress, which was well correlated with marked proteolysis of Bid (Fig. 6A). Incubation with the specific caspase-3 inhibitor Z-DEVD-fmk clearly reduced Bid proteolysis at 4 h, as indicated by the low levels of nBid-Ub detected with Myc antibodies or the low levels of nBid-Ub and nBid-2Ub detected with cleaved caspase-3 antibodies (Fig. 6A). The cleavage of caspase-3, supposedly induced by caspase-9, was unchanged in the presence of the caspase-3 inhibitor as expected, but it was almost completely blocked with the broad caspase inhibitor Z-VAD-fmk. Accordingly, Bid proteolysis was completely blocked by Z-VAD-fmk (Fig. 6A, last lane), suggesting that Bid might be cleaved by caspase-9 or other caspases activated by osmstress. Of note, Smac/DIABLO and cytochrome c release induced by osmstress at 4 h were slightly reduced in the presence of the caspase-3 inhibitor and more clearly in the presence of Z-VAD-fmk (Fig. 6A, lower blots). We have previously reported that osmstress induces rapid Smac/DIABLO release from the mitochondria to the cytosol and that Z-VAD-fmk delays osmstress-induced apoptosis (2). Altogether, the above results suggest that low amounts of Bid are cleaved by a caspase or another protease, not yet identified, and when caspase-3 is fully active, a massive proteolysis of Bid occurs at later times, which can be easily detected by Western blot. The proteolysis of Bid induced by osmstress was accelerated by co-expression of Bax in oocytes expressing Bid and Bcl-\(\chi\) (Fig. 6B), confirming that changes in the equilibrium between pro- and anti-apoptotic members of the Bcl-2 family can modify the kinetics of osmstress-induced apoptosis.

We have previously reported that hyperosmotic shock induces early calpain activation, which contributes to regulate osmstress-induced apoptosis (2), and calpains have been reported to proteolyze Bid at the N terminus (33). As shown in Fig. 6C, the calpain inhibitor MDL28170 only slightly reduced Bid proteolysis at 4 h after osmstress, measured as the amount of nBid-Ub. This must be a consequence of partial caspase inhibition (Fig. 6C, lower graph). As a control,.Z-VAD-fmk efficiently blocked Bid proteolysis and caspase-3 activation. In conclusion, hyperosmotic shock induces early Bid cleavage at low amounts by a caspase or a protease, not yet identified, and massive Bid cleavage at later times mainly by caspase-3.

**Caspase-3-dependent Cleavage of Bid at Asp-52 Engages a Positive Feedback Loop Increasing Cytochrome c Release and Caspase-3 Activation**—We have described that osmstress induces two important modifications in Bid, ubiquitination and cleavage. It has been reported that three sites (Lys-18/21/37) at the N terminus of Xenopus Bid are important for ubiquitination (15). In addition, Xenopus Bid has been reported to be cleaved by caspases at Asp-52 (7, 15). Therefore, to investigate the function of Bid ubiquitination and cleavage in hyperosmotic shock-induced apoptosis, wild type Bid, triple mutant Bid-K18R/K21R/K37R (Bid-nonUb, with mutation of amino acids Lys-18, Lys-21, and Lys-37 to Arg), or mutant Bid-D52N (mutation of Asp-52 to Asn) were expressed in Xenopus oocytes in combination with Bcl-\(\chi\), and exposed to hyperosmotic shock. As shown in Fig. 7A, Bid-D52N was not proteolyzed in the oocytes exposed to osmstress, in contrast to wild type Bid or Bid-nonUb. Proteolysis of wild type Bid generated a 15-kDa fragment corresponding to nBid-Ub (detected with Myc antibodies), which was not observed after proteolysis of Bid-nonUb, as expected. nBid-2Ub and nBid-Ub were also detected with cleaved caspase-3 antibodies (Fig. 7A, lower blot) in the oocytes expressing Bid but not in the oocytes expressing Bid-D52N or Bid-nonUb. nBid was difficult to detect in the blots, suggesting that this fragment is unstable in the oocyte (data not shown). Ubiquitination analysis by Western blot indicated that Bid-nonUb presented a clear reduction of the mono- and bi-ubiquitinated bands compared with wild type Bid or Bid-D52N in untreated oocytes (Fig. 7B, upper graph). The bi-ubiquitinated N-terminal fragment (nBid-2Ub) generated by osmotic shock was also reduced in Bid-nonUb, compared with wild type Bid (Fig. 7B, upper blot). Despite this marked reduction, low levels of ubiquitination were still detected in the Bid-nonUb mutant (Fig. 7B), as reported previously by other authors (15). Bid-nonUb proteolysis was apparently faster than wild type Bid pro-
teolysis (Fig. 7A, 4 h, upper panel), but this may be due to the absence of nBid-2Ub after proteolysis of Bid-nonUb (Fig. 7B), because nBid-2Ub overlaps with Bid in Western blot detection, as described before. Importantly, expression of mutant Bid-nonUb did not modify cytochrome c release and caspase-3 activation induced by osmostress, compared with wild type Bid, whereas mutant BidD52N clearly reduced cytochrome c release and caspase-3 activation induced by osmostress (Fig. 7, A and C). The differences between Bid and BidD52N were statistically significant (Fig. 7D). This result clearly indicates that Bid cleavage induced by caspases, but not Bid ubiquitination, regulates osmostress-induced apoptosis through activation of caspase-3, therefore creating a positive feedback loop. Injection of truncated C-terminal Bid (53–184 amino acids), also known as tBid, induced high caspase-3 activity in oocytes co-injected with Bcl-xL, even without hyperosmotic shock treatment (Fig. 7, C and D). On the contrary, co-injection of nBid (1–52 amino acids) with Bcl-xL did not have any effect on osmostress-induced apoptosis compared with Bcl-xL-injected oocytes (Fig. 7C).

FIGURE 6. Hyperosmotic shock induces marked proteolysis of Bid by caspase-3. A, hyperosmotic shock-induced Bid proteolysis is blocked by caspase inhibitors. Oocytes were injected with cRNA combination FTX4-Bcl-xL + FTX5-Bid and 18 h later were exposed to osmotic shock (300 mM sorbitol) for 4 h in the presence or absence of caspase inhibitors. Samples were collected at different times to obtain cytosolic fractions and analyzed by Western blot and caspase-3 activity assay giving value 1 to non-treated oocytes. Samples incubated in the presence of caspase inhibitors (*, Z-DEVD-fmk; **, Z-VAD-fmk) were analyzed 4 h after hyperosmotic shock treatment. B, Bax expression accelerates Bid proteolysis induced by hyperosmotic shock. Oocytes were injected with H2O or with two cRNA combinations (FTX4-Bcl-xL + FTX5-Bid or FTX4-Bcl-xL + FTX5-Bid + FTX4-Bax) and 18 h later were exposed to osmotic shock (300 mM sorbitol). Samples were collected at the indicated times, and cytosolic fractions were analyzed by Western blot with Myc antibodies. C, calpain inhibitor (MDL28170) does not alter Bid proteolysis induced by osmostress. Oocytes were injected with H2O, cRNA FTX4-Bcl-xL, or with the combination FTX4-Bcl-xL and FTX5-Bid and 18 h later were exposed to hyperosmotic shock (300 mM sorbitol) for 4 h in the presence or absence of pan-caspase inhibitor Z-VAD-fmk or calpain inhibitor MDL28170. Samples were collected at 0 and 4 h, and cytosolic fractions were analyzed by Western blot with Myc or cytochrome c (CC) antibodies and caspase-3 activity assay (lower graph) giving value 1 to non-treated water-injected oocytes. The results presented are representative of three independent experiments. AMPK, AMP-activated protein kinase. AU, arbitrary units.
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**Discussion**

**JNKs, Phosphorylation and Proteolysis**—Here, we show that sustained activation of JNK1-1 and JNK1-2 accelerates hyperosmotic shock-induced apoptosis in *Xenopus* oocytes. In addition, proteolysis of JNK1-2 by caspase-3 at later times engages a positive feedback loop increasing the release of cytochrome c release and caspase-3 activation. Sustained activation of JNK might induce apoptosis through different mechanisms. It has been reported that JNK induces the phosphorylation of Bax sequestration proteins 14-3-3, which promotes Bax translocation to mitochondria (34). However, we have not observed any significant change in Bax distribution during osmostress-in-
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Osmostress-induced apoptosis in Xenopus oocytes is achieved through the proteolysis of Bid and the generation of truncated jBid (9). However, the proteolytic site reported in human Bid that is responsible for the generation of jBid (9) is not conserved in Xenopus. Moreover, oocytes exposed to hyperosmotic shock or expressing a constitutively active MKK7 did not produce another N-terminal fragment of Bid different from nBid. This work demonstrates that osmostress induces the caspase-3-dependent cleavage of Xenopus JNK1-2 into a JNK1-1-like protein. Scanning via the on-line software Caspase-3 (31) showed two potential caspase-3 recognition sites in JNK1-2 at Asp-385 and Asp-412. In humans, JNK1 and JNK2 are proteolyzed by caspase-3 at Asp-413 and Asp-410, respectively (24), at a site identical to Xenopus JNK1-2 recognition site Asp-412 (SDTD). However, our results clearly demonstrate that cleavage of Xenopus JNK1-2 occurs only at site Asp-385 (AVTD). Interestingly, the mutant JNK1-2 D412A seems to be proteolyzed faster than wild type JNK1-2. It might be possible that the sequence at Asp-412 in Xenopus serves as a binding site for caspase-3, thus reducing the cleavage of JNK1-2 at the optimal site Asp-385. Alternatively, the sequence at Asp-412 might be protected from cleavage by some modifications at amino acids near the cleavage site (e.g., phosphorylation) or by the interaction with other proteins. Cleavage of JNK1-2 by caspase-3 positively regulates cytochrome c release and caspase-3 activity induced by osmotic stress, thus creating a positive feedback loop. It seems that the positive feedback comes from the generation of the JNK1-1-like protein. Scanning via the on-line software Cascleave (31) showed two potential caspase-3 recognition sites in JNK1-2 at Asp-385 and Asp-412. In humans, JNK1 and JNK2 are proteolyzed by caspase-3 at Asp-413 and Asp-410, respectively (24), at a site identical to Xenopus JNK1-2 recognition site Asp-412 (SDTD). However, our results clearly demonstrate that cleavage of Xenopus JNK1-2 occurs only at site Asp-385 (AVTD). Interestingly, the mutant JNK1-2 D412A seems to be proteolyzed faster than wild type JNK1-2. It might be possible that the sequence at Asp-412 in Xenopus serves as a binding site for caspase-3, thus reducing the cleavage of JNK1-2 at the optimal site Asp-385. Alternatively, the sequence at Asp-412 might be protected from cleavage by some modifications at amino acids near the cleavage site (e.g., phosphorylation) or by the interaction with other proteins. Cleavage of JNK1-2 by caspase-3 positively regulates cytochrome c release and caspase-3 activity induced by osmotic stress, thus creating a positive feedback loop. It seems that the positive feedback comes from the generation of the JNK1-1-like fragment (amino acids 1-385), because overexpression of JNK1-2A385 increased caspase-3 activity compared with JNK1-1-2. Further studies are necessary to elucidate the mechanism in detail.

Bcl-xL and Bid Co-expression in Xenopus Oocytes—Bcl-2 family members are central proteins in regulating mitochondrial permeability in apoptosis. Here, we show that Xenopus oocytes overexpressing the anti-apoptotic protein Bcl-xL present a marked reduction of cytochrome c release and caspase-3 activation induced by hyperosmotic shock. On the contrary, ectopic expression of the pro-apoptotic Bcl-2 member Bid induces oocyte apoptosis without any stress stimulation. Although it is reported that Bid is located in the cytosol, and the full activity of Bid is not exerted until proteolytic cleavage by caspase-8 (6), full-length Bid can still translocate to mitochondria, and it is sufficient to induce apoptosis of cultured cells (35, 36). Co-expression of Bcl-xL with Bid blocks the apoptosis induced by Bid but not the apoptosis induced by osmotic stress. This allowed us to express high levels of both pro- and anti-apoptotic proteins to study some modifications induced by osmotic stress that would be difficult to detect due to the small amounts of endogenous proteins present in the oocytes. In addition, there are no good commercial antibodies to detect Xenopus Bcl-2 family members, but ectopic expression of the proteins with a Myc tag at the N terminus can be easily detected. Thus, we observed that overexpressed Bid is located in the cytosol, and the mitochondria and hyperosmotic shock induced Bid proteolysis in both pools. We also demonstrate that cleavage of Bid induced by osmotic stress occurs after residue Asp-52, in the conserved caspase-8/9/10 site ETD, is markedly reduced in oocytes incubated with the specific caspase-3 inhibitor, and is completely blocked in the presence of Z-VAD-fmk. Therefore, it seems that most of Bid proteolysis is due to caspase-3 activity, but another caspase can also participate, to a minor extent, in Bid proteolysis.

Caspase-3-dependent Proteolysis of Bid—It has been reported that caspase-3 can cleave human Bid at residue Asp-60, and cell extracts depleted of caspase-3 or extracts of MCF-7 cells that are devoid of caspase-3 due to a deletion in exon3 of the caspase-3 gene fail to induce Bid cleavage (8). The cleavage site in Xenopus Bid is similar to the cleavage activation site in Xenopus caspase-3, and therefore caspase-9 could cleave both proteins. It has also been reported that Xenopus Bid can be proteolyzed by Xenopus caspase-8 and caspase-10β (7). Altogether, the above data indicate that the ETD site present in Xenopus Bid can be recognized by caspase-3, caspase-8, caspase-9, and caspase-10. Xenopus caspase-8, however, is mainly expressed at stages 15/16 in early embryogenesis, with very low levels, if any, in stage VI oocytes (7). During apoptosis, Bid can be cleaved not only by caspasess, but also by granzyme B, calpains, and cathepsins (5). We have reported that calpain inhibitors, but not cathepsin inhibitors, delay osmotic stress-induced apoptosis in Xenopus oocytes (2). We show here that calpain inhibitor ML-28170 only slightly decreases Bid proteolysis, probably due to the reduction in caspase-3 activation. The time course analysis of Bid cleavage induced by osmotic stress indicates that low amounts of Bid are proteolyzed very early after hyperosmotic shock by a caspase or a protease, whereas most of Bid is cleaved by caspase-3 at later times. Candidates for early proteolysis of Bid are caspase-10, which is highly expressed in stage VI oocytes (7), and caspase-9. Future experiments will address the role of these caspases in early Bid cleavage induced by osmotic stress. The C-terminal product of Bid cleaved at residue Asp-52, tBid, is more active than full-length Bid in inducing cytochrome c release and caspase-3 activation. Although overexpression of Bcl-xL can block the apoptotic function of wild type Bid in the oocytes, a tiny amount of tBid (1/100 of wild type Bid) is enough to induce apoptosis without any stress stimulation (data not shown). Therefore, osmotic stress-induced Bid cleavage mediated by initiator caspases or another protease could produce enough tBid to induce cytochrome c release and caspase-3 activation, which in turn generates more tBid, thus creating a positive feedback loop. Indeed, we demonstrate that expression of Bid mutated at the cleavage caspase site ETD (BidD52N) clearly reduces the release of cytochrome c and caspase-3 activity induced by osmotic stress compared with wild type Bid.

Mono- and Bi-ubiquitination in Xenopus Bid—Here, we show that a significant portion of Bid is mono- and bi-ubiquitinated in the cytosol. The mitochondria only presented a slight increase of mono- and bi-ubiquitinated Bid after hyperosmotic shock treatment (data not shown). Not only ubiquitinated full-length Bid but also ubiquitinated N-terminal Bid fragments obtained by proteolysis of Bid are quite stable. Proteolysis of Bid and Bid-Ub in the cytosol generates N-terminal fragments that are ubiquitinated and accumulated in the cytosol and the mitochondria as nBid-Ub and nBid-2Ub. It is difficult to know the role of mono- and bi-ubiquitination in Bid, but in other pro-
Teins it has been reported that these modifications can alter the localization in the cell, the interaction with other proteins, and their function (37). Human Bid is subject to autoinhibition in the absence of stimuli, because the N- and C-terminal fragments bind each other through interactions of the BH3-like region at the N terminus and the BH3 region at the C terminus (10). Unconventional ubiquitination and degradation of human nBid is required to increase its apoptotic activity (14). Removal of nBid has been suggested to increase the number of exposed hydrophobic residues in tBid thereby facilitating binding of the protein to membranes (38). However, in Xenopus Bid the BH3-like region at the N-terminal fragment is not conserved. Therefore, mono- and bi-ubiquitination of the N-terminal fragment in Xenopus Bid could be a protective mechanism to avoid its interaction with the mitochondrial membrane to induce cytochrome c release in the absence of any stressful stimulus. However, the triple mutant Bid (K18R/K21R/K37R), with a marked reduction in ubiquitination, did not have a differential effect on osmostress-induced apoptosis compared with wild type Bid. More studies are necessary to address the role of Bid ubiquitination in Xenopus oocytes.

Integration of Different Pathways in Osmostress-induced Apoptosis—We have previously reported that hyperosmotic shock induces rapid calpain activation, Smac/DIABLO release, and JNK/p38 activation in Xenopus oocytes, all contributing to cytochrome c release and caspase-3 activation (2). Here, we show that Bcl-xL overexpression protects oocytes from hyperosmotic shock-induced apoptosis, indicating that cytochrome c release from the mitochondria is crucial for caspase-3 activation. When cytochrome c is released and reaches a threshold level, caspase-3 is activated and induces JNK1-2 proteolysis at Asp-385 and massive proteolysis of Bid at Asp-52, which in turn induce more cytochrome c release and caspase-3 activity engaging two positive feedback loops (Fig. 8). It has been reported that caspase-3 increases calpain activation (39, 40), and our previous results also indicated that caspase-3 increased Smac/DIABLO release (2) and p38 phosphorylation (3). Therefore, hyperosmotic shock induces the activation of different pathways that converge on the activation of caspase-3, which engages an irreversible apoptotic program through activation of multiple positive feedback loops.

FIGURE 8. Model for osmostress-induced apoptosis in Xenopus oocytes. Hyperosmotic stress induces rapid calpain/JNK/p38 activation and tiny amounts of Bid cleavage by an unknown protease/caspase. High levels of Smac/DIABLO and low amounts of cytochrome c are released from the mitochondria at this early stage, but they are not sufficient to activate caspase-3. Sustained activation of these pathways (during 2 to 4 h) collectively leads to a marked release of cytochrome c promoting caspase-3 activation, which in turn induces more Smac/DIABLO release, calpain, and p38 activation, and cleavage of JNK1-2 and Bid. These events positively regulate cytochrome c release and caspase-3 activation resulting in an irreversible apoptotic process. In contrast, overexpression of Bcl-xL protects oocytes from osmostress-induced apoptosis via inhibiting cytochrome c release.

Author Contributions—J. M. L. conceived the study. J. Y. performed the experiments shown in Figs. 3–7. J. Y. and J. M. L. designed and analyzed the experiments shown in Figs. 3–7. N. B. M. performed the experiments shown in Fig. 1. N. B. M. and J. M. L. performed the experiments shown in Fig. 2 and designed and analyzed the experiments shown in Figs. 1 and 2. J. Y. and J. M. L. wrote the paper.

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