

Metabolic Regulation of Oocyte Cell Death through the CaMKII-Mediated Phosphorylation of Caspase-2

Leta K. Nutt,^{1,5} Seth S. Margolis,^{1,5} Mette Jensen,^{1,3} Catherine E. Herman,^{1,2,3} William G. Dunphy,⁴ Jeffrey C. Rathmell,^{1,2,3} and Sally Kornbluth^{1,*}

¹Department of Pharmacology and Cancer Biology

²Department of Immunology

³Sarah Stedman Center for Nutrition and Metabolism
Duke University Medical Center
Durham, North Carolina 27710

⁴Division of Biology
California Institute of Technology
Pasadena, California 91125

Summary

Vertebrate female reproduction is limited by the oocyte stockpiles acquired during embryonic development. These are gradually depleted over the organism's lifetime through the process of apoptosis. The timer that triggers this cell death is yet to be identified. We used the *Xenopus* egg/oocyte system to examine the hypothesis that nutrient stores can regulate oocyte viability. We show that pentose-phosphate-pathway generation of NADPH is critical for oocyte survival and that the target of this regulation is caspase-2, previously shown to be required for oocyte death in mice. Pentose-phosphate-pathway-mediated inhibition of cell death was due to the inhibitory phosphorylation of caspase-2 by calcium/calmodulin-dependent protein kinase II (CaMKII). These data suggest that exhaustion of oocyte nutrients, resulting in an inability to generate NADPH, may contribute to oocyte apoptosis. These data also provide unexpected links between oocyte metabolism, CaMKII, and caspase-2.

Introduction

Vertebrate female reproduction is limited by the oocyte stockpiles acquired during embryonic development. Apoptotic loss of oocytes over a lifetime leads ultimately to loss of fertility (Tilly, 2001). In addition, pathological insults or chemotherapeutic treatment can accelerate oocyte death, resulting in premature oocyte depletion and sterility (Perez et al., 1997). Despite the importance of oocyte survival in determining female fertility, the molecular pathways governing the timing of apoptosis in these cells are not fully understood.

Genetic analyses in mice and in vitro experiments using murine oocytes have provided a framework in which to understand the molecular underpinnings of oocyte apoptosis. Death of these cells can be prevented by caspase inhibitors or antiapoptotic Bcl-2 proteins and induced by overexpression of either caspases or proapoptotic Bcl-2 family members (Morita et al., 1999; Morita and Tilly, 1999; Kim and Tilly, 2004; Morita et al., 2000). Conclusions from such studies

have been borne out by analyses of knockout mice in which Bax-deficient animals exhibited a reduction in oocyte apoptosis, with a consequent delay in oocyte depletion (Perez et al., 1999), while mice lacking Bcl-2 or Bcl-xL had a decreased oocyte reserve (Ratts et al., 1995; Watanabe et al., 1997).

Insight into oocyte apoptosis has come from studies of knockout mice deficient in caspase-2. Surprisingly, these mice developed normally, with one striking exception: female mice were endowed with an excess of oocytes. Moreover, caspase-2-deficient oocytes were markedly resistant to cell death induced by chemotherapeutics (Bergeron et al., 1998). The precise role of caspase-2 in cell death has been controversial, but recent studies demonstrate that it can act upstream of the mitochondria in a number of settings to trigger cytochrome c release (Guo et al., 2002; Lassus et al., 2002; Robertson et al., 2002). Caspase-2 is activated by binding to oligomerizing adaptor proteins. In response to various stressors, caspase-2 is recruited into high-molecular-weight complexes reminiscent of, though distinct from, the Apaf-1/caspase-9 apoptosome (Read et al., 2002). The caspase-2 prodomain can recruit an adaptor protein, RAIDD, which, when overexpressed, induces caspase-2 activation (Duan and Dixit, 1997). Recently it was reported that p53 induces expression of PIDD, a protein which promotes formation of a caspase-2 activation complex containing PIDD, caspase-2, and RAIDD (Tinel and Tschopp, 2004). It is not known whether PIDD or RAIDD regulates caspase-2 in the oocyte.

Although genetic analyses and microinjection studies have provided critical information for understanding oocyte apoptosis, most vertebrate oocytes are not amenable to biochemical analysis due to their small size and limited abundance. However, over a decade ago, Newmeyer et al. reported that extracts prepared from eggs of the frog, *Xenopus laevis*, could, upon prolonged incubation at room temperature, spontaneously recapitulate many events of apoptosis, including mitochondrial cytochrome c release, caspase activation, and nuclear fragmentation (Newmeyer et al., 1994). Importantly, this in vitro apoptosis could be inhibited by antiapoptotic Bcl-2 proteins and caspase inhibitors, suggesting that at least some aspects of germ-cell apoptosis are faithfully recapitulated in this system, thereby providing a biochemically manipulable setting in which to understand germ-cell apoptosis (Kluck et al., 1997).

Despite the ease of manipulation of *Xenopus* eggs and oocytes and the manifestation of apoptotic markers, it was not clear what might be driving apoptosis in this system. Although initial reports suggested that the timing of hormone administration used to elicit egg laying might determine the susceptibility of eggs to apoptosis, research in the intervening years has failed to establish a firm correlation between the rates of apoptosis in *Xenopus* eggs and the hormonal regimen used to obtain the eggs. Hence, we were driven to consider other features of the oocyte/egg that might contribute

*Correspondence: kornb001@mc.duke.edu

⁵These authors contributed equally to this work.

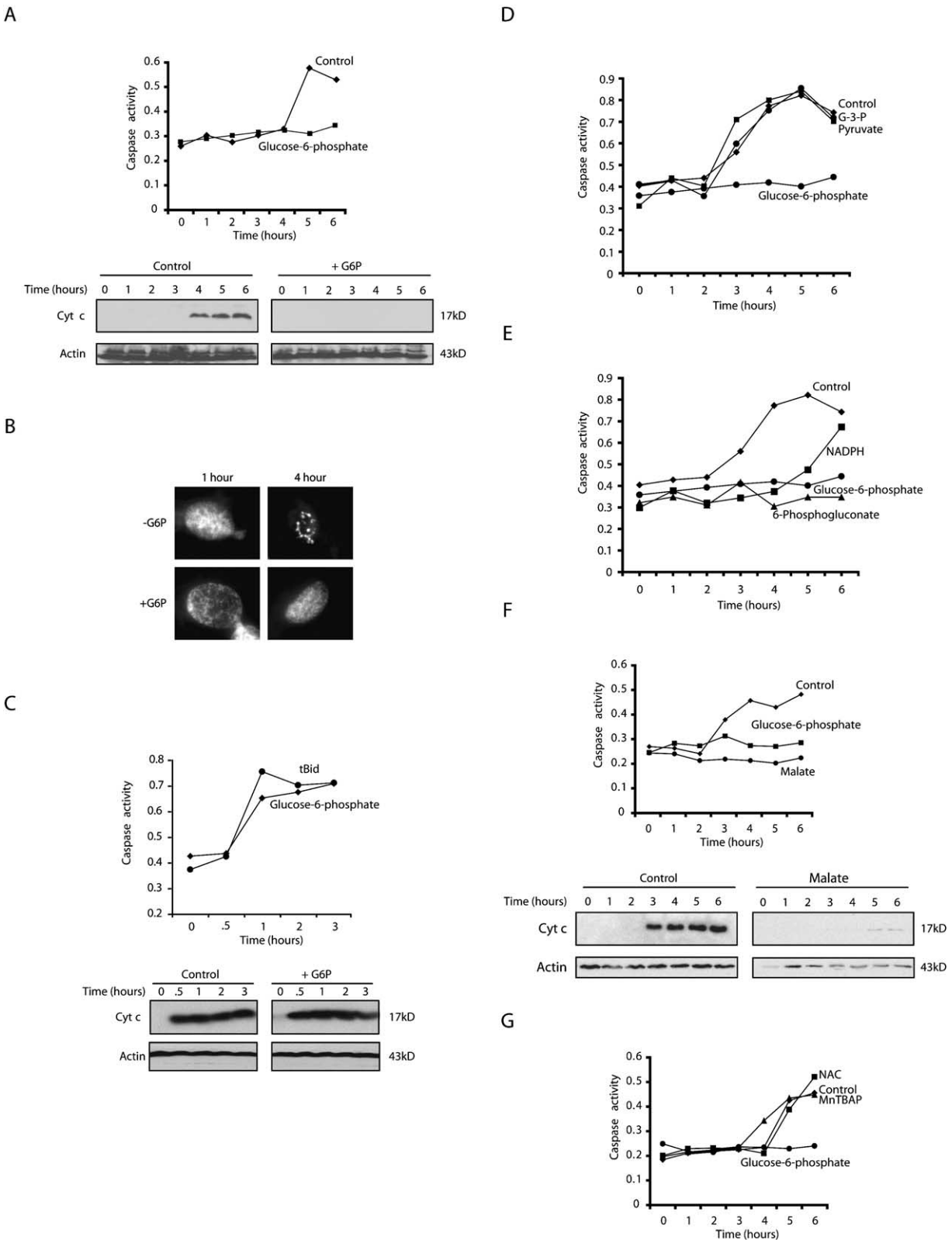


Figure 1. NADPH is a Potent Inhibitor of Egg Apoptosis

(A) *Xenopus* egg extracts supplemented with G6P or buffer were analyzed for caspase-3 activity at various time points using the caspase substrate Ac-DEVD-pNA. Substrate cleavage was measured spectrophotometrically at 405 nm. Cytochrome c release was measured in parallel by filtering extract through a 0.1 μ m microfilter. The filtrate, lacking mitochondria, was analyzed by SDS-PAGE and immunoblotting with anti-cytochrome c antibody. Blots were reprobbed with anti-actin antibody.

to cell-death regulation. In this regard, we were interested in the fact that oocytes are uniquely endowed with a large stockpile of nutrients in the form of both yolk proteins and glycogen stores, which are used to sustain early embryonic development.

Numerous links have been established between metabolism and apoptosis. For example, the Bcl-2 family protein Bad associates with and regulates glucokinase/hexokinase 4 (Danial et al., 2003). Moreover, AKT requires glucose uptake to promote cell survival, and this effect is exerted in part through targeting of hexokinase to the mitochondria (Majewski et al., 2004; Rathmell et al., 2003). In lymphocytes and neurons, growth-factor withdrawal leads to a drop in glucose uptake and glycolytic rates prior to commitment to cell death (Deckwerth and Johnson, 1993; Deshmukh et al., 1996; Rathmell et al., 2000; Vander Heiden et al., 2001). Moreover, artificial maintenance of glycolysis confers some resistance to cell death induced by cytokine withdrawal, suggesting that active growth-factor signaling is required to maintain sufficient metabolism to prevent death (Rathmell et al., 2001, 2003). In addition, glucose-6-phosphate dehydrogenase activity, which promotes the metabolism of glucose through the pentose phosphate pathway, can protect CHO cells from death induced by ionizing radiation (Tuttle et al., 2000). Conversely, inhibition of both glycolytic and pentose phosphate pathways can promote the apoptotic death of some cultured cells (Comin-Anduix et al., 2002; Tian et al., 1999; Tuttle et al., 2000). Thus, it was attractive to hypothesize that nutrient stores in the oocyte might support cell survival through modulation of metabolic pathways. According to such a scenario, depletion of energy stores within oocytes over time would result either in loss of a critical survival pathway or the de novo engagement of a cell-death pathway.

Here, using the facile biochemistry provided by the *Xenopus* system, we report the identification and characterization of a novel caspase-2 regulatory pathway responsive to the metabolic state of the oocyte/egg. We demonstrate that glucose-6-phosphate (G6P) sufficient to drive continual operation of the pentose phosphate pathway can greatly prolong germ-cell survival. Moreover, our data indicate that NADPH generation by this pathway is critical for promoting survival and that a surfeit of NADPH promotes a calcium/calmodulin-dependent protein kinase II (CaMKII) dependent inhibitory phosphorylation of caspase-2. Mutant caspase-2 resistant to CaMKII phosphorylation can circumvent this metabolism-dependent suppression of apoptosis, both in egg extracts and intact oocytes. These data, which are fully consistent with the reported requirement for caspase-2 in mouse oocyte apoptosis, link the operation of a specific metabolic pathway to the direct

CaMKII-mediated regulation of caspase-2, thereby providing insight into the control of germ-cell life and death.

Results

G6P Inhibits Apoptosis in *Xenopus* Egg Extracts

If nutrient depletion contributed to apoptotic induction in *Xenopus* eggs and oocytes, we hypothesized that boosting glucose-utilizing metabolic pathways might forestall apoptosis in this system. Accordingly, we added G6P directly to the in vitro egg extract system and monitored cleavage of the model substrate, DEVD-pNA, as a measure of caspase-3 activity. As shown in Figure 1A, the caspase-3 activation observed following incubation of the extract at room temperature was entirely suppressed by G6P addition. Note that G6P, which in oocytes is derived largely from gluconeogenesis or glycogenolysis, was used in these experiments rather than glucose due to the presence of very low hexokinase activity in these extracts (C.E.H. and L.K.N., unpublished data). We also found that G6P could inhibit apoptotic fragmentation of sperm nuclei added to egg extracts (Figure 1B). Consistent with the critical role for mitochondria in the induction of *Xenopus* egg apoptosis, the suppressive effects of G6P could be seen at the level of cytochrome c release (Figure 1A, lower panel). Moreover, the inhibitory effect of G6P on caspase activation and cytochrome c release could be overridden by addition of truncated Bid (tBid) to forcibly promote cytochrome c release (Figure 1C). These data indicate that G6P-containing extracts were still capable of activating caspases and that the observed block to apoptosis following G6P treatment occurred upstream of or at the level of the mitochondria.

Pentose-Phosphate-Pathway Intermediates Suppress Cell Death

In *Xenopus* eggs/oocytes, G6P is used for glycogen deposition or is metabolized through the pentose phosphate pathway. Indeed, the fate of radiolabeled G6P has been followed in amphibian oocytes, and little is metabolized by glycolysis (Dworkin and Dworkin-Rastl, 1989). Consistent with this, we found that glycolytic intermediates, including glyceraldehyde-3-phosphate and pyruvate, had no effect on caspase-3 activation in egg extracts (Figure 1D). In contrast, 6-phosphogluconate, a pentose-phosphate-pathway intermediate, could mirror the ability of G6P to inhibit apoptosis (Figure 1E). These data suggested that some feature of pentose-phosphate-pathway operation could suppress apoptosis. A key metabolic by-product of the pentose phosphate pathway is NADPH, produced when G6P is metabolized to 6-phosphogluconolactone and 6-phos-

(B) Extracts treated as in (A) were supplemented with sperm nuclei and stained with Hoechst dye to detect DNA by fluorescence microscopy. Shown are representative nuclei at the indicated times.

(C) Truncated Bid (tBid) was added to extract treated with G6P. Caspase-3 activity and cytochrome c release were measured as in (A).

(D) Buffer, glyceraldehyde-3-phosphate (G3P), pyruvate, or G6P was added to extract, and caspase-3 activity was measured.

(E) Pentose phosphate intermediates (NADPH, 6-phosphogluconate) or G6P was added to extract, and caspase-3 activity was measured.

(F) Malate was added to extracts, and caspase-3 activity and cytochrome c release were measured.

(G) A superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) or a precursor to glutathione, N-acetyl-L-cysteine (NAC), was added to extracts, and caspase activity was measured.

phogluconate is converted to ribulose-5-phosphate. Hypothesizing that production of NADPH might be the relevant consequence of G6P addition for apoptotic inhibition, we sought to generate NADPH using an alternative route and to examine the effects of direct NADPH addition to extracts. Addition of malate, which promotes NADPH production in concert with malic enzyme, potently inhibited mitochondrial cytochrome c release and caspase activation in the egg extract (Figure 1F). Moreover, despite its relative instability in solution, NADPH could markedly suppress caspase activation (Figure 1E). These data suggest that G6P flux through the pentose phosphate pathway, leading to NADPH production, can potently suppress egg extract apoptosis. Although NADPH can act as a reducing agent, we do not believe that it is this feature of NADPH function that is responsible for apoptotic suppression as neither the SOD mimetic MnTBAP, the electron scavenger NAC, nor other reducing agents (TEMPO or reduced glutathione) could suppress extract apoptosis (Figure 1G and data not shown).

Pentose-Phosphate-Pathway Inhibition Induces Apoptosis in Egg Extracts and Oocytes

Since hyperstimulation of the pentose phosphate pathway or NADPH addition inhibited caspase activation, we speculated that inhibition of the endogenous pentose phosphate pathway in egg extracts should accelerate apoptosis, particularly if the spontaneous extract apoptosis relied upon nutrient depletion during incubation. To test this, we treated extracts with the G6P dehydrogenase inhibitor, dehydroisoandrosterone (DHEA), to prevent entry of endogenous G6P into the pentose phosphate pathway (Schwartz and Pashko, 2004). This treatment accelerated apoptosis, largely eliminating the lag normally seen before caspase activation, as would be expected if pentose-phosphate-pathway function were important for extract "survival" (Figure 2A). We also examined extracts to determine whether endogenous G6P was depleted over time upon incubation. For this purpose, extracts were incubated at room temperature and assayed for caspase activation (Figure 2B, top panel). Aliquots of extract withdrawn at the start of the incubation (0 hr) or just prior to caspase activation (2 hr) were incubated with a vast excess of G6P dehydrogenase and NADP and assayed over time for generation of NADPH, which, under these circumstances, depended solely on the available G6P. As shown in Figure 2B (middle panel), G6P levels (as measured by the rate of NADPH generation) dropped substantially within the 2 hr of incubation. Note also that NADPH generation remained high in extracts supplemented with excess G6P (Figure 2B, lower panel).

We wished to extend these observations to whole oocytes to determine if the pentose phosphate pathway was important for suppressing death in these intact cells. For this, we developed a visual assay for oocyte death. Normally, oocytes contain a light-colored vegetal half and a dark, uniformly pigmented animal half. When induced to undergo maturation with progesterone, oocytes exhibit a white spot resulting from nuclear-envelope breakdown and clearance of surrounding pigment granules. We noted that a similar but

considerably larger white spot appeared at the animal poles of oocytes injected with cytochrome c to induce caspase activation (C. Holley, personal communication). These oocytes did not exhibit elevated Cdc2/cyclin kinase activity characteristic of maturing oocytes but rather showed high levels of caspase-3 activity (data not shown). Morphological events of apoptosis have been observed in oocytes by others, both in *Xenopus* and in other species (Bagowski et al., 2001; Bhuayan et al., 2001; Braun et al., 2003; Demirci et al., 2001, 2002, 2003; Voronina and Wessel, 2001). Interestingly, we had observed that oocytes taken freshly out of a virgin female frog are remarkably refractory to apoptosis. With the idea that this resistance might stem from continual functioning of the pentose phosphate pathway, we soaked fresh oocytes in a DHEA-containing solution and monitored the appearance of large white spots at the animal poles. As shown in the micrographs in Figure 2C and the graph in Figure 2D, 200 μ M DHEA rapidly killed oocytes, while control treated oocytes remained healthy. These data were corroborated in experiments where cytochrome c was released from mitochondria in DHEA-treated but not control oocytes (Figure 2E). Importantly, this acceleration of oocyte death occurred at even lower doses of DHEA (100 μ M) and could be reversed by simultaneous treatment with the cell permeant D-methylmalate, which would be expected to promote NADPH generation within oocytes (Figure 2F). These data demonstrate that pentose-phosphate-pathway inhibition promotes rapid oocyte death, suggesting that this pathway is normally critical for oocyte viability.

Caspase-2 Is a Target of NADPH-Mediated Apoptotic Inhibition

Since G6P (or NADPH) could inhibit apoptosis, we wished to identify the target of this inhibition. As mentioned above, genetic analyses in mice had implicated caspase-2 as an important constituent of oocyte apoptotic pathways (Bergeron et al., 1998). To determine if caspase-2 was affected by G6P, we first examined processing of radiolabeled caspase-2 during spontaneous apoptosis. As shown in Figure 3A, addition of G6P, pentose-phosphate-pathway intermediates, malate, or NADPH all prevented caspase-2 processing, while glycolytic intermediates had no effect. Moreover, G6P inhibited endogenous caspase-2 processing, as detected by immunoblotting (Figure 3H). Caspase-2 processing in untreated extracts was inhibitable by the baculoviral caspase inhibitor p35 and the peptide inhibitor of caspase-2, VDVAD-fmk, but was not inhibited by Bcl-xL, which prevented cytochrome c release and caspase-3 activation (Figures 3B, 3C, and 3E). Interestingly, treatment with p35 or VDVAD-fmk also inhibited cytochrome c release, consistent with the involvement of a caspase (potentially caspase-2) upstream of mitochondria in this system (Figures 3D and 3E). Moreover, neither caspase-2 processing nor cytochrome c release could be inhibited by the pancaspase inhibitor, z-VAD-fmk, consistent with previous reports that caspase-2 is markedly less sensitive to zVAD than other caspases (Garcia-Calvo et al., 1998) (Figures 3B and 3D). Thus, G6P could inhibit the z-VAD-insensitive, p35- and

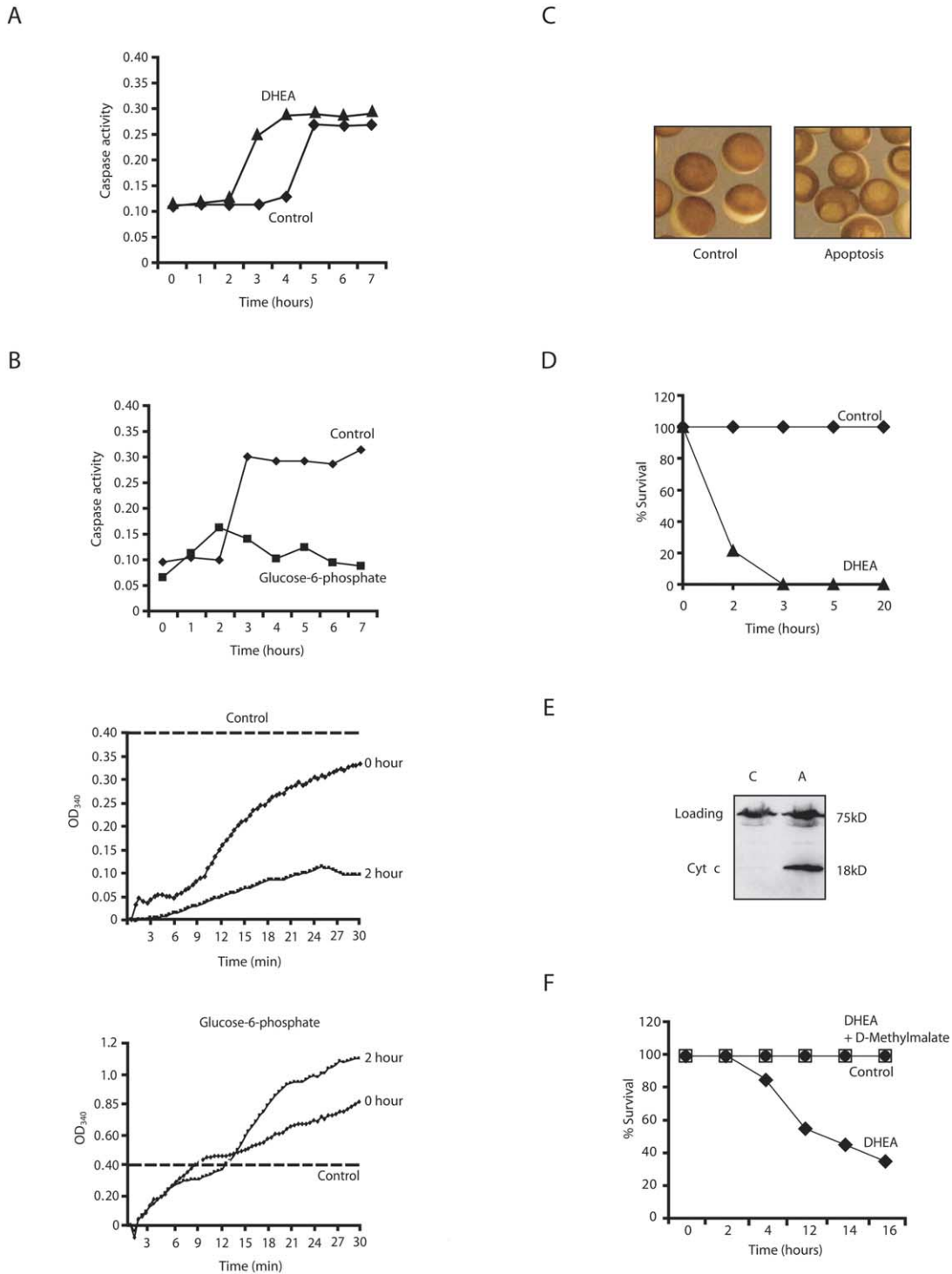


Figure 2. Inhibition of G6P Dehydrogenase Induces Apoptosis

(A) Extracts supplemented with either dehydroisoandrosterone (DHEA) or buffer were analyzed for caspase-3 activity. Shown is a representative experiment repeated on three separate batches of oocytes with similar results.

(B) Upper panel: extract was incubated at room temperature and assayed for caspase activity. Middle panel: samples withdrawn at 0 and 2 hr of incubation were assayed for G6P levels by monitoring NADPH production over time (absorbance at 340 nm) in the presence of excess G6P dehydrogenase and NADP. Lower panel: addition of excess G6P maintained NADPH production at all times tested. Dotted line indicates the fact that the scales are different in the extracts \pm G6P, as NADPH was produced at much higher rates in the presence of excess G6P.

(C) Oocytes treated with DHEA or buffer are shown in representative micrographs.

(D) Percent survival of oocytes treated with buffer or DHEA.

(E) Cytochrome c release was measured in parallel by filtering aliquots of lysed oocytes through a 0.1 μ m microfilter and analyzing the filtrate by anti-cytochrome c immunoblotting.

(F) Percent survival of oocytes treated with buffer, DHEA, or DHEA and cell-permeable D-methyl malate.

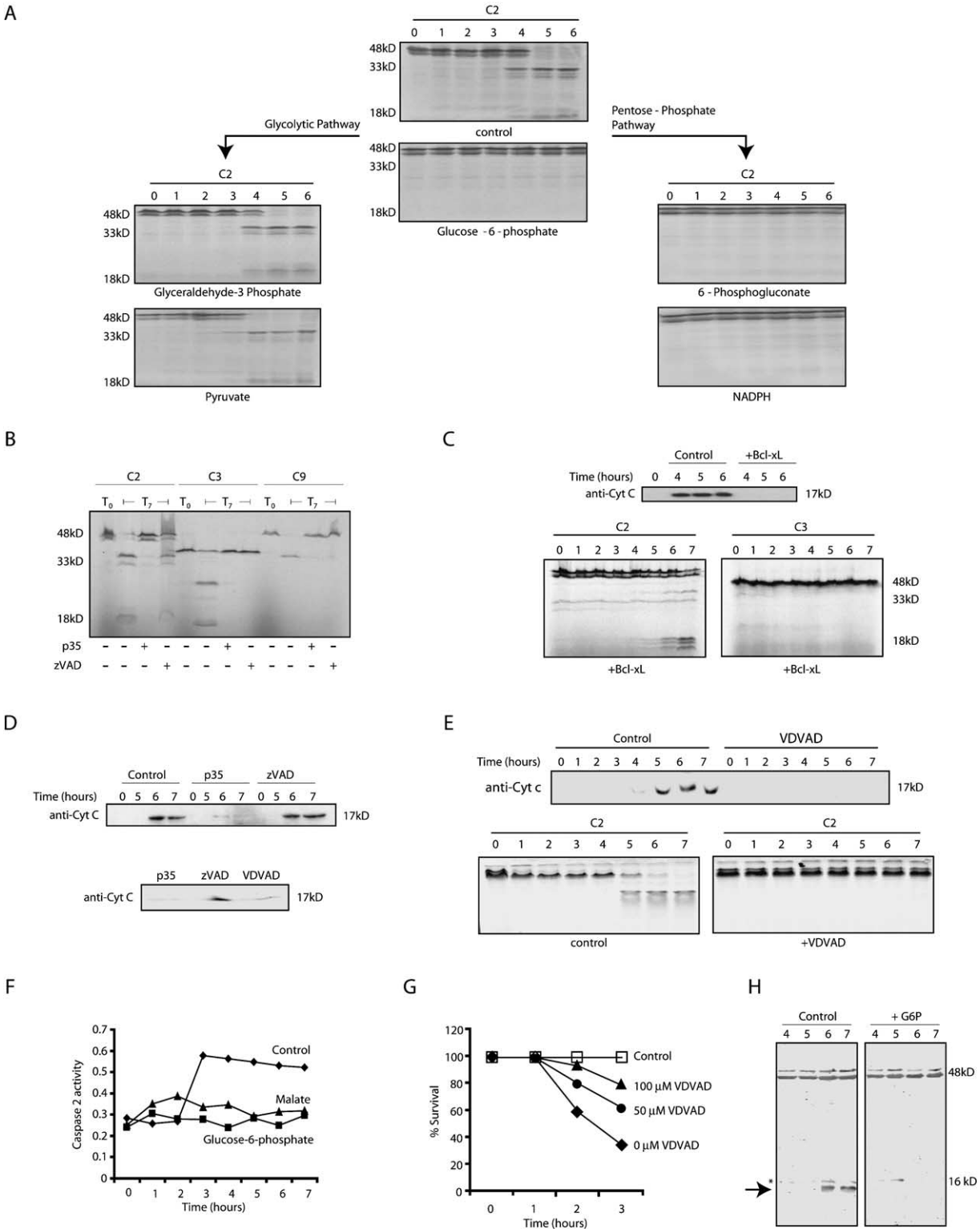


Figure 3. Pentose Phosphate Metabolites Act Upstream of Cytochrome c Release

(A) Egg extract was incubated with metabolic substrates, and samples were analyzed for processing of in vitro-translated caspase-2.
 (B) Extract was incubated at room temperature in the presence of caspase inhibitors (zVAD, 100 μ M; VDVAD, 100 μ M; or p35) and radiolabeled procaspases. Samples from time 0 (T_0) and 7 hr (T_7) were analyzed by SDS-PAGE for processing of in vitro-translated caspase-2 (C2), -3 (C3), and -9 (C9).
 (C) Extract was incubated at room temperature \pm Bcl-xL, and samples were analyzed every hour for in vitro-translated C2 and C3 processing by SDS-PAGE and for cytochrome c release.

VDVAD-sensitive processing of caspase-2, most likely catalyzed by caspase-2 itself. Consistent with these observations, G6P blocked cleavage of the peptide substrate VDVAD-pNA (Figure 3F). In addition, VDVAD, which inhibited both caspase-2 processing and cytochrome c release in egg extracts, could block the DHEA-induced apoptosis of oocytes (Figure 3G). These data agree with the idea that caspase-2 is a critical inhibitory target of pentose-phosphate-pathway metabolites.

G6P and NADPH Prevent Activation of Caspase-2 by RAIDD

Caspase-2 is activated by oligomerization through binding of its CARD to adaptors (Duan and Dixit, 1997). Although our data suggested that G6P and NADPH could inhibit caspase-2, it was not clear that this inhibition was exerted at the level of caspase-2 rather than indirectly through inhibition of an upstream signaling component. To address this, we wished to promote direct caspase-2 oligomerization and determine if this, too, could be inhibited by G6P. The adaptor RAIDD binds to the caspase-2 CARD and has been implicated in caspase-2 activation in several settings. We have found that GST-RAIDD bound to glutathione Sepharose can, upon addition to egg extracts, directly promote caspase-2 processing (Figure 4A). Even this caspase-2 autoprocessing was inhibited by G6P or NADPH, suggesting that NADPH can interfere with direct RAIDD-induced caspase-2 activation (Figure 4A). Moreover, G6P or NADPH decreased the total amount of caspase-2 that could be retrieved from extracts in association with Sepharose-RAIDD, suggesting that these metabolites could interfere with adaptor-caspase-2 complex formation (Figure 4B). Similar results were obtained using *in vitro*-translated human caspase-2, whose processing was also inhibited by G6P and VDVAD in the egg extract (Figures 4C–4E). These data suggest that metabolic inhibition of caspase-2 can be exerted directly at the level of oligomerization/activation.

G6P and NADPH Promote Caspase-2 Phosphorylation by CaMKII

Since G6P and NADPH blocked activation of caspase-2 by RAIDD, we suspected that either these proteins were posttranslationally modified to prevent their interaction or a CARD binding inhibitor was recruited in the presence of G6P. Experiments to detect proteins bound to either GST-RAIDD or the GST-caspase-2 prodomain did not reveal any potential inhibitors bound only in the presence of G6P (data not shown). Therefore, we speculated that the CARD-containing regions

of these proteins might be posttranslationally modified. Thus, we looked to see whether GST-RAIDD or the GST-caspase-2 prodomain was phosphorylated in G6P-treated extracts. Although we saw no evidence of RAIDD phosphorylation, G6P addition greatly stimulated phosphorylation of the caspase-2 prodomain, derived from either human or *Xenopus* caspase-2 (Figure 5A and data not shown). The caspase-9 prodomain was not phosphorylated under these conditions (Figure 5A). Note that prolonged exposure of kinase-assay gels revealed basal phosphorylation of the caspase-2 prodomain in untreated extracts, but this was markedly stimulated by G6P (Figures 5A and 5B). Moreover, endogenous pro-caspase-2 immunoprecipitated from egg extracts incubated with [γ -³²P]ATP was also phosphorylated, and this phosphorylation was stimulated by G6P (Figure 5C).

To identify the kinase (or kinases) phosphorylating the caspase-2 prodomain, we repeated the experiment in Figure 5B in the presence of a battery of kinase inhibitors. Inhibition of several kinases previously implicated in apoptotic regulation, including PKC, PKA, AKT, and MEK, had no effect on G6P-stimulated caspase-2 phosphorylation (Figure 5D and data not shown). However, UCN-01 blocked phosphorylation of the caspase-2 prodomain (Figure 5D). Although UCN-01 is a broad-spectrum kinase inhibitor, it has been used extensively in *Xenopus* egg extracts to inhibit both Chk1 and CaMKII (Graves et al., 2000; Hutchins et al., 2003). The inability of caffeine, an inhibitor of Chk1-activating kinases, to override G6P suggested Chk1 might not play a role in this pathway (Figure 5D). Indeed, Chk1 immunodepletion produced no loss of G6P-stimulated prodomain phosphorylation (Figure 5E). Conversely, treatment of egg extracts with the general CaMK inhibitor, KN93, led to a substantial loss of G6P-stimulated caspase-2 phosphorylation (Figure 5F). Moreover, depletion of extracts using calmodulin Sepharose or treatment of extracts with the calmodulin inhibitors W7 or W13 markedly diminished G6P-triggered phosphorylation of caspase-2, consistent with the involvement of a calmodulin binding protein in this pathway (Figures 5G and 6D). Moreover, excess calcium stimulated and EGTA inhibited phosphorylation of the caspase-2 prodomain, consistent with the involvement of a calcium-regulated kinase (Figure 6C).

To determine if CaMKs could phosphorylate the caspase-2 prodomain, we incubated the GST prodomain *in vitro* with [γ -³²P]ATP and either CaMKI, CaMKII, or CaMKIV. Only CaMKII could phosphorylate the recombinant caspase-2 prodomain (CaMKII could also phosphorylate the human caspase-2 prodomain), although all kinases were active against their model peptide sub-

(D) Upper panel: egg extract was incubated at room temperature and treated with or without p35 and zVAD-fmk. Samples were taken every hour, mitochondria were removed by filtration, and the remaining cytosol was immunoblotted with anti-cytochrome c antibody. Lower panel: same as above, except a 7 hr time point is shown also in the presence of VDVAD.

(E) Upper panel: samples were treated as in (D), but in the presence of VDVAD. Lower panel: processing of radiolabeled caspase-2 was monitored \pm VDVAD.

(F) Cleavage of VDVAD-pNA was monitored in extracts containing buffer, malate, or G6P.

(G) Oocytes were treated with DHEA as in Figure 2A, with or without prior soaking in buffer containing the indicated concentrations of VDVAD.

(H) Cleavage of endogenous caspase-2 was monitored \pm G6P by anti-caspase-2 immunoblotting using a mix of C20 and H119 antibodies from Santa Cruz, which recognize only the full-length 48 kDa protein and the 12 kDa cleavage fragment, indicated by the arrow. The asterisk indicates a background band present in all lanes.

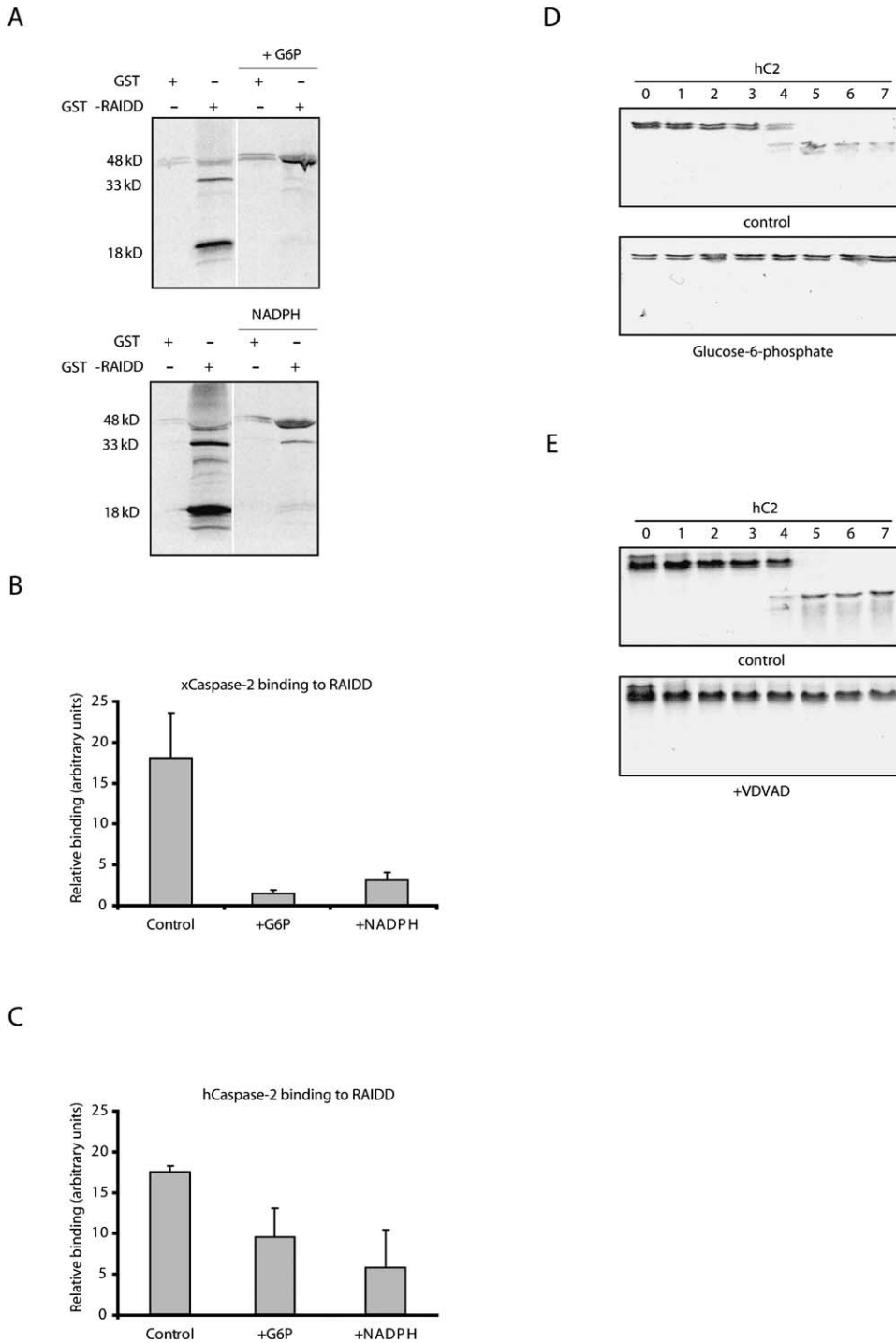


Figure 4. Antiapoptotic Effects of NADPH Are Exerted at the Level of Caspase-2

Error bars indicate the standard deviation of triplicate samples.

(A) GST-RAIDD or GST bound to glutathione Sepharose was dipped into extract \pm NADPH or G6P in the presence of radiolabeled procaspase-2. Beads were retrieved by centrifugation, boiled in SDS sample buffer, and resolved by SDS-PAGE for autoradiography. Procaspase-2 not bound to beads was also unprocessed in the presence of G6P or NADPH (data not shown).

(B) GST-RAIDD Sepharose was dipped into extracts with *in vitro*-translated caspase-2 in the presence and absence of NADPH. Beads were washed extensively, and radiolabeled caspase-2 binding was measured by phosphorimager.

(C) The same experiment in (B) was repeated using *in vitro*-translated human, rather than *Xenopus*, caspase-2.

(D) Processing of radiolabeled human procaspase-2 supplemented into egg extracts was monitored in the presence and absence of G6P.

(E) The same as in (D), but \pm VDVAD.

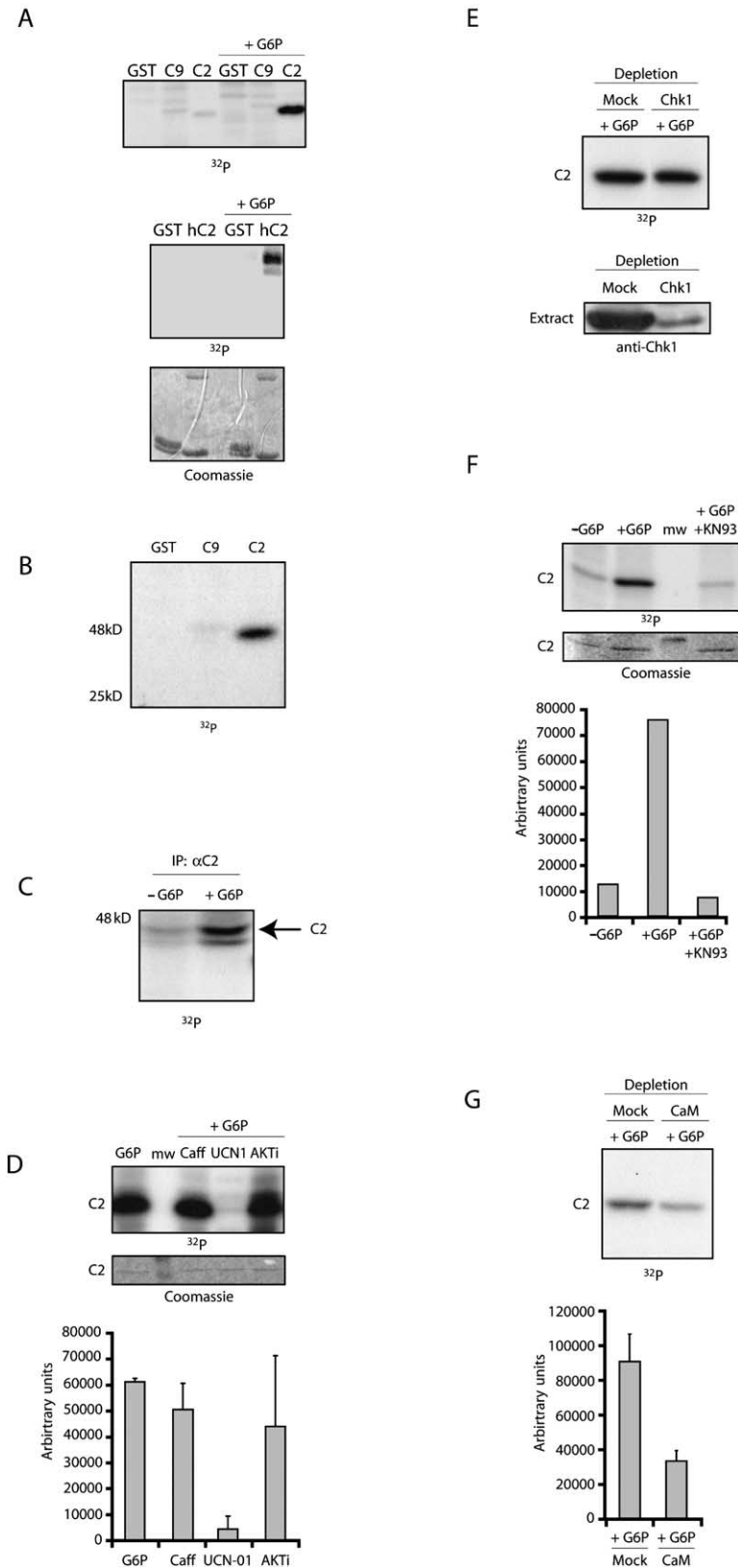


Figure 5. G6P/NADPH Induces Phosphorylation and Inactivation of Caspase-2

Error bars indicate the standard deviation of triplicate samples.

(A) Upper panel: the GST prodomain of *Xenopus* caspase-2 (C2) or -9 (C9) was bound to glutathione Sepharose and dipped into cytosolic extracts \pm G6P and [γ - 32 P]ATP. Bead bound proteins were washed and analyzed by SDS-PAGE and phosphorimaging. Lower panel: similar results were obtained with the human caspase-2 prodomain.

(B) The experiment in (A) was repeated minus G6P and overexposed to detect basal caspase-2 phosphorylation.

(C) Anti-*Xenopus* caspase-2 prepared against a C-terminal 20 amino acid peptide or preimmune antibodies were bound to protein A beads and dipped into extract \pm G6P and [γ - 32 P]ATP. Samples were resolved by SDS-PAGE and detected by autoradiography.

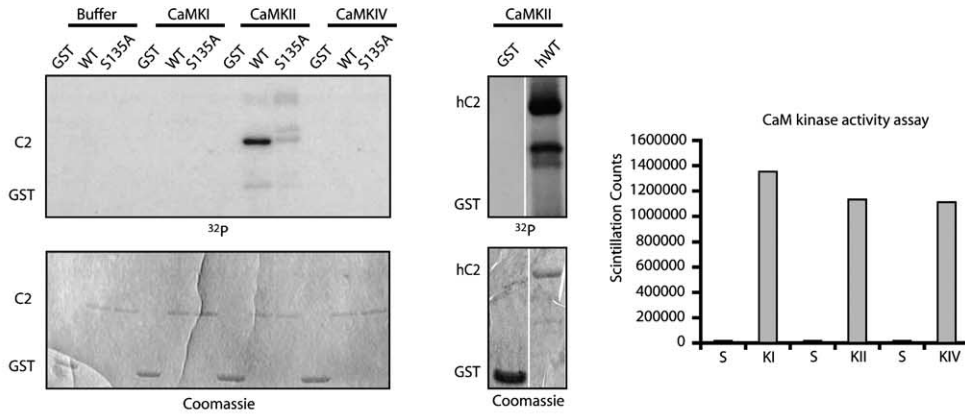
(D) The experiment in (A) was repeated with 5 mM caffeine, UCN-01 (1 μ M), or Akt inhibitor (20 μ M). Sepharose bound GST proteins were washed and analyzed by SDS-PAGE and quantitated by phosphorimaging.

(E) Egg extracts were depleted with anti-Chk1 or control antibody and incubated with G6P. Samples were washed and analyzed by SDS-PAGE and autoradiography (upper panel) or anti-Chk1 immunoblotting (lower panel).

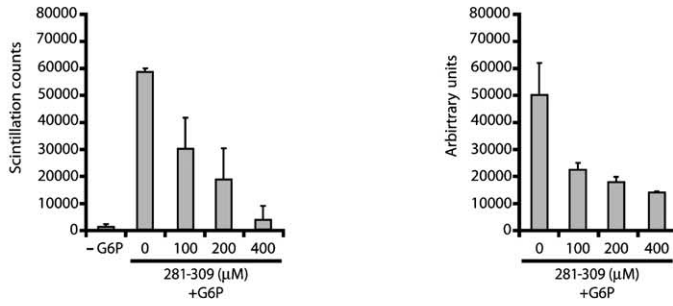
(F) The experiment in (A) was repeated with 5 μ M KN-93. Sepharose bound GST proteins were washed and analyzed by SDS-PAGE and quantitated by phosphorimaging.

(G) Egg extracts were depleted with calmodulin Sepharose or control Sepharose and then incubated with G6P. Caspase-2 prodomain phosphorylation was monitored as in (A).

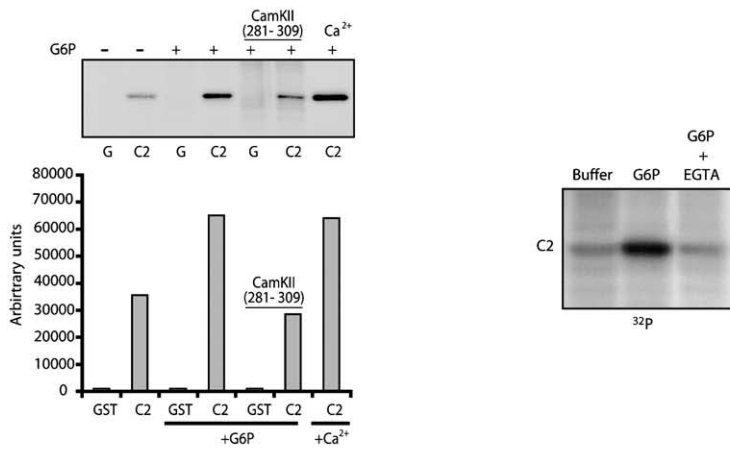
A



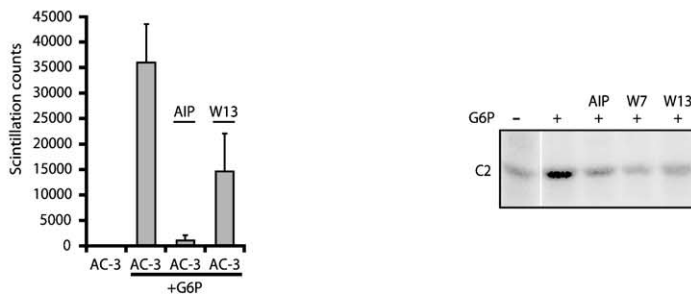
B



C



D



strates (Figure 6A, right). More importantly, two specific peptide inhibitors of CaMKII (Ca²⁺/calmodulin kinase II inhibitor 281-309 and autocalmitide-2-related inhibitory peptide) could prevent G6P-induced phosphorylation of both a model CaMKII substrate and the caspase-2 prodomain (Figures 6B–6D). These data strongly suggest that CaMKII mediates G6P-stimulated caspase-2 phosphorylation.

Abrogation of Caspase-2 Phosphorylation Prevents Metabolite-Mediated Survival

Given the involvement of CaMKII in caspase-2 phosphorylation, we scanned the caspase-2 prodomain for sequence motifs characteristic of CaMK phosphorylation sites and identified Ser73 and Ser135 as candidate sites; these were individually changed to Ala. When we assayed these mutants in egg extracts, we found that both G6P and NADPH-stimulated prodomain phosphorylation were abrogated by the S135A mutation but were unaffected by Ser73 alteration (Figure 7A). Ser135 mutation also prevented in vitro phosphorylation by CaMKII (Figure 6A). To determine if this site was critical for metabolic suppression of caspase-2, we produced mRNA and in vitro-translated proteins for full-length wild-type, S135A, and S73A caspase-2. As shown in Figure 7B, the S135A protein was processed even in the presence of G6P. More importantly, mutation of Ser135 to Ala not only overrode the G6P-mediated block to spontaneous caspase-3 activation in egg extracts but also overrode the inhibition of RAIDD-induced processing and binding to caspase-2 (Figures 7C and 7D and data not shown). Strikingly, the S135A mutant also induced apoptosis in oocytes (Figure 7E). Consistent with a role for CaMKII in suppressing caspase-2-mediated death of intact oocytes, prior injection of the CaMKII peptide inhibitor allowed efficient cell-death induction by subsequent injection of the wt procaspase-2 mRNA (Figure 7F). These data demonstrate that metabolic suppression of caspase-2 in eggs and oocytes is exerted through CaMKII-mediated phosphorylation of the caspase-2 prodomain.

Discussion

Although it has long been appreciated that oocytes die by apoptosis, the signaling pathways governing death

of these cells have not yet been fully described (Tilly, 2001). Here we have provided evidence for a molecular pathway linking metabolic processes with the survival of oocytes/eggs. The target of this pathway, caspase-2, has been previously implicated in oocyte apoptosis (Bergeron et al., 1998). We have found that stimulation of the pentose phosphate pathway, leading to NADPH generation, can restrain caspase-2 via the action of CaMKII, thereby promoting oocyte/egg survival.

Oocyte/Egg Metabolic Pathways and Apoptosis

Studies on metabolic flux in *Xenopus* oocytes have demonstrated that glycolysis is low in these cells and that the majority of carbon that could enter glycolytic pools is, instead, rapidly converted to glycogen (Dworkin and Dworkin-Rastl, 1989). However, this glycogen is not metabolized until after gastrulation and does not serve as an energy source to support early embryogenesis (Cohen, 1954; Jaeger, 1945). Rather, it has been proposed that amino acids derived from yolk-protein breakdown can be partially oxidized to malate in mitochondria, which is then oxidized to oxaloacetate and converted to phosphoenolpyruvate to generate G6P (Dworkin and Dworkin-Rastl, 1989; Kovacevic and Morris, 1972; Maller, 1985; Reitzer et al., 1979). According to this scenario, yolk proteins serve as the source of energy for the oocyte and early embryo. Our observations suggest that maintenance of G6P levels from adequate yolk-protein stores can prevent death of oocytes and eggs. It is worth noting that the amount of G6P required to achieve full inhibition of apoptosis in egg extracts is quite high (i.e., greater than 2.5 mM). Consistent with previous measurements of radiolabeled G6P flux in amphibian oocytes, we believe that the vast majority of the added G6P is shunted directly into glycogen synthesis and that only a small amount is available to enter the pentose phosphate pathway (Dworkin and Dworkin-Rastl, 1989; Ureta et al., 2000). Within the animal, such levels could be maintained as long as yolk-protein stores were continually feeding G6P production.

Because stimulation of pentose-phosphate-pathway metabolism, malate, or NADPH could substitute for G6P to inhibit apoptosis, our data suggest that it is NADPH generation that transmits the antiapoptotic signal. Addition of NADH or stimulation of NADH production did not have similar effects, pointing to the speci-

Figure 6. CaMKII Phosphorylates the Prodomain of Caspase-2

Error bars indicate the standard deviation of triplicate samples.

(A) Left: in vitro kinase assays were performed with either buffer, CaMKI, CaMKII, or CaMKIV and the GST-fused prodomain of caspase-2 (wt or S135A). Prodomain phosphorylation was analyzed by SDS-PAGE, and the gel was stained with Coomassie blue to show equal loading. Middle: the same assay was performed using CaMKII and the human caspase-2 prodomain. Right: the activity of each kinase was tested against its cognate peptide substrate and compared to substrate (S) alone in the absence of kinase.

(B) Left panel: extract was incubated \pm G6P, [γ -³²P]ATP, and the peptide substrate Syntide-2 (Syn2) \pm various doses of a CaMKII peptide inhibitor (CaMKII 281-309). Substrate trapped on filter paper was washed extensively and scintillation counted. Right panel: the same extracts were tested using the GST-caspase-2 prodomain as substrate by the same protocol as in Figure 5A. Shown is the quantitation of GST-caspase-2 prodomain phosphorylation from three gels exposed to the indicated inhibitor doses.

(C) Upper left panel: a representative gel showing inhibition of caspase-2 phosphorylation by the 281-309 peptide (as in the right-hand panel of [B]) and Ca²⁺-stimulated phosphorylation of GST (G) and the GST prodomain of caspase-2 (C2). Lower left panel: quantitation of the gel. Right panel: the assay in Figure 5A was repeated in the presence of EGTA.

(D) Using the same conditions as in (B), another CaMKII inhibitor (5 μ M AIP) was tested using its peptide substrate AC-3 (left panel). Right panel: calmodulin inhibitors (10 μ M W13 and 10 μ M W7) were tested in the same manner as in (C) using the GST-caspase-2 prodomain as substrate.

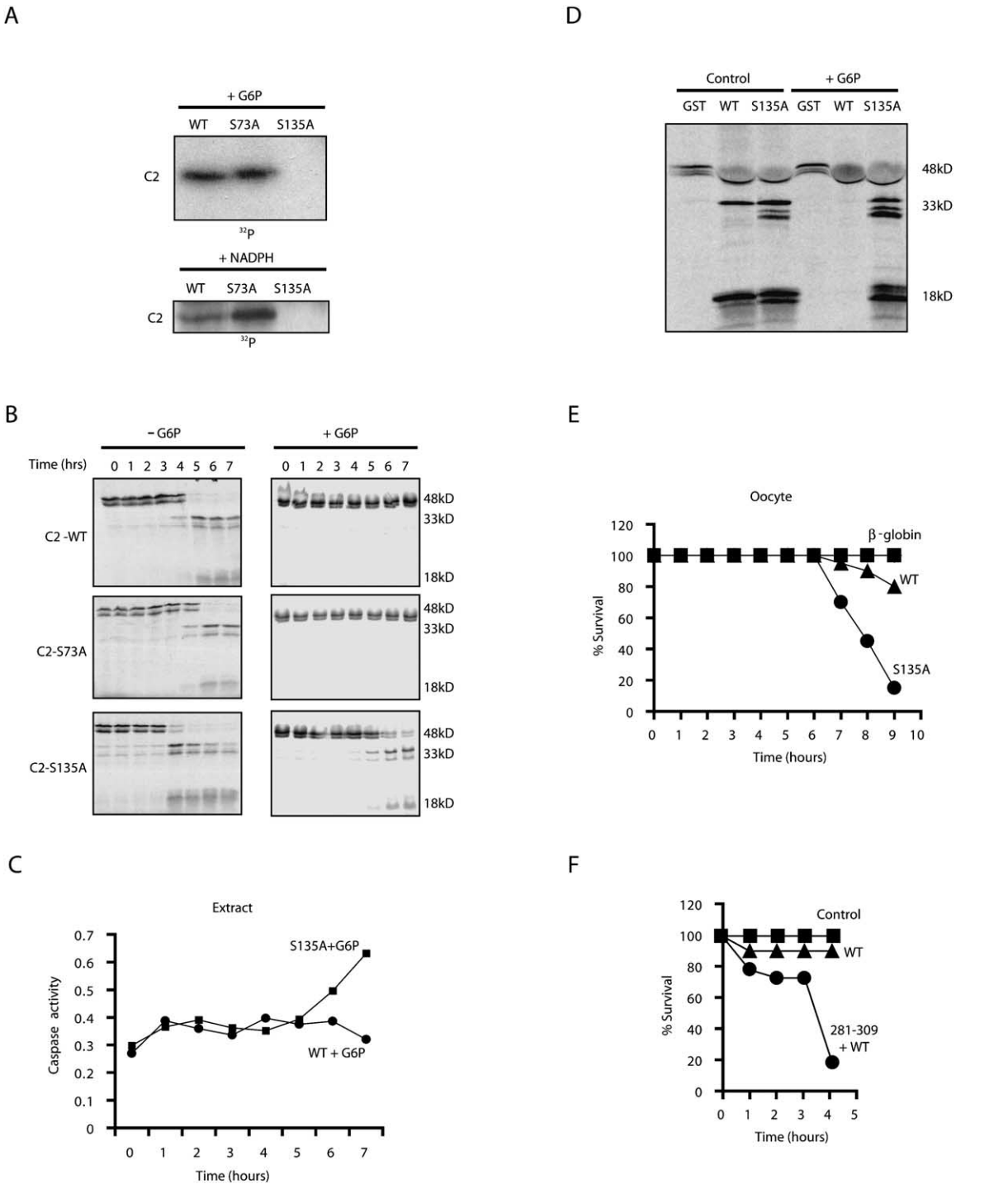


Figure 7. Mutation of S135 of Caspase-2 to Ala Abrogates the Protective Effects of NADPH

(A) The wt, S73A, and S135A caspase-2 prodomains fused to GST were bound to glutathione Sepharose and dipped into cytosolic extracts ± G6P, ± NADPH, and [γ -³²P]ATP. Sepharose bound proteins were washed, eluted with sample buffer, and resolved by SDS-PAGE.

(B) Extracts supplemented with wt, S135A, or S73A radiolabeled procaspase-2 were incubated at room temperature and treated with or without G6P. Samples were taken at the indicated times and analyzed by SDS-PAGE.

(C) Procaspase-2 S135A or wt mRNA and G6P were added to a translationally competent egg extract, and samples were taken to measure cleavage of Ac-DEVD-pNA.

(D) GST-RAIDD was added to extracts with in vitro-translated wt or S135A procaspase-2 ± G6P for 1 hr. Samples were taken to measure caspase-2 processing by SDS-PAGE.

(E) Oocytes were injected with mRNA encoding wt or S135A procaspase-2 or β-globin. Cell death was quantitated as in Figure 2. Shown is a representative experiment repeated on three oocyte batches with similar results.

(F) Oocytes were injected with the 281-309 CaMKII inhibitory peptide, subsequently injected with wt procaspase-2-encoding mRNA, and monitored as in (E).

ficity of our results (Figures 1D and 3A and data not shown). Also consistent with the involvement of pentose-phosphate-pathway operation are our observations that inhibition of G6P dehydrogenase could accelerate caspase activation in both egg extracts and oocytes. Although the initiator of spontaneous death in the *Xenopus* egg system has not been previously identified, our data suggest that an active signaling system is engaged to keep these cells alive and that the default pathway, when key metabolites cannot be maintained, is cell death.

Although it is attractive to speculate that nutrient stockpiles serve as a timer for oocyte survival, changes in pentose-phosphate-pathway flux might also result from age- or hormone-related alterations in enzyme activities. Indeed, age-related decreases in G6P dehydrogenase activity have been observed in mouse oocytes, and it has been suggested that, even if such oocytes survive, they may develop poorly postfertilization (de Schepper et al., 1987).

Caspase-2 Is a Target for the Antiapoptotic Action of NADPH

A previous report that caspase-2 was required for oocyte apoptosis led us to investigate caspase-2 as a potential target of metabolic regulation (Bergeron et al., 1998). In addition to our observation that caspase-2 processing was inhibited by G6P or NADPH, the fact that a caspase-2 mutant resistant to phosphorylation could override the effects of G6P in egg extracts as well as the natural resistance of intact oocytes to apoptosis strongly suggests that caspase-2 is an important target of metabolic survival pathways.

It is not clear how caspase-2 is activated in oocytes. However, based on the ability of RAIDD-mediated activation to be regulated by NADPH and G6P, we hypothesize that an endogenous adaptor protein binding to the caspase-2 prodomain promotes activation; phosphorylation of the prodomain could block access of the adaptor, preventing activation. Consistent with such a possibility, caspase-2 binding to GST-RAIDD was substantially reduced by G6P and NADPH addition.

CaMKII Phosphorylates and Inhibits Caspase-2

Based on calmodulin Sepharose depletion, kinase assays, and inhibitor studies, we conclude that CaMKII maintains caspase-2 phosphorylation in response to pentose-phosphate-pathway operation. Although this phosphorylation was stimulated by G6P, we note that basal phosphorylation could be observed upon longer exposure of kinase assays. We suggest that basal phosphorylation can keep caspase-2 suppressed and that dephosphorylation occurs prior to entry of extracts into apoptosis. Consistent with this, the GST-caspase-2 prodomain prephosphorylated with CaMKII loses its radioactive labeling immediately preceding caspase-3 activation in the extract (L.K.N. and S.S.M., unpublished data). We were able to maintain this phosphorylation through the simple provision of metabolites.

Although our data demonstrate that CaMKII activity is required for maintaining caspase-2 suppression in response to nutrients, it is not yet clear how NADPH levels are translated into sustained phosphorylation. It

is possible that a caspase-2-directed phosphatase is regulated to antagonize constitutive CaMKII-mediated caspase-2 phosphorylation supported by ambient calcium levels in the egg/oocyte. However, the fact that CaMKII phosphorylation of model peptides (which we might not expect to be targeted for dephosphorylation by the same phosphatases as the prodomain) was increased following G6P/NADPH treatment suggests that these metabolites may increase CaMKII activation or activity. It is possible that a phosphatase controlling CaMKII activity (through regulation of the CaMKII-activating phosphorylation at Thr286 as observed in other settings) is regulated by NADPH (Blitzer et al., 1998). It is not clear whether pathways regulating calcium storage or utilization may also be regulated by NADPH to control CaMKII activation. In any event, the spontaneous activation of the S135A mutant caspase-2 (even in cytosol lacking mitochondria; data not shown) demonstrates that maintenance of this phosphorylation is critical to keep eggs/oocytes alive. Because processing of the S135A mutant was slightly slower in the presence of G6P (see Figure 7B), it may be that additional modes of caspase-2 regulation (or targets other than caspase-2) contribute to G6P-mediated apoptotic suppression. However, S135 is clearly a major target of this regulatory pathway. Moreover, because there is still a lag time until apoptosis in the presence of the S135A mutant, pathways in addition to the CaMKII-caspase-2 pathway may contribute to the normal suppression of apoptosis in oocytes/eggs. Although further experiments will be required to fill in the precise regulatory details, this work has revealed key elements of a previously unknown pathway linking oocyte metabolism to caspase-2 through the action of CaMKII. This work may help to explain why oocytes are lost through apoptosis as females age, thereby providing potential therapeutic targets for the maintenance of oocyte viability and fertility.

Experimental Procedures

Preparation of *Xenopus* Oocytes and Extracts

Egg extracts were prepared as in Smythe and Newport (1991). Stage VI oocytes were prepared from ovaries manually excised from PMSG-primed frogs, digested with 2.8 units of liberase in OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES [pH 7.5]) for 1.5 hr at room temperature, washed extensively with OR-2 buffer, and stored in OR-2 buffer + 1% fetal bovine serum + 0.2% gentamicin at 18°C.

Caspase Assays and Metabolite Addition

To measure caspase activity, 3 μ l extract aliquots were incubated with 90 μ l DEVDase buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the substrate Ac-DEVD-pNA (200 μ M) at 37°C for 30–60 min. Absorbance was measured at 405 nm. For some experiments, extracts were treated with G6P (2.5–10 mM), NADPH (5–10 mM), or other metabolites (all 10 mM). Other reagents were used at 100 μ M (zVAD), 500 nM (Bcl-xL), and 25 nM (tBid).

Xenopus Oocyte Apoptosis Assays

For pentose-phosphate-pathway inhibition studies, ~50 stage VI *Xenopus* oocytes were incubated with vehicle, 100–200 μ M DHEA, and/or 2.5 mM methyl malate in OR-2 buffer at 16°C. For caspase-2 potency studies, 40 ng of wt, S73A, and S135A mutant caspase-2 or β -globin-encoding mRNA was injected into oocytes and incubated in OR-2 buffer at 16°C. To measure cytochrome c release,

oocytes were manually lysed in 10 μ l lysis buffer/oocyte (20 mM HEPES [pH 7.5], 20 mM β -glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM PMSF, 10 μ g aprotinin/leupeptin) and spun at 14,000 \times g for 5 min. Samples were filtered through a 0.1 μ m ultrafree-MC filter and immunoblotted for cytochrome c.

Protein Expression and Reagents

Xenopus caspase-2 was amplified by PCR using the primers 5'-CGCGGATCCATGCTGGGAGGCATGCAGCAAC-3' and 5'-GACGGATCCTCACTTGGGAGCCCGTTGCTTGG-3'. Purified PCR products were digested and cloned into the BglII site of pSP64T, an expression vector with flanking 5' and 3' β -globin UTRs. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate point mutants in caspase-2 in pSP64T. The S73A primers were 5'-GCAGAGCATTGCAGAATGCTGCAAAAGCACGTGGACCCC-3' and its complement. The S135A primers were 5'-GCAGAGAGTACAGGAAGAGGCTATTGATGATGGAGATGG-3' and its complement.

To produce radiolabeled proteins, pSP64T-caspase-2, -3, and -9 templates were translated in TNT reticulocyte lysates (Promega) containing 1 μ Ci/ μ l 35S-Translabel according to manufacturer's instructions. mRNA for microinjection was generated using the Stratagene mRNA capping kit.

The wt, S73A, and S135A caspase-2 prodomains were amplified by PCR using the pSP64T-caspase-2 clones as templates with the primers 5'-CGCGGATCCATGCTGGGAGGCATGCAGCAAC-3' and 5'-TGCTCTAGATCATGGACCATCTCCATCATCAATAG-3'. PCR products were cloned into BamHI and XbaI sites of pGEXKG. pGEXKG caspase-2 and RAIDD proteins were expressed in BL21 *E. coli* as in Evans et al. (1997). GST-BCL-xL was a gift from M. Hardwick. GST-human caspase-9 prodomain was expressed as in Deming et al. (2004).

His-p35-encoding baculovirus was produced using the Invitrogen baculovirus expression system, and proteins were purified on a nickel chelate column, eluted with 200 mM imidazole, and dialyzed overnight into egg lysis buffer (ELB) (250 mM sucrose, 2.5 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 10 mM HEPES [pH 7.7]).

Kinase Assays and Depletions

One to two micrograms of recombinant GST-prodomain fusion proteins were incubated in egg extract with 20 μ Ci [γ - ^{32}P]ATP and various metabolites in the presence or absence of CaMK inhibitors for 1 hr at room temp and retrieved on glutathione Sepharose. Similar extracts were treated with caspase-2 antibody bound to protein A sepharose to precipitate endogenous phosphorylated caspase-2. Samples were washed, eluted with SDS-PAGE sample buffer, and resolved by SDS-PAGE for autoradiography and quantitation by phosphorimager.

For in vitro phosphorylation, recombinant CaM kinases and 1–2 μ g GST prodomains were incubated in kinase buffer (25 mM HEPES [pH 7.5], 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 200 μ M ATP, 20 μ Ci/reaction [γ - ^{32}P]ATP, 1 μ M *A. nidulans* CaM, 1 mM $CaCl_2$, 0.1% Tween 20) for 1 hr at room temperature. Reactions were stopped with SDS-PAGE sample buffer and resolved by SDS-PAGE for quantitation by phosphorimager.

CaM kinase activity was tested in 50 μ l kinase buffer (as above) for 1 hr at room temperature. CaMKI and CaMKIV were assayed using 200 μ M ADR1G (LKKLRRASFSGG) as a substrate; 60 μ M Syntide-2 (PLARTLSVAGLPGKK) was used for CaMKII. To measure CaMKII activity in the egg extract, 60 μ M Syntide-2 and 50 μ M autocamtide were added to extract in the presence or absence of CaMK inhibitors with 20 μ Ci/reaction [γ - ^{32}P]ATP. Reactions were terminated by spotting 20 μ l onto p81 phosphocellulose filters followed by washing in 75 mM phosphoric acid. Dried filters were scintillation counted.

Egg extracts were depleted of CaM kinase by three 30 min rounds of incubation with calmodulin Sepharose or control protein A sepharose at 4°C.

RAIDD Assays

GST or GST-RAIDD proteins linked to glutathione Sepharose were added to 100 μ l egg extract supplemented with energy mix, various metabolites or drugs, and ^{35}S -labeled wt or S135A caspase-2.

Samples were incubated at room temp for 1 hr. GST or GST-RAIDD beads were pelleted and washed three times with ELB supplemented with 300 mM NaCl and 0.1% Tween 20. Samples were eluted with sample buffer and resolved by SDS-PAGE for autoradiography.

Acknowledgments

We thank A.R. Means, C. Newgard, J. Chandra, F. Chow, and T. Ribar for helpful discussions. We thank D. Ribar for excellent technical assistance, D. Vandré and D. Weitzel for UCN-01, and V. Dixit for RAIDD. This work was supported by NIH RO1s GM 56518 and CA 102727 to S.K. L.K.N. was supported by an individual NRSA. J.C.R. is the recipient of a Howard Temin Career Development award, a Sidney Kimmel Foundation Scholar award, and a V Foundation Scholarship. S.S.M. is a recipient of the George H. Hitchings Fund for Health Research and Science Education of the Triangle Community Foundation.

Received: May 2, 2005

Revised: July 8, 2005

Accepted: July 27, 2005

Published: October 6, 2005

References

- Bagowski, C.P., Xiong, W., and Ferrell, J.E., Jr. (2001). c-Jun N-terminal kinase activation in *Xenopus laevis* eggs and embryos. A possible non-genomic role for the JNK signaling pathway. *J. Biol. Chem.* 276, 1459–1465.
- Bergeron, L., Perez, G.I., Macdonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K.E., Flaws, J.A., Salter, J.C., et al. (1998). Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev.* 12, 1304–1314.
- Bhuyan, A.K., Varshney, A., and Mathew, M.K. (2001). Resting membrane potential as a marker of apoptosis: studies on *Xenopus* oocytes microinjected with cytochrome c. *Cell Death Differ.* 8, 63–69.
- Blitzer, R.D., Connor, J.H., Brown, G.P., Wong, T., Shenolikar, S., Iyengar, R., and Landau, E.M. (1998). Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. *Science* 280, 1940–1942.
- Braun, T., Dar, S., Vorobiov, D., Lindenboim, L., Dascal, N., and Stein, R. (2003). Expression of Bcl-x(S) in *Xenopus* oocytes induces BH3-dependent and caspase-dependent cytochrome c release and apoptosis. *Mol. Cancer Res.* 1, 186–194.
- Cohen, A.I. (1954). Studies on glycolysis during the early development of the *Rana pipiens* embryo. *Physiol. Zool.* 27, 128–141.
- Comin-Anduix, B., Boros, L.G., Marin, S., Boren, J., Callol-Massot, C., Centelles, J.J., Torres, J.L., Agell, N., Bassilian, S., and Cascante, M. (2002). Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. *J. Biol. Chem.* 277, 46408–46414.
- Danial, N.N., Gramm, C.F., Scorrano, L., Zhang, C.Y., Krauss, S., Ranger, A.M., Datta, S.R., Greenberg, M.E., Licklider, L.J., Lowell, B.B., et al. (2003). BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature* 424, 952–956.
- Deckwerth, T.L., and Johnson, E.M., Jr. (1993). Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* 123, 1207–1222.
- Deming, P.B., Schafer, Z.T., Tashker, J.S., Potts, M.B., Deshmukh, M., and Kornbluth, S. (2004). Bcr-Abl-mediated protection from apoptosis downstream of mitochondrial cytochrome c release. *Mol. Cell Biol.* 24, 10289–10299.
- Demirci, B., Lornage, J., Salle, B., Frappart, L., Franck, M., and Guerin, J.F. (2001). Follicular viability and morphology of sheep ovaries after exposure to cryoprotectant and cryopreservation with different freezing protocols. *Fertil. Steril.* 75, 754–762.

- Demirci, B., Salle, B., Frappart, L., Franck, M., Guerin, J.F., and Lornage, J. (2002). Morphological alterations and DNA fragmentation in oocytes from primordial and primary follicles after freezing-thawing of ovarian cortex in sheep. *Fertil. Steril.* 77, 595–600.
- Demirci, B., Lornage, J., Salle, B., Poirel, M.T., Guerin, J.F., and Franck, M. (2003). The cryopreservation of ovarian tissue: uses and indications in veterinary medicine. *Theriogenology* 60, 999–1010.
- de Schepper, G.G., van Noorden, C.J., and Houtkooper, J.M. (1987). Age-related changes of glucose-6-phosphate dehydrogenase activity in mouse oocytes. *Histochem. J.* 19, 467–470.
- Deshmukh, M., Vasilakos, J., Deckwerth, T.L., Lampe, P.A., Shivers, B.D., and Johnson, E.M., Jr. (1996). Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *J. Cell Biol.* 135, 1341–1354.
- Duan, H., and Dixit, V.M. (1997). RAIDD is a new 'death' adaptor molecule. *Nature* 385, 86–89.
- Dworkin, M.B., and Dworkin-Rastl, E. (1989). Metabolic regulation during early frog development: flow of glycolytic carbon into phospholipids in *Xenopus* oocytes and fertilized eggs. *Dev. Biol.* 132, 524–528.
- Evans, E.K., Kuwana, T., Strum, S.L., Smith, J.J., Newmeyer, D.D., and Kornbluth, S. (1997). Reaper-induced apoptosis in a vertebrate system. *EMBO J.* 16, 7372–7381.
- Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W., and Thornberry, N.A. (1998). Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J. Biol. Chem.* 273, 32608–32613.
- Graves, P.R., Yu, L., Schwarz, J.K., Gales, J., Sausville, E.A., O'Connor, P.M., and Piwnicka-Worms, H. (2000). The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. *J. Biol. Chem.* 275, 5600–5605.
- Guo, Y., Srinivasula, S.M., Druilhe, A., Fernandes-Alnemri, T., and Alnemri, E.S. (2002). Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J. Biol. Chem.* 277, 13430–13437.
- Hutchins, J.R., Dikovskaya, D., and Clarke, P.R. (2003). Regulation of Cdc2/cyclin B activation in *Xenopus* egg extracts via inhibitory phosphorylation of Cdc25C phosphatase by Ca(2+)/calmodulin-dependent protein [corrected] kinase II. *Mol. Biol. Cell* 14, 4003–4014.
- Jaeger, L. (1945). Glycogen utilization by the amphibian gastrula in relation to invagination and induction. *J. Cell. Comp. Physiol.* 25, 97–120.
- Kim, M.R., and Tilly, J.L. (2004). Current concepts in Bcl-2 family member regulation of female germ cell development and survival. *Biochim. Biophys. Acta* 1644, 205–210.
- Kluck, R.M., Bossy-Wetzell, E., Green, D.R., and Newmeyer, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275, 1132–1136.
- Kovacevic, Z., and Morris, H.P. (1972). The role of glutamine in the oxidative metabolism of malignant cells. *Cancer Res.* 32, 326–333.
- Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002). Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297, 1352–1354.
- Majewski, N., Nogueira, V., Bhaskar, P., Coy, P.E., Skeen, J.E., Gottlob, K., Chandel, N.S., Thompson, C.B., Robey, R.B., and Hay, N. (2004). Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol. Cell* 16, 819–830.
- Maller, J.L. (1985). Regulation of amphibian oocyte maturation. *Cell Differ.* 16, 211–221.
- Morita, Y., and Tilly, J.L. (1999). Oocyte apoptosis: like sand through an hourglass. *Dev. Biol.* 213, 1–17.
- Morita, Y., Perez, G.I., Maravei, D.V., Tilly, K.I., and Tilly, J.L. (1999). Targeted expression of Bcl-2 in mouse oocytes inhibits ovarian follicle atresia and prevents spontaneous and chemotherapy-induced oocyte apoptosis in vitro. *Mol. Endocrinol.* 13, 841–850.
- Morita, Y., Perez, G.I., Paris, F., Miranda, S.R., Ehleiter, D., Haimovitz-Friedman, A., Fuks, Z., Xie, Z., Reed, J.C., Schuchman, E.H., et al. (2000). Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. *Nat. Med.* 6, 1109–1114.
- Newmeyer, D.D., Farschon, D.M., and Reed, J.C. (1994). Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 79, 353–364.
- Perez, G.I., Knudson, C.M., Leykin, L., Korsmeyer, S.J., and Tilly, J.L. (1997). Apoptosis-associated signaling pathways are required for chemotherapy-mediated female germ cell destruction. *Nat. Med.* 3, 1228–1232.
- Perez, G.I., Robles, R., Knudson, C.M., Flaws, J.A., Korsmeyer, S.J., and Tilly, J.L. (1999). Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. *Nat. Genet.* 21, 200–203.
- Rathmell, J.C., Vander Heiden, M.G., Harris, M.H., Frauwirth, K.A., and Thompson, C.B. (2000). In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol. Cell* 6, 683–692.
- Rathmell, J.C., Farkash, E.A., Gao, W., and Thompson, C.B. (2001). IL-7 enhances the survival and maintains the size of naive T cells. *J. Immunol.* 167, 6869–6876.
- Rathmell, J.C., Fox, C.J., Plas, D.R., Hammerman, P.S., Cinalli, R.M., and Thompson, C.B. (2003). Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol. Cell Biol.* 23, 7315–7328.
- Ratts, V.S., Flaws, J.A., Kolp, R., Sorenson, C.M., and Tilly, J.L. (1995). Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology* 136, 3665–3668.
- Read, S.H., Baliga, B.C., Ekert, P.G., Vaux, D.L., and Kumar, S. (2002). A novel Apaf-1-independent putative caspase-2 activation complex. *J. Cell Biol.* 159, 739–745.
- Reitzer, L.J., Wice, B.M., and Kennell, D. (1979). Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J. Biol. Chem.* 254, 2669–2676.
- Robertson, J.D., Enoksson, M., Suomela, M., Zhivotovsky, B., and Orrenius, S. (2002). Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *J. Biol. Chem.* 277, 29803–29809.
- Schwartz, A.G., and Pashko, L.L. (2004). Dehydroepiandrosterone, glucose-6-phosphate dehydrogenase, and longevity. *Ageing Res. Rev.* 3, 171–187.
- Smythe, C., and Newport, J.W. (1991). Systems for the study of nuclear assembly, DNA replication, and nuclear breakdown in *Xenopus laevis* egg extracts. *Methods Cell Biol.* 35, 449–468.
- Tian, W.N., Braunstein, L.D., Apse, K., Pang, J., Rose, M., Tian, X., and Stanton, R.C. (1999). Importance of glucose-6-phosphate dehydrogenase activity in cell death. *Am. J. Physiol.* 276, C1121–C1131.
- Tilly, J.L. (2001). Commuting the death sentence: how oocytes strive to survive. *Nat. Rev. Mol. Cell Biol.* 2, 838–848.
- Tinel, A., and Tschopp, J. (2004). The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 304, 843–846.
- Tuttle, S., Stamato, T., Perez, M.L., and Biaglow, J. (2000). Glucose-6-phosphate dehydrogenase and the oxidative pentose phosphate cycle protect cells against apoptosis induced by low doses of ionizing radiation. *Radiat. Res.* 153, 781–787.
- Ureta, T., Fernandez, W.Y., Centelles, J.J., and Cascante, M. (2000). In vivo measurements of control coefficients for hexokinase and glucose-6-phosphate dehydrogenase in *Xenopus laevis* oocytes. *FEBS Lett.* 475, 145–149.
- Vander Heiden, M.G., Plas, D.R., Rathmell, J.C., Fox, C.J., Harris, M.H., and Thompson, C.B. (2001). Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol. Cell Biol.* 21, 5899–5912.
- Voronina, E., and Wessel, G.M. (2001). Apoptosis in sea urchin oocytes, eggs, and early embryos. *Mol. Reprod. Dev.* 60, 553–561.
- Watanabe, M., Shirayoshi, Y., Koshimizu, U., Hashimoto, S., Yonehara, S., Eguchi, Y., Tsujimoto, Y., and Nakatsuji, N. (1997). Gene transfection of mouse primordial germ cells in vitro and analysis of their survival and growth control. *Exp. Cell Res.* 230, 76–83.