The metabolism of the *Xenopus laevis* egg provides a cell survival signal. We found previously that increased carbon flux from glucose-6-phosphate (G6P) through the pentose phosphate pathway in egg extracts maintains NADPH levels and calcium/calmodulin regulated protein kinase II (CaMKII) activity to phosphorylate caspase 2 and suppress cell death pathways. Here we show that the addition of G6P to oocyte extracts inhibits the dephosphorylation/inactivation of CaMKII bound to caspase 2 by protein phosphatase 1. Thus, G6P sustains the phosphorylation of caspase 2 by CaMKII at Ser-135, preventing its dephosphorylation/inactivation of CaMKII and apoptosis. Furthermore, these findings suggest novel approaches to disrupt the suppressive effects of the abnormal metabolism on cell death pathways.

Altered metabolism is well established as a contributing factor in many disease processes, including cancer, diabetes, infertility, and heart disease (1–4). However, studying the direct consequences of altered metabolic regulation in mammalian cells is cumbersome because introducing intermediate metabolites directly into the cells is not feasible. It is also difficult to do biochemistry in the limited amount of extract obtained from mammalian cell systems. Interestingly, the increased rates of glycolysis and lactic acid accumulation in neoplastically transformed cells have also been reported in newly fertilized invertebrate eggs, even under highly aerobic conditions (5). Thus, it has been suggested that studies of the more biochemically tractable *Xenopus* oocyte system may provide novel insights into tumor cell metabolism (1, 6). Indeed, we found that addition of G6P to *Xenopus* egg extracts leads to increased NADPH production via the pentose phosphate pathway, which enhances inhibitory phosphorylation of caspase 2 by CaMKII, promoting oocyte survival (2). Thus, the metabolic status of oocytes plays a key role in cell death regulation.

The four CaMKII isoforms (α, β, γ, and δ) form a family of multifunctional serine/threonine protein kinases that are important in many signaling cascades, from learning and memory to regulating the exit from mitosis. CaMKII plays a crucial role in cancer cell survival as well. Overexpression of CaMKII confers resistance to apoptosis induced by doxorubicin (7), and the CaMKII inhibitor KN-93 induces prostate cancer cell death (8). CaMKII exists as either a homo- or heterododecamer (9). Ca2+- and calmodulin-stimulated autophosphorylation at Thr-286/287, the canonical CaMKII activation pathway, results in formation of a constitutively active form of CaMKII that is essential for normal signaling. However, our previous study (2) found that activation of CaMKII by NADPH is independent of an increase in cytosolic Ca2+, suggesting that metabolism can regulate CaMKII via a novel non-canonical pathway.

In this study, we interrogated the mechanisms underlying metabolic regulation of CaMKII. We found that CaMKII activation was through metabolic inhibition of PP1 activity.

### EXPERIMENTAL PROCEDURES

**Reagents**—Reagents were used as described previously (2, 10). Purified calmodulin (pig brain) was purchased from EMD Millipore. Purified PP1 γ (rabbit skeletal muscle) was purchased from GloboZymes. Microcystin-LR was purchased from Enzo Life Sciences and conjugated to *N*-hydroxysuccinimide-activated CH-Sepharose 4B as described by Moorehead *et al.* (11).

**Recombinant Protein Cloning and Expression**—N-terminally GST-tagged (pGEX-KG) *Xenopus* PP1 (α, β, and γ), calmodulin regulated protein kinase II; PP1, protein phosphatase 1; OA, okadaic acid.

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lin, CaMKIIα (TT305/6AA), and rat neurabin were expressed in, and purified from, BL21 Escherichia coli as described previously by Evans et al. (12). Xenopus caspase 2 constructs were cloned into pGEX-KG and pSP64T as described previously by Nutt et al. (2). Xenopus PP1 (H9251, H9252, and H9253) were amplified from Xenopus RNA by RT-PCR using the SuperScript III one-step PCR system (Invitrogen). Sequences for Xenopus PP1 isoforms were obtained from Xenbase (13). The primers used were as follows: PP1/H9251, 5'-ATAGAATTCTAATGGGGGACGGAGAAAATTT-3' (forward) and 5'-ATAGTCGACTTATTATTTCTTTGCTTGTTTGTGAT-3' (reverse); and PP1/H9252, 5'-ATAGAATTCTAATGGGCAGATGTTGACAAGCTAAA-3' (forward) and 5'-ATAGTCGACTTATTATTTCTTTGCTTGTTTGTGAT-3' (reverse). Purified PCR products were digested and cloned into pGEX-KG using EcoRI/SalI (PP1/H9251), XhoI/HindIII (PP1/H9252), and EcoRI/XhoI (PP1/H9253). Purified PCR products were digested and cloned into pGEX-KG using BamHI/EcoRI. The QuickChange site-directed mutagenesis kit (Agilent) was used to create point mutations in CaMKIIα in pGEX-KG. The

FIGURE 1. Activity and dephosphorylation of CaMKII is regulated metabolically. A, G6P inhibits apoptosis in egg extracts. Egg extracts were incubated in the absence or presence of G6P, and samples were taken at the indicated times and analyzed for caspase 3/7 activation using the caspase 3/7 GLO assay (Promega). 

B, egg extract contains multiple isoforms of CaMKII. Egg extract was treated with or without G6P, and samples were taken and immunoblotted for pan-CaMKII and pCaMKII Thr-286/287. GAPDH was used as a loading control.

C, G6P maintains phosphorylation of endogenous CaMKII Thr-286/287. Egg extracts were incubated at room temperature in the absence (upper panel) or presence (lower panel) of G6P. Samples were taken at the indicated times and immunoblotted for CaMKIIα and pCaMKII Thr-286/287. n = > 3 independent experiments. 

D, G6P inhibits recombinant CaMKIIα Thr-286 dephosphorylation. GST-CaMKIIα (TT305/6AA) bound to glutathione-Sepharose was incubated in egg extract in the presence of G6P and [γ-32P]ATP. Beads were washed and then incubated in a fresh aliquot of egg extract in the absence or presence of G6P. At the indicated times, samples were taken and analyzed for GST-CaMKIIα (TT305/6AA) phosphorylation status by SDS-PAGE, Coomassie Blue staining, and autoradiography. n = > 3 independent experiments.
TT305/6AA primers were 5'-GGCCATCTGGCTGCAATG-CTGCTACCTGCTGCAACTCG-3' and its complement. Xenopus calmodulin cDNA (pCMV-SPORT6) was purchased from Open Biosystems (catalog no. MXL1736-9507481). Calmodulin was amplified from this cDNA using the following primers: calibration, 5'-TATGGATCTACGGCGACTGTTGCTTTTGC-3' (forward) and 5'-TATAAGCTTCTATTGCAATCTCAT-3' (reverse). The purified PCR product was digested and cloned into pGEX-KG using BamHI/HindIII. Sequencing analysis confirmed the identity of all constructs. Purified mouse CaMKIIα and rat GST-neurabin were generated as described previously (14, 15). The construct to express N-terminally FLAG-tagged Xenopus caspase 2 was a gift from Dr. Sally Kornbluth (Duke University, NC).

**Mass Spectrometry Analysis of Recombinant Proteins**—Mass spectrometry analysis of GST full-length caspase 2 (C2), GST-active C2, and GST-CaMKII (TT305/6AA) proteins was performed. When purified from *E. coli*, GST full-length C2, GST-

### Table 1: Analysis of Recombinant Proteins

| Time (hr) | Sepharose | MC-LR |
|-----------|-----------|-------|
| 0         | 50        | 50    |
| 1         | 50        | 50    |
| 2         | 50        | 50    |
| 3         | 50        | 50    |
| 4         | 50        | 50    |

**Figure A**: Western blot analysis of GST-CaMKII (TT305/6AA) expression in Sepharose and MC-LR.

**Figure B**: Western blot analysis of GST-CaMKII (TT305/6AA) expression in Sepharose and MC-LR.

**Figure C**: Caspase 3/7 activity over time.

**Figure D**: Western blot analysis of GST full-length caspase 2 (C2) and GST-active C2 in 1nM and 10μM Okadaic Acid.

**Figure E**: Western blot analysis of GST-CaMKII (TT305/6AA) in 10μM Okadaic Acid.

**Figure F**: Graph of Caspase 3/7 activity over time.
active C2, and GST-CaMKII (TT305/6AA) produce two bands: one at the predicted size and one at a lower molecular weight size than expected. In each case, following mass spectrometric analysis, the lower molecular weight band was confirmed as a C-terminal truncation of the full-length recombinant protein. The expected and truncated sizes of the GST-caspase proteins were as follows: full-length GST-caspase 2, ~74 kDa; truncated full-length GST-caspase 2, ~56 kDa; GST-active caspase 2, ~56 kDa; truncated GST-active caspase 2 (without the p12 fragment), ~45 kDa; and GST-Pro C2, ~44 kDa.

Peptide Synthesis and Sepharose Coupling—Calmodulin binding peptide (KKRRWKKNFIAVSAANRFKKISSSGAL), corresponding to the calmodulin binding domain of myosin light chain kinase, was synthesized by the Macromolecular Synthesis Laboratory Core of St. Jude Children’s Research Hospital. Calmodulin binding peptide was coupled covalently to Sepharose 4B at its NH₂ terminus via a hexanoic acid linker.

Caspase 3/7 Assay—At the indicated time points, 3 μl of egg extract was added to each well of a 96-well plate on ice containing 100 μM NaCl, 0.1% CHAPS, 10 mM EDTA, and 10% glycerol. After all time points were taken, 5 μl of the caspase GLO 3/7 luminescent substrate (caspase GLO 3/7 assay, Promega) was added to each well. Plates were incubated at 37 °C for 15 min. Luminescence values were measured using 3-s integrated counting.

In Vitro Translated Caspase 2 Activation—Analysis of in vitro translated caspase 2 activation was performed as described previously (2, 10).

Kinase Assays—Kinase assays were performed as described previously (2, 10). A modified in vitro kinase assay using endogenous CaMKII and GST-Pro C2 as bait and substrate was also carried out by first incubating GST-Pro C2 in egg extract for 45 min at room temperature to bind endogenous CaMKII. GST-Pro C2 (bound to CaMKII) was then collected, washed in egg lysis buffer (ELB) (10 mM HEPES (pH 7.7), 250 mM sucrose, 25 mM MgCl₂, 1 mM DTT, and 50 mM KCl), and incubated in kinase buffer (25 mM HEPES (pH 7.5), 0.5 mM DTT, 10 mM MgCl₂, 0.1% (v/v) Tween 20, and 50 μM ATP) with 5 μCi of [γ-32P]ATP or with and without 500 μM okadaic acid. Beads were washed and analyzed for GST-Pro C2 phosphorylation as described above.

RESULTS

Dephosphorylation of CaMKII Is Regulated Metabolically—Previous studies in Xenopus oocytes have shown that sustained levels of G6P drive the pentose phosphate pathway and NADPH production to promote oocyte survival (2). We confirmed this early finding by showing that addition of G6P to egg extracts prevented the activation of caspase 3/7, markers of the activation of apoptotic pathways (Fig. 1A). This inhibition of apoptosis is associated with sustained phosphorylation of caspase 2 at an inhibitory CaMKII site, suggesting that CaMKII activation of apoptotic pathways (Fig. 1A).

Inhibition or dephosphorylation of PP1 inhibits CaMKII dephosphorylation and apoptosis. A, depletion of PP1 and PP2A inhibits recombinant CaMKII activation (Thr-286 dephosphorylation). Upper panel, GST-CaMKII (TT305/6AA) was phosphorylated in egg extract in the presence of G6P as in Fig. 1A. Beads were collected, washed, and then incubated in egg extract depleted with Sepharose or microcystin-Sepharose (MC-LR). At the indicated times, samples were taken and analyzed by SDS-PAGE, Coomassie Blue staining, and autoradiography. Lower panel, depletion of PP1 and PP2A was confirmed by immunoblotting for PP1β, PP2A, and GAPDH as a loading control. n = > 3 independent experiments. B, depletion of PP1 and PP2A inhibits endogenous CaMKII activation (Thr-286 dephosphorylation). Upper panel, egg extracts were depleted as in A. Samples were taken at the indicated times and immunoblotted for CaMKII and pCaMKII Thr-286/287. Lower panel, depletion of PP1 and PP2A was confirmed by immunoblotting for PP1β, PP2A, and GAPDH as a loading control. n = 3 independent experiments. C, depletion of PP1 and PP2A inhibits apoptosis. At the indicated times, samples were taken from egg extract depleted with Sepharose or MC-LR and analyzed for caspase 3/7 (C3/7) activation using a caspase 3/7 GLO assay (Promega). n = > 3 independent experiments. D, inhibition of PP1 and PP2A with okadaic acid inhibits recombinant CaMKII activation (Thr-286 dephosphorylation). Left panel, GST-CaMKII (TT305/6AA) was phosphorylated in egg extract in the presence of G6P as in Fig. 1A. Beads were collected, washed, and then incubated in a fresh aliquot of egg extracts containing 1 mM or 10 μM okadaic acid. Samples were taken at the indicated times and analyzed as A. Right panel, the same experiment as displayed in the left panel, conducted independently. n = > 3 independent experiments. E, inhibition of PP1 and PP2A with okadaic acid inhibits endogenous CaMKII activation (Thr-286/287 dephosphorylation). Egg extracts were incubated at room temperature in the presence of 10 μM okadaic acid. Samples were taken at the indicated times and immunoblotted for CaMKII and pCaMKII Thr-286/287. n = 2 independent experiments. F, inhibition of PP1 and PP2A with okadaic acid inhibits apoptosis. Egg extracts were incubated in the absence or presence of okadaic acid (1 mM or 10 μM), and samples were taken at the indicated times and analyzed for C3/7 activation using a caspase 3/7 GLO assay (Promega). n = 3 independent experiments.
that the three bands recognized by the phospho-Thr-286/287 antibody likely correspond to different CaMKII isoforms because a similar pattern of bands is detected by immunoblotting using a pan-CaMKII antibody that recognizes the α, β, γ, and δ isoforms. Note that the mobility of some bands detected using the pan-CaMKII antibody is sensitive to G6P, consistent with a change in CaMKII phosphorylation. Using the phospho-Thr-286/287-specific antibody to determine CaMKII activity in the extract revealed that the endogenous CaMKII isoforms were phosphorylated persistently at Thr-286/287 in the presence, but not the absence, of G6P (Fig. 1C) (16). Because CaMKII autophosphorylation was maintained, these data suggest that addition of G6P inhibits the phosphatase responsible for dephosphorylating Thr-286/287. To test if this was the case, we radiolabeled GST-CaMKII protein in which other autophosphorylation sites (Thr-305 and Thr-306) had been replaced with Ala, and then tested it as a substrate for protein phosphatases in egg extracts in the presence or absence of added G6P. As shown in Fig. 1D, G6P inhibits the dephosphorylation of both radiolabeled CaMKII bands (explanation of the two bands is provided under

**Figure 1**

**A** 

GST MC-LR Neu 37-37-37-37 anti-PP1α anti-PP1β anti-PP1γ anti-PP2A anti-GST

**B** 

GST | Neu 15 30 45 60 15 30 45 60 37 37 37 37 anti-PP1α anti-PP1β anti-PP1γ anti-PP2A anti-Actin

**C** 

Caspsae 37/ Activity

**D** 

GST 0 Neu 0 GST 5 Neu 5 GST 6 Neu 6 Time (hrs) IVT 35S C2

**E** 

GST 0 .5 1 2 4 MW Neu 0 .5 1 2 4

**F** 

GST 0 2 4 Neu 0 2 4 anti-Thr286/287 pCamKII anti-CamKII α anti-CamKII γ

**Metabolic Regulation of CaMKII and Caspases**

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PP1 Dephosphorylates CaMKII—Previous studies have shown that PP1, PP2A, and PP2C are capable of dephosphorylating Thr-286/287 in CaMKII in brain extracts (17). To begin to identify the metabolically regulated phosphatases responsible for CaMKII dephosphorylation in egg extracts, we depleted both PP2A and PP1 from egg extracts (lacking added G6P) using a microcystin-Sepharose affinity resin (18) (Fig. 2A), which has a similar affinity for PP1 and PP2A (19). Notably, the dephosphorylation of radiolabeled GST-CaMKII by PP1/PP2A-depleted egg extracts was diminished substantially relative to extracts depleted using control Sepharose beads (Fig. 2A). Furthermore, PP1/PP2A depletion sustained the Thr-286/287 autophosphorylation of endogenous CaMKII (Fig. 2B) and prevented the activation of caspase 3/7 (C).

To investigate the relative importance of PP1 and PP2A, we compared the effects of adding 1 mM or 10 μM okadaic acid (OA) to egg extracts. OA has a biphasic dose-response curve, selectively inhibiting PP2A at nanomolar concentrations but also inhibiting PP1 at micromolar concentrations (20). CaMKII was still rapidly dephosphorylated in the presence of 1 mM OA, whereas 10 μM OA substantially attenuated CaMKII dephosphorylation (Fig. 2D). Furthermore, the dephosphorylation of endogenous CaMKII at Thr-286/287 and the activation of caspase 3/7 were also suppressed following PP1/PP2A inhibition by 10 μM, but not by using 1 mM OA (Fig. 2, E and F). These data are consistent with a predominant role for PP1 in the dephosphorylation of Thr-286/287 in GST-CaMKII and the initiation of apoptosis in egg extracts.

PP1 catalytic subunits are targeted to discrete subcellular compartments and regulated by interactions with proteins that typically contain a canonical PP1-binding motif with a consensus R/K-V/I-X-F sequence. To specifically deplete PP1 from egg extracts, we used a GST fusion protein containing the consensus R/K-V/I-X-F sequence. To deplete PP1 from egg extracts, we used a GST fusion protein containing the consensus R/K-V/I-X-F sequence and flanking sequences from neurabin, a neuronal PP1-targeting subunit (21). Although neurabin shows selectivity for mammalian PP1 (21), such as present in the egg extracts. Fig. 3A shows that OA has a biphasic dose-response curve, selectively for mammalian PP1 isoforms, but not PP2A, by GST-neurabin. Actin was used as a loading control.

PP1 Dephosphorylates CaMKII—Metabolic Regulation of CaMKII and Caspases

FIGURE 3. Depletion of PP1 inhibits apoptosis and dephosphorylation of CaMKII. A, recombinant neurabin binds all isoforms of PP1 but not PP2A. GST or GST-neurabin (Neu) bound to glutathione-Sepharose or microcystin conjugated to Sepharose (MC-LR) was incubated in egg extract. The beads were washed, eluted, and immunoblotted for PP1α, PP1β, and PP1γ, and GST as a loading control. n = 3 independent experiments. B, depletion of PP1 inhibits caspase 3/7 activation. Egg extracts were depleted of PP1 with indicated concentrations of GST-neurabin. Immunoblotting confirmed the selective depletion of PP1 isoforms, but not PP2A, by GST-neurabin. As used as a loading control. C, samples were taken from egg extracts depleted with GST or GST-neurabin at the indicated times and analyzed for caspase 3/7 activation using a caspase 3/7 Glo assay (Promega). n = 2 independent experiments. D, depletion of PP1 inhibits caspase 2 processing. Egg extracts were depleted of GST or GST-neurabin bound to glutathione-Sepharose. Upper panel, egg extract depleted with GST or GST-neurabin was incubated in vitro-translated (IVT) 32P-labeled full-length caspase 2 (C2). Samples were taken at the indicated times and analyzed for C2 processing by SDS-PAGE and autoradiography. Processing of caspase 2 is indicated by cleavage of full-length caspase 2 (~45 kDa) to lower molecular weight cleavage products (~30 kDa and ~15 kDa). Note the 6-h time point in GST 6 versus Neu 6 (lanes 5 and 6). Lower panel, selective depletion of PP1, but not PP2A, was confirmed by immunoblotting for PP1β, PP2A, and GAPDH as a loading control. n = 3 independent experiments. E, depletion of PP1 inhibits recombinant CaMKIIa Thr-286 dephosphorylation. Upper panel, GST-CaMKIIa (TT305/6AA) bound to glutathione-Sepharose was incubated in egg extract in the presence or absence of G6P. The calmodulin-Sepharose bound recombinant CaMKIIa and PP1 (10). Because CaMKII associates comparably to non-phosphorylated CaMKII in the absence of G6P or to Thr-286/287 autophosphorylated CaMKII in the presence of G6P, as predicted (Fig. 4A). Notably, the FLAG-caspase 2 also bound to calmodulin-Sepharose in the presence or absence of G6P. To better understand the interaction of CaMKII with caspase 2, egg extracts were incubated with GST-caspase 2 (full-length), and complexes isolated using glutathione-Sepharose were probed for endogenous CaMKIIa. Fig. 4B shows that the endogenous CaMKIIa binds to GST-caspase 2.
To determine whether CaMKII directly interacts with caspase 2, we incubated *Xenopus* GST-caspase 2 (full-length) with various concentrations of purified mammalian CaMKIIα. Fig. 4C shows that CaMKIIα directly binds to caspase 2. Furthermore, we found that purified CaMKIIα directly interacted with GST fusion proteins containing either the pro-domain of caspase 2 (GST-Pro C2) or the catalytic active domain of caspase 2 (GST-Active C2) (Fig. 4D). However, binding at lower concentrations of CaMKIIα was detected using GST-Pro C2 but not when using GST-active C2. Furthermore, although calmodulin does not directly bind to caspase 2 (data not shown), calmodulin appears to be...
associated indirectly with the caspase 2 complex (Fig. 4A), perhaps via an interaction with CaMKII.

We have shown previously that PP1 binds constitutively to caspase 2 in the egg extracts. To examine whether caspase 2 preferentially binds to PP1 isoforms, we spiked egg extracts with in vitro-translated caspase 2 and then added recombinant PP1α, PP1β, or PP1γ (fused to GST). Protein complexes were isolated using glutathione-Sepharose. Both endogenous CaMKIIα and the in vitro-translated caspase 2 bound all three isoforms of PP1 (Fig. 4, E and F). Furthermore, the in vitro-translated 35S-labeled caspase 2 associated with all three PP1 catalytic isoforms in the absence of the egg extract (Fig. 4G). Moreover, untagged purified mammalian PP1γ associated directly with GST-caspase 2 (full-length protein) (Fig. 4H). Note that the GST-caspase 2 migrates as two bands: one of the predicted size and a second smaller band (see “Experimental Procedures”). These results establish that all three PP1 isoforms interact directly with caspase 2 (10). Taken together, the data in Fig. 4 suggest that both CaMKII and PP1 bind to caspase 2.
Metabolic Regulation of CaMKII and Caspases

A G6P-induced Factor Inhibits PP1-mediated Dephosphorylation of CaMKII Thr-286/287—To investigate the metabolic regulation of PP1-mediated dephosphorylation of CaMKII, we developed an in vitro dephosphorylation assay using recombinate caspase 2 and endogenous Casp-2, the protein complexes bound to GST-Pro C2 (WT or S315A) retrieved from egg extracts using glutathione-Sepharose was washed with an isotonic buffer and then mixed with a kinase buffer containing [γ-32P]ATP (see “Experimental Procedures”). The WT but not S315A Pro C2 was efficiently phosphorylated in a Ca2+-dependent manner (Fig. S4A). The WT and S315A GST-Pro C2 proteins bound similar amounts of endogenous CaMKII (data not shown), demonstrating that the lack of phosphorylation does not result from a lack of CaMKII binding to the mutant GST-Pro C2 domain. Furthermore, depletion of either CaMKII or calmodulin from the extracts prevented the phosphorylation of the WT GST-Pro C2 protein incubated in egg extract in the presence of Ca2+ (Fig. S5B).

Egg extracts were then incubated at room temperature with GST-Pro C2 in the presence of G6P, and complexes were isolated at various times using glutathione-Sepharose. As expected, Thr-286/287-autophosphorylated CaMKII was detected in the complex, and sustained incubation of the complex in the G6P-enriched extract did not result in substantial dephosphorylation of the isolated CaMKII (Fig. 5C). However, incubation of this isolated complex in buffer at room temperature resulted in the time-dependent dephosphorylation of the CaMKII (Fig. 5D). Taken together, these data suggest that G6P, or a downstream metabolite, inhibits the phosphatase. Notably, the dephosphorylation of CaMKII bound to GST-Pro C2 was inhibited by 10 μM okadaic acid (Fig. 5E), suggesting the involvement of PP1 and/or PP2A. To further identify the identity of phosphatases responsible for CaMKII dephosphorylation in this complex, GST-Pro C2 complexes were isolated from G6P-treated egg extracts that had been depleted of both PP1 and PP2A using microcystin-Sepharose or of PP1 alone using GST-neurabin. Notably, Thr-286/287-phosphorylated CaMKII was readily detected bound to GST-Pro C2, but the time-dependent dephosphorylation in the isolated complex was essentially abrogated completely by depletion of PP1 and PP2A (Fig. 5F) or PP1 alone (G). Together, these data show that G6P, or a downstream metabolite, can inhibit the PP1-mediated dephosphorylation of CaMKII at Thr-286/287 in complexes associated with GST-Pro C2.

**DISCUSSION**

It is well established that apoptosis is regulated by the phosphorylation of several proteins by multiple protein kinases. However, the counter-regulatory roles of protein phosphatases in regulating cell survival are less well understood. Previous studies have found that PP2A or PP1 can directly activate pro-apoptotic Bcl-2 family proteins by dephosphorylation in an endoplasmic reticulum stress-dependent or glucose deprivation-dependent cell death, respectively (23, 24). In addition, the net activity of the proapoptotic protein Bad depends on the balance between suppression because of PKA phosphorylation and activation by PP1 dephosphorylation in a complex coordinated by the A kinase-anchoring protein Wiskott-Aldrich family member WAVE-1 (23, 25). Here we provide evidence that PP1 regulation of CaMKII and caspase 2 is important in modulating caspase activation in response to the addition of G6P.

We interpret our data to indicate that G6P, or the sustained production of a soluble metabolic signal(s), blocks PP1 from dephosphorylating CaMKII in the complex with caspase 2. In extracts lacking a G6P supplement, the absence of this metabolic signal allows a default cell death pathway to be initiated by PP1-mediated dephosphorylation of CaMKII in the caspase 2 complex. This finding is consistent with the original hypothesis of Martin Raff, published in 1992, stating that, “cells constitutively express all of the proteins required to undergo programmed cell death (PCD) and undergo PCD unless continuously signaled not to” (26). However, direct addition of G6P or NADPH (a major product of the pentose phosphate pathway) to the isolated complex failed to protect CaMKII from dephosphorylation (data not shown). Thus, it will be important for...
future studies to identify the molecule(s) that provide this metabolic signal.

The molecular basis for the assembly of this complex remains to be elucidated. PP1 specificity is typically conferred by a targeting subunit that directs the catalytic subunit to its substrates by altering its subcellular localization, modifying the substrate selectivity and/or modulating the activity (27). PP1 activity and specificity may also be modulated by additional posttranslational modifications (28). When dephosphorlating the activation of caspase 2 by dephosphorylation of Ser-135, we were able to show that the PP1 catalytic subunit binds constitutively to caspase 2 and was responsible for dephosphorylation and activation of caspase 2 (10). Although we showed that PP1 binds to caspase 2 directly, caspase 2 seems to lack a clearly identified canonical PP1-binding motif. Thus, further studies will be required to understand the basis for this interaction.

Similarly, the biological roles of CaMKII are also influenced by its interactions with other proteins. Work in the CNS has shown that CaMKII can interact with other proteins by diverse mechanisms (29, 30). Our data show that CaMKII also interacts with the caspase 2-PP1 complex. Within this complex, CaMKII can phosphorylate the critical modulatory site Ser-135 in caspase 2 and also serves as a substrate for the bound PP1. Interestingly, studies in the brain indicate that the availability of CaMKII to protein phosphatases is modulated by subcellular targeting. Soluble CaMKII is dephosphorylated preferentially by PP2A, whereas CaMKII associated with the postsynaptic density is a preferential substrate for PP1 (31). Moreover, PP1 has a gatekeeping role in modulating synaptic CaMKII activation (32), and more recent studies have shown that CaMKII and PP1 coassemble with the synaptic scaffolding protein spinophilin (21, 22, 33). Our demonstration of a complex with caspase 2 provides a novel mechanism for targeting PP1 to regulate CaMKII that appears to play an important role in the recruitment of apoptotic pathways in oocytes.

Interestingly, the activity of PP1 toward CaMKII in the complex with caspase 2 appears to be suppressed by the metabolic signal that might be generated following the addition of G6P to the egg extracts. This provides a non-canonical mechanism for calcium-independent activation of CaMKII that suppresses apoptosis in oocytes. It is interesting to note that the metabolism of oocytes resembles the abnormal metabolism of tumor cells, which are also resistant to cell death. Thus, developing a better understanding of the molecular mechanisms underlying these links might suggest novel strategies for therapeutic intervention directed at blocking this metabolic “brake” on apoptosis via PP1 and CaMKII to promote the death of transformed cells.

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