Metabolic Control of Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II (CaMKII)-mediated Caspase-2 Suppression by the B55β/Protein Phosphatase 2A (PP2A)*

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**Background:** CaMKII autophosphorylates at Thr\(^{286}\) in cell-free lysates supplemented with glucose-6-phosphate (G6P).

**Results:** The B55β and C subunits of PP2A interact with and dephosphorylate CaMKII at novel sites following addition of G6P.

**Conclusion:** B55β/PP2A is critical for stimulating CaMKII in the presence of G6P.

**Significance:** This work identifies a novel role for a specific phosphatase complex in controlling CaMKII.

High levels of metabolic activity confer resistance to apoptosis. Caspase-2, an apoptotic initiator, can be suppressed by high levels of nutrient flux through the pentose phosphate pathway. This metabolic control is exerted via inhibitory phosphorylation of the caspase-2 prodomain by activated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII). We show here that this activation of CaMKII depends, in part, on dephosphorylation of CaMKII at novel sites (Thr\(^{393}\)/Ser\(^{395}\)) and that this is mediated by metabolic activation of protein phosphatase 2A in complex with the B55β targeting subunit. This represents a novel locus of CaMKII control and also provides a mechanism contributing to metabolic control of apoptosis. These findings may have implications for metabolic control of the many CaMKII-controlled and protein phosphatase 2A-regulated physiological processes, because both enzymes appear to be responsive to alterations in glucose metabolism via the pentose phosphate pathway.

Apoptosis is a programmed form of cell death executed by caspase proteases. Multiple studies have implicated caspase-2 in cell stress-induced apoptosis (i.e. via DNA damage, endoplasmic reticulum stress, or heat shock) (1–5). Acting upstream of mitochondria in the intrinsic pathway (6), caspase-2 leads to cleavage of the pro-apoptotic Bcl-2 family member, Bid, to promote mitochondrial outer membrane permeabilization (7, 8).

In the *Xenopus* egg extract system, caspase-2 has also been tied to metabolic control of apoptosis (9–11). We have reported that caspase-2 is important for recapitulating apoptotic events in this system and that its activity can be modulated by controlling the metabolic status of the egg extracts. Specifically, incubation of extracts at room temperature reduced levels of pentose phosphate pathway (PPP)-generated NADPH, and supplementation of extracts with NADPH or PPP stimulatory glucose-6-phosphate (G6P) greatly delayed caspase-2 activation and ensuing apoptotic events (9). Biochemical analyses revealed that metabolic inhibition of caspase-2 was caused by inhibitory phosphorylation within the caspase-2 prodomain at Ser\(^{135}\) (*Xenopus* numbering). Using kinase inhibitors and immunodepletions, we found that this phosphorylation was catalyzed by the Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II (CaMKII) and that CaMKII activity was elevated following G6P or NADPH treatment of extracts (9).

Four highly similar isoforms exist of CaMKII, which is an important mediator of many Ca\(^{2+}\)-induced signaling pathways (12–15). Each isoform contains a catalytic domain near the N terminus, an autoregulatory domain, and a C-terminal association domain (16). When inactive, pseudosubstrate sequences bind and inhibit the catalytic domains (17). Ca\(^{2+}\)/CaM binding disrupts catalytic and autoinhibitory domain interaction, activating the kinase and allowing access to an autophosphorylation site (Thr\(^{286}\), α isoform) (18). Once activated, within the holoenzyme, one subunit phosphorylates an adjacent subunit at Thr\(^{286}\) when both are bound to Ca\(^{2+}\)/CaM (19). Once phosphorylated on Thr\(^{286}\), the Ca\(^{2+}\)/CaM off-rate drops over 1000-fold, stabilizing CaMKII activity (20). Therefore, the autophosphorylation of Thr\(^{286}\) can be used as an indicator of CaMKII activation. Following Ca\(^{2+}\)/CaM dissociation, Thr(Phospho)\(^{269}\) CaMKII remains active, and further autophosphorylation occurs at Thr\(^{305}\), Thr\(^{306}\), and Ser\(^{314}\) (21, 22).

Recently, the Nutt laboratory reported that CoA, generated in *Xenopus* egg extracts in the presence of abundant nutrients, binds to and activates CaMKII (23). We show here that nutrient-driven CaMKII activation additionally requires release of a “brake.” Specifically, we identify two novel sites of CaMKII phosphorylation (Thr\(^{393}\)/Ser\(^{395}\) on the *Xenopus* γ isoform L subunit and Thr\(^{371}\)/Ser\(^{373}\) on the human homolog) located within the association domain, whose phosphorylation falls in

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§ The abbreviations used are: PPP, pentose phosphate pathway; CaMKII, calmodulin-dependent protein kinase II; CaM, calmodulin; PP2A, protein phosphatase 2A; G6P, glucose-6-phosphate.
the presence of high G6P levels. Dephosphorylation of these sites, catalyzed by protein phosphatase 2A (PP2A), is necessary (albeit not sufficient) for metabolic activation of CaMKII. In addition, nutrient-driven PP2A targeting to CaMKII is driven by metabolically regulated interaction of CaMKII with the PP2A targeting subunit B55δ. Furthermore, this mechanism of CaMKII regulation is conserved in mammalian cells. Together, these findings provide insight into metabolic control of apoptosis and define a new mechanism for controlling CaMKII, a protein critical for cell signaling in response to multiple stimuli.

**EXPERIMENTAL PROCEDURES**

**Preparation of Xenopus Egg Extracts and Nutrient Treatment—Xenopus** egg extracts were prepared as previously described (24). G6P was prepared as a 1 M solution in water. Extracts were prepared at 4 °C, treated with G6P at a final concentration of 20 mM, and incubated at room temperature.

**Cell Culture and Nutrient Treatment—HEK 293T cells** were grown in DMEM with 10% FBS medium at 37 °C. Before treatment, cells were starved with glucose-free DMEM with 10% dialyzed FBS medium containing no d-glucose and sodium pyruvate at 37 °C for 12 h and then treated with or without 25 mM d-glucose (Sigma) for another 12 h. Cells were lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT, and 1% Nonidet P-40 with 5 μg/ml aprotinin/leupeptin and 100 μM PMSF and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Mixture Tablets from Roche, 20× on ice.

**siRNA Transfection—Lipofectamine RNAiMAX** (Invitrogen) was used for siRNA transfection. PP2A-B55δ siRNA was purchased from Santa Cruz Biotechnology to knock down B55δ in 293T cells. Control siRNA was designed to target a nonmammalian protein, firefly luciferase (5′-CGUACCGGAAUAUCUGCA-3′).

**Plasmids and Protein Preparation—Xenopus** CaMKIIα was amplified by PCR using the primers 5′-GGGGAATTCAATGGCCACTACCAAGACAGGCTC-3′ and 5′-GGGCTCAGATGCATCGAGGAGGGGCGTCTGTCG-3′. Purified PCR products were cloned into the EcoRI and Xhol sites of pENTR3C. The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate point mutants in CaMKIIα in pENTR3C. The T393A/S395A primers were 5′-CAGATGGGATAAAAGGATCAGGAGGCGTTGGTCAAC-3′ and its complement. The T393D/S395D primers were 5′-CAGATTGGGATAAAAGGATCAGGAGGCGTTGGTCAAC-3′ and its complement. The Gateway LR Clonase II enzyme mix (Invitrogen) was used to transfer CaMKIIα wild type and mutants from pENTR3C into pDEST24. Bacmid DNAs were produced by transforming pDEST24 plasmids into MAX Efficiency DH10Bac Chemically Competent Competent E. coli (Invitrogen) and transferred into SF9 cells using Cellfectin II reagent (Invitrogen).

**Antibodies, Immunoprecipitation, and Immunodepletion—** The following antibodies were used in this study: anti-CaMKII Thr(P)286 (Abcam), anti-CaMKII (BD Transduction Laboratories), anti-PP2A catalytic subunit (Millipore), anti-PP5 (BD Transduction Laboratories), anti-PPP2R1A (Abcam), anti-PPP2R2B (Abcam), anti-pan B56 (Millipore), and anti-GST (Santa Cruz Biotechnology). Proteins were measured by Western blot using LI-COR Biosciences Odyssey software or ECL method with HRP secondary antibody.

For immunoprecipitation, two micrograms of anti-CaMKIIα (Sigma) or Mouse control IgG were incubated with 20 μl of Dynabead protein G (Invitrogen) slurry overnight at 4 °C. 100 μl of Xenopus egg extracts treated with or without G6P were incubated with beads for 1 h at 4 °C. Beads were washed four times with wash buffer (described below) and eluted with SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE for immunoblotting.

The wash buffer for immunoprecipitation: 300 mM NaCl and 0.1% Triton X-100 (Sigma) in 1X egg lysis buffer (10 mM HEPES, 50 mM KCl, 2.5 mM MgCl2, 1 mM DTT, and 0.25 mM sucrose, pH 7.7).

For immunodepletion, 10 μg of PPP2R2B antibody (0.25 μg/μl) or rabbit control IgG were incubated with 100 μl of Dynabead protein A (Invitrogen) overnight at 4 °C. Beads were washed and divided into three equal parts. 90 μl of Xenopus egg extracts were incubated with beads for 1 h at 4 °C. This step was repeated three times.

**Phospho-antibody Purification—** The following peptides were synthesized to generate and purify phospho-antibody against Thr393 and Ser395 of Xenopus CaMKIIα: [H]-VHNATDGKGTSTECN-[NH2] (non-phospho-peptide), [H]-VHNATDGKGTSTF-[NH2] (Thr(P)393 peptide), and [H]-VHNATDGKGTSTF-[NH2] (Ser(P)395 peptide). Sera were run through a column of non-phospho-peptide conjugated with UltraLink Biosupport (Thermo Scientific) and were then purified on a column of phospho-peptide.

**Gel Filtration Chromatography—Xenopus** egg extracts were treated with G6P or water and co-treated with okadaic acid or DMSO. After incubation at room temperature for 4 h, the extracts were centrifuged at 200,000 × g and were fractionated on Superose 6 (GE Healthcare Life Sciences).

**Nano-Flow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS) Analysis—Xenopus egg extracts** were treated with G6P or water, and CaM-Sepharose was added (Agilent Technologies). After incubation, the CaM-Sepharose beads were collected by centrifugation and washed four times with 1× egg lysis buffer containing 500 mM NaCl and 0.5% Triton X-100 and then eluted with RapiGest SF Surfactant (Waters).

Following buffer exchange into 50 mM ammonium bicarbonate, pH 8.0, samples were subjected to a standardized reduction/alkylation procedure followed by overnight trypsin digestion according to the in-solution tryptic digestion protocol established by the Duke Center for Genomic and Computational Biology. Peptides were then either analyzed directly by LC-MS/MS analysis or subjected to a phosphopeptide enrichment with a 200 μl of TiO2, Protea Tip (Protea Bio) as per the manufacturer’s recommended protocol. Samples were then subjected to LC-MS/MS analysis using a Waters NanoAcquity UPLC equipped with a 1.7-μm BEH130 C18 75-μm inner diameter × 250-mm reversed phase column employing a 90-min gradient at 300 nl/min from 5% acetonitrile, 0.1% formic acid to 40% acetonitrile, 0.1% formic acid. Eluting peptides were analyzed on a Thermo LTQ-Orbitrap XL mass spectrometer set to acquire a precursor MS scan in the Orbitrap from m/z 400–2000 with r = 60,000 at m/z 400 and a target AGC setting of 1e6 ions. MS/MS spectra of the five most abundant...
precursor ions were acquired either in the Orbitrap with 
\[ r/H11005 \]
7500 at \[ m/z \] with a target AGC setting of 2e5 ions for nonenriched samples or in the ion trap with a target AGC setting of 1e3 for enriched samples. Max fill times were set to 1000 ms for full MS scans and either 500 ms for Orbitrap MS/MS scans or 250 ms for ion trap MS/MS scans with minimum MS/MS triggering thresholds of 5000 counts. For all experiments, fragmentation occurred in the LTQ linear ion trap with a CID energy setting of 35%, and a dynamic exclusion of 60 s was employed for previously fragmented precursor ions.

Raw LC-MS/MS data files were processed in Mascot distiller (Matrix Science) and then submitted to independent Mascot searches (Matrix Science) against an Trembl database (v 40.14 Xenopus laevis taxonomy, 12,530 forward entries) containing both forward and reverse entries of each protein. Search tolerances for LTQ-Orbitrap XL data were 5 ppm for precursor ions and 0.02 Da for Orbitrap product ions or 0.8 Da for ion trap product ions using full trypsin specificity. Carbamidomethylation (\[ +57.0214 \text{ Da on Cys} \]) was set as a fixed modification, whereas oxidation (\[ +15.9949 \text{ Da on Met} \]) and phosphorylation (\[ +79.9663 \text{ Da on Ser, Thr, and Tyr} \]) were considered variable modifications. All searched spectra were imported into Scaffold (v4.0, Proteome Software), and mascot ion scoring thresholds of 26 (p 0.05 Mascot identity score was 14.0) were set to achieve a false discovery rate of 0.0%. Probability of correct phosphorylation modification localization to a specific Ser, Thr, or Tyr residue was performed by submitting each MS/MS spectrum to the AScore algorithm with AScores of >15 or >19, indicating a >90% or >99% probability of correct localization, respectively.

Relative quantitation was performed in Skyline (v1.4.1, University of Washington) by applying the full MS precursor extracted ion chromatogram function to integrate and measure peak areas (area under the curve) of each identified phosphopeptide. Reported area under the curve measurements for each phosphopeptide were the sum of the monoisotopic peak extracted ion chromatogram, as well as the second and third isotopomer extracted ion chromatogram. To adjust for slight variations in starting CaMKII prior to TiO2 enrichment, the average area under the curve of three nonphosphorylatable (i.e. did not contain a STY) peptides from the nonenriched LC-MS samples were used to generate a correction factor.

**Kinase Assay—**40 \( \mu \text{L} \) of glutathione-Sepharose containing 4 \( \mu \text{g} \) of recombinant GST tagged caspase-2 prodomain or GST-only fusion proteins were incubated with Xenopus egg extracts supplemented with \[ \gamma^{32}\text{P-ATP} \] and treated with or without G6P. Samples were resolved by SDS-PAGE and detected by autoradiography. CB, Coomassie Blue. C, glucose-starved 293T cells treated with or without glucose (25 mM) were lysed and analyzed for CaMKII Thr286 autophosphorylation. D, glucose-starved 293T cells treated with or without glucose and with or without dehydroepiandrosterone (DHEA) were lysed and analyzed for CaMKII Thr286 autophosphorylation.

**RESULTS**

**CaMKII Activity Is under Metabolic Control—**As we reported previously, treatment of Xenopus egg extracts with G6P should elevate the kinase activity of CaMKII (9). We first tested the autophosphorylation of Thr286, an indicator of CaMKII activation. By directly treating egg extracts with or without G6P, we discovered that G6P increased phosphorylation of Thr286 on CaMKII, a known site of CaMKII autophosphorylation (Fig. 1A; note that the multiple phosphorylated bands are
likely due to multiple isoforms and allelic variants in the pseudotetraploid *X. laevis*). Although the total CaMKII antibody used here recognized predominantly the α isoform, with increased exposure time, additional CaMKII bands became evident, corresponding to multiple bands of Thr\(^{286}\) phosphorylation. This suggests that the observed increase in Thr\(^{286}\) phosphorylation following G6P phosphorylation is likely generalizable to multiple CaMKII isoforms. Additionally, as we reported previously, the *Xenopus* caspase-2 prodomain (which we showed to be a CaMKII substrate (9)) added to egg extracts was more heavily phosphorylated in the presence of G6P, again consistent with the notion that the kinase activity of CaMKII is up-regulated by G6P (Fig. 1B). As also reported, CaMKII can physically bind the caspase-2 prodomain, which is stimulated by G6P treatment (25). Interestingly, an up-regulation of Thr\(^{286}\) phosphorylation was also observed in human 293T cells after glucose starvation and resupplementation (Fig. 1C). As we reported in *Xenopus* egg extracts, the increased activity of CaMKII depended on the activity of the PPP; glucose-induced Thr\(^{286}\) phosphorylation was reduced by co-treatment of 293T cells with dehydroepiandrosterone, an allosteric inhibitor of glucose-6-phosphate dehydrogenase, suggesting that the regulation of Thr\(^{286}\) phosphorylation by glucose is also through the PPP (Fig. 1D).

**The Phosphorylation Status of CaMKII Is Altered by G6P Treatment of Egg Extracts**—Although phosphorylation of Thr\(^{286}\) on CaMKII is stabilized following G6P treatment (25), it was not determined whether other modifications of CaMKII, upstream of activation, are modulated by metabolism. To examine the status of CaMKII in G6P-treated egg extracts, we resolved G6P-treated and untreated extracts by gel filtration and examined the profile of CaMKII fractionation. As shown in Fig. 2A, the apparent molecular weight of the CaMKII complex was increased following G6P treatment. It is possible that this G6P-induced shift in the CaMKII fractionation profile is caused by the association of additional proteins with CaMKII, because even in the untreated extract, the molecular weight of CaMKII was above 600,000, consistent with the dodecameric (potentially active) form; note that the shift might also be caused by the incorporation of other, higher molecular weight CaMKII isoforms into the CaMKII holoenzyme.

To further investigate the mechanism of CaMKII activation, we looked for post-translational modifications of CaMKII induced by G6P treatment. Endogenous CaMKII was precipitated from extracts treated with either G6P or buffer using CaM-Sepharose. These precipitates were then analyzed by mass spectrometry (MS). The predominant isoform identified was CaMKII\(^{\Delta} \)L subunit. MS analysis identified several phosphorylation sites on CaMKII that were responsive to G6P addition. As expected, we observed an increase in phosphorylated Thr\(^{287}\) (Thr\(^{286}\) on CaMKII\(^{\Delta} \)) phosphorylation (Fig. 2C). More importantly, as shown in Fig. 2 (D and E), we identified two sites on CaMKII, Thr\(^{393}\) and Ser\(^{395}\), whose phosphorylation was decreased in response to G6P treatment. These data suggested that regulated dephosphorylation of these two sites could influence G6P-induced CaMKII activation.

Based on these observations, we also performed the gel filtration experiment described in Fig. 2A with or without the phosphatase inhibitor, okadaic acid. Although G6P treatment alone induced an upshift in the apparent molecular weight of CaMKII, as expected, co-treatment with the phosphatase inhibitor okadaic acid largely abrogated this up-shift, suggesting that some okadaic acid-inhibitable phosphatase(s) might be required for the observed G6P-induced CaMKII molecular weight upshift (Fig. 2B). Combined with the MS analysis result, these data suggested the possible involvement of some phosphatase(s) in G6P-induced CaMKII activation.

**G6P Treatment Increases Binding of PP2A to CaMKII**—To determine whether a phosphatase is involved in CaMKII activation, CaM-Sepharose was used to pull down endogenous CaMKII from *Xenopus* egg extracts treated with or without G6P. Precipitates were immunoblotted for PP1, PP2A, and PP5. Although there was constitutive binding between PP1 and CaMKII, consistent with a previous report (26), this association did not appear to be regulated metabolically (Fig. 3A). Only PP2A exhibited increased interaction with CaM-Sepharose following G6P treatment. PP2A did not bind directly to CaM in the extract because prior removal of all CaM-associated proteins, including CaMKII (via CaM-Sepharose), did not affect total PP2A levels but prevented G6P-inducible binding of PP2A to CaM-Sepharose (Fig. 3, B and C). Furthermore, endogenous PP2A could be co-immunoprecipitated with anti-CaMKII antibody at greater levels in the presence of G6P (Fig. 3D; note that G6P treatment appeared to increase background CaMKII immunoprecipitation in the presence of G6P, but the PP2A interaction was elevated specifically in the presence of G6P). Together, these data strongly suggest that CaMKII interacts with PP2A, and this interaction is up-regulated by G6P-stimulated metabolism.

**B55\(^{\beta}\) Targets PP2A to Regulate CaMKII Activation**—Functional PP2A is a multiprotein complex containing a catalytic (C) subunit, a scaffolding (A) subunit, and a regulatory (B) subunit. The B subunit typically determines substrate specificity. Four different B subunit families have been identified: B (PR55), B\(^{\prime}\) (B56), B\(^{\prime \prime}\) (PR72), and B\(^{\prime \prime \prime}\) (PR93). The B55 and B56 families have been implicated in cell proliferation/death in several settings (27, 28). We identified a B subunit subtype in the PR55 family, B55\(^{\beta}\) whose interaction with CaM-Sepharose was up-regulated upon G6P treatment. B56 isoforms were unaffected (Fig. 4A). In agreement, G6P could stimulate the interaction of recombinant GST-tagged B55\(^{\beta}\) protein with multiple CaMKII isoforms (Fig. 4B). In addition, recombinant CaMKII\(^{\gamma}\) (the isoform identified in the MS analysis) was added into egg extracts. Although G6P treatment increased background protein-protein interactions (GST alone could pull down some CaMKII protein), we were still able to see that G6P could significantly stimulate the interaction between B55\(^{\beta}\) and the recombinant CaMKII\(^{\gamma}\) protein (Fig. 4C). Taken together, these data suggest that a complex containing CaMKII, PP2A C subunit, A subunit, and B55\(^{\beta}\), forms in the presence of abundant nutrients, potentially contributing to the metabolic activation of CaMKII, and that this increased protein complex formation stimulated by G6P seems to occur with multiple CaMKII isoforms.

To determine whether B55\(^{\beta}\) was required for the observed increase Thr\(^{286}\) phosphorylation of CaMKII following G6P treatment, we immunodepleted B55\(^{\beta}\) from egg extracts and monitored G6P-induced alterations in CaMKII phosphorylation. As shown in Fig. 4D, B55\(^{\beta}\) was largely depleted from egg...
extracts without significant depletion of the PP2A C subunit. The depleted and undepleted extracts were treated with or without G6P, and the Thr\textsuperscript{286} autophosphorylation of CaMKII was monitored. Compared with undepleted extracts, the depleted extracts showed significantly diminished Thr\textsuperscript{286} autophosphorylation in the presence of G6P (Fig. 4E). In addition, by using the GST-tagged \textit{Xenopus} caspase-2 prodomain as a substrate, we were able to monitor the ability of B55\beta-depleted and undepleted extracts to phosphorylate caspase-2, treated with or without G6P. We found that the undepleted extracts could phosphorylate caspase-2 in the presence of G6P, whereas this phosphorylation was largely diminished by B55\beta depletion (Fig. 4F). Because Thr\textsuperscript{286} autophosphorylation and caspase-2 phosphorylation were both indicators of CaMKII activation, both measures indicated diminished CaMKII activation in the B55\beta-depleted extracts, suggesting that B55\beta is critical for G6P-driven CaMKII activation in the \textit{Xenopus} egg extract system. In addition, the fact that the immunodepletion of B55\beta diminished multiple Thr\textsuperscript{286} autophosphorylation bands suggests that B55\beta can regulate multiple CaMKII isoforms (Fig. 4E). Moreover, when endogenous B55\beta was knocked down by B55\beta siRNA in 293T cells, the glucose-driven stimulation of CaMKII Thr\textsuperscript{286} autophosphorylation was suppressed, suggesting that the role of B55\beta in regulating CaMKII is evolutionarily conserved (Fig. 4G).

**FIGURE 2.** Modification of CaMKII by metabolism. A, \textit{Xenopus} egg extracts were treated with or without G6P for 4 h at room temperature and centrifuged at 200,000 \( \times \) g. Cytosolic extracts were fractionated by gel filtration chromatography, and fractions were immunoblotted for CaMKII. B, \textit{Xenopus} egg extracts treated with or without G6P and okadaic acid (OA) were fractionated and analyzed as in A. C–E, post-translational modifications of CaMKII were identified by MS analysis. Upper panels, change of phosphorylation with or without G6P. Lower panels, tandem mass spectra used for identification and localization of phosphorylation modifications on phosphopeptide containing corresponding site. C, Thr\textsuperscript{287} phosphorylation levels with or without G6P. D, Ser\textsuperscript{395} phosphorylation levels with or without G6P. E, Thr\textsuperscript{393} phosphorylation levels with or without G6P. WB, Western blot.
PP2A Dephosphorylates Ser\textsuperscript{395} on CaMKII in the Presence of G6P—Because the MS analysis showed that Thr\textsuperscript{393} and Ser\textsuperscript{395} of CaMKII were dephosphorylated in the presence of G6P, we postulated that these sites might be targeted by B55\(\beta\)-PP2A. To assess this, we generated phospho-antibodies recognizing each of these sites on the \textit{Xenopus} CaMKII\(\gamma\) isoform. Although we were unable to produce a high quality antibody specific for phosphorylated Thr\textsuperscript{393}, we were successful with Ser(P)\textsuperscript{395}. As shown in Fig. 5A, WT CaMKII\(\gamma\) protein (SF9, baculovirus produced) exhibited basal phosphorylation of Ser\textsuperscript{395}. However, the S395A mutation greatly diminished antibody recognition, attesting to its phospho-specificity. To confirm that the phosphorylation level of Ser\textsuperscript{395} was down-regulated by G6P, we incubated \textit{Xenopus} egg extract with or without G6P, precipitation endogenous CaMKII with CalM-Sepharose, and immunoblotted with the phospho-antibody. As shown in Fig. 5B, phosphorylation of Ser\textsuperscript{395} was down-regulated in the presence of G6P (note that the \(\gamma\) is the loading control as the Ser\textsuperscript{395} antibody was designed based on a \(\gamma\) phosphopeptide sequence). Additionally, when B55\(\beta\) was depleted from the \textit{Xenopus} egg extract, the G6P effect was inhibited, strongly suggesting that Ser\textsuperscript{395} was dephosphorylated by the B55\(\beta\)-PP2A (Fig. 5C). Although the Ser\textsuperscript{395} antibody was unable to recognize the CaMKII\(\alpha\) isoform, because B55\(\beta\) immunodepletion could suppress the activation of all CaMKII isoforms, it is quite possible that Ser\textsuperscript{344} (the equivalent residue to Ser\textsuperscript{395} in the \(\alpha\) isoform) is also dephosphorylated by B55\(\beta\)-PP2A.

The Phosphorylation Status of Thr\textsuperscript{287} and Ser\textsuperscript{395} Affects CaMKII Activation—To determine whether B55\(\beta\)-mediated dephosphorylation of Ser\textsuperscript{395} is critical for nutrient-driven activation of CaMKII, we first added CaMKII\(\gamma\) WT, T393A/S395A mutant, and T393D/S395D mutant proteins into \textit{Xenopus} egg extract, treated with or without G6P, and monitored Thr\textsuperscript{287} (Thr\textsuperscript{286} for CaMKII\(\alpha\)) autophosphorylation. As shown in Fig. 5D, after only 10 min of incubation with G6P, the T393A/S395A mutant exhibited higher phosphorylation of Thr\textsuperscript{287}, compared with WT CaMKII. Consistent with these data, G6P-induced stimulation of CaMKII Thr\textsuperscript{287} autophosphorylation was significantly dampened by the T393D/S395D mutations (which may not perfectly mimic phosphorylation) (Fig. 5E). In addition, an \textit{in vitro} kinase assay was performed, incubating CaMKII\(\gamma\) T393A/S395A or T393D/S395D with calmodulin and the \textit{Xenopus} caspase-2 prodomain. We found that the CaMKII T393A/S395A was more able than the T393D/S395D to phosphorylate the caspase-2 prodomain, consistent with the idea that the dephosphorylation of Thr\textsuperscript{393} and Ser\textsuperscript{395} is important for CaMKII activation (Fig. 5F).

DISCUSSION
Although calcium is a central regulator of CaMKII, previous work from our lab has shown that the centrifugal removal of Ca\textsuperscript{2+} stores from \textit{Xenopus} egg extracts does not impede G6P-mediated activation of CaMKII, suggesting that increased available Ca\textsuperscript{2+} does not underlie nutrient-dependent CaMKII activation. Studies of CaMKII regulation have largely focused on Thr\textsuperscript{286} autophosphorylation after Ca\textsuperscript{2+}/CaM binding. Indeed, G6P treatment of egg extract impairs PP1-mediated dephosphorylation of Thr\textsuperscript{286} (25). However, this is unlikely to drive
CaMKII activation because this phosphorylation is a result of activation. It has also been reported that CoA generation is increased downstream of G6P addition to egg extracts (though the mechanism underlying this increase is not clear) and that CoA can directly bind to and activate CaMKII. We have now identified an additional metabolically regulated break to CaMKII activation that must be lifted for G6P to robustly activate CaMKII via dephosphorylation of Thr393/Ser395.

In addition to Thr393/Ser395 phosphorylation, MS analysis also identified several previously uncharacterized sites with increased phosphorylation in the presence of G6P: Ser311, Ser326, Ser333, and Thr421 (data not shown). Although we have not yet investigated these sites, they may contribute to full CaMKII activation. The T393D/S395D mutant of CaMKII is less potently activated by G6P than WT CaMKII, consistent with a requirement for phosphorylation of additional sites for full activation. Furthermore, the T393A/S395A mutant is not spontaneously active, so there must be additional metabolic input for full activation, likely CoA (the high levels of exogenous CoA used in the published experiments may have forced activation of CaMKII despite the brake (23)).

Effects of G6P-stimulated Dephosphorylation on CaMKII—Interestingly, most of the sites whose phosphorylation was altered by nutrient status are located in the association domain of CaMKII, responsible for self-association (29, 30). However, our gel filtration results suggest that oligomerization itself is not affected by phosphorylation. Rather, G6P appears to shift the molecular weight of the full holoenzyme, suggesting association of additional factors. Importantly, the molecular weight shift was largely abrogated by treatment with okadaic acid, consistent with the idea that dephosphorylation of Thr393/Ser395 might be required. It is attractive to speculate that Thr393/Ser395 dephosphorylation might allow binding of additional regulatory factors to the CaMKII association domain. Interestingly, one variant of CaMKIIγ (CaMKIIγ-G-2) contains within its association domain a targeting sequence essential for ERK
activation and contractility of smooth muscle cells, suggesting that the association domain can perform roles other than self-association (31).

Regulation of Thr393/Ser395 Phosphorylation—Our data demonstrate a previously unknown role for the B55/H9252 targeting subunit of PP2A in regulating CaMKII. Often, the B subunit not only targets PP2A to the correct substrate(s) but also serves as a key locus of regulation. For example, Chk1-mediated phosphorylation B56 can inhibit PP2A-mediated dephosphorylation and activation of the Cdc25 mitotic regulator during DNA damage checkpoint signaling (27). Although it is possible that the CamKII-B55 association is regulated at the level of CamKII modification, we speculate that B55 may be differentially modified in a nutrient-dependent manner to alter targeting of PP2A to CamKII. Such a modification could be sensitive to CoA levels.

Thr393 and Ser395 are phosphorylated in the egg extract before any treatment. Therefore, Xenopus egg extracts must contain a kinase(s) directed against these sites. Because Thr393/Ser395 phosphorylation decreases prior to CamKII activation, these residues are clearly not autophosphorylation sites. The relevant kinase is not currently known but may provide an additional locus of metabolic regulation if its activity is high in the presence of abundant nutrients and low upon their depletion. Future experiments will be directed toward the identification and characterization of kinase(s) directed toward these sites.

Metabolic Regulation of CamKII in Other Signaling Pathways—CamKII is ubiquitously expressed and has a diverse array of substrates. For example, CamKII regulates the contractility of smooth muscle cells, and reduced contractile force has been observed to be associated with altered metabolism. Although this decrease in force has been associated with impaired Ca2+ flux, metabolically controlled dephosphorylation of Thr393 and Ser395 might also control the activation of CamKII in smooth muscle cells. It will be interesting to determine whether these sites play a
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similar role in regulating the highly abundant CaMKIIα isoform in neurons, which is important for long term potentiation. Similarly, B55β may control CaMKII in other physiological paradigms of regulation or metabolic control, warranting further investigation in other physiological settings.

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