THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 52, pp. 35882–35890, December 26, 2014

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

Received for publication, June 9, 2014, and in revised form, November 3, 2014 Published, JBC Papers in Press, November 4, 2014, DOI 10.1074/jbc.M114.585844

Bofu Huang[‡], Chih-Sheng Yang[‡], Jeffrey Wojton[‡], Nai-Jia Huang[‡], Chen Chen[‡], Erik J. Soderblom[§], Liguo Zhang[¶1], and Sally Kornbluth[‡2]

From the [‡]Department of Pharmacology and Cancer Biology, the [§]Proteomics and Metabolomics Core Facility, and the [¶]Division of Medical Oncology, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27705

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 metabolized 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[3] 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, $Thr(P)^{286}$ 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

* This work was supported, in whole or in part, by National Institutes of Health Grant RO1 GM080333.
[1] To whom correspondence may be addressed. Tel.: 919-613-8625; E-mail: liguo.zhang@duke.edu.
[2] To whom correspondence may be addressed. Tel.: 919-684-2631; E-mail: sally.kornbluth@duke.edu.

[3] 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 B55β. 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 nutrient 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 aprotonin/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-B55β siRNA was purchased from Santa Cruz Biotechnology to knock down B55β in 293T cells. Control siRNA was designed to target a nonmammalian protein, firefly luciferase (5′-CGUACGCGGAAUACUUCGA-3′).

Plasmids and Protein Preparation—Xenopus CaMKIIγ was amplified by PCR using the primers 5′-GGGGAATTCATGGCCACTACCCAGACTTGCACC-3′ and 5′-GGGCTCGAGTCACTGGAGAGGGGCTGCTGGTGC-3′. Purified PCR products were cloned into the EcoRI and XhoI sites of pENTR3C. The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate point mutants in CaMKIIγ in pENTR3C. The T393A/S395A primers were 5′-CAGATGGGATAAAAGGATCAGCAGAGGCTTGCAACACCACCACTGAAG-3′ and its complement. The T393D/S395D primers were 5′-CAGATGGGATAAAAGGATCAGACGAGGATTGCAACACCACCACTGAAG-3′ and its complement. The Gateway LR Clonase II enzyme mix (Invitrogen) was used to transfer CaMKIIγ wild type and mutants from pENTR3C into pDEST24. Bacmid DNAs were produced by transforming pDEST24 plasmids into MAX Efficiency DH10Bac Chemically Competent *Escherichia coli* (Invitrogen) and transfected 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 1× egg lysis buffer (10 mM HEPES, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 0.25 M 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 Thr^{393} and Ser^{395} of *Xenopus* CaMKIIγ: [H]-VHNATDGIKGSTESCN-[NH_2] (non-phospho-peptide), [H]-VHNATDGIKGS-Thr(P)-ESCN-[NH_2] (Thr(P)^{393} peptide), and [H]-VHNATDGIKGSTE-Ser(P)-CN-[NH_2] (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 TiO_2 Protea Tip (Protea Bio) as per the manufacturer's recommended protocol. Samples were then subjected to LC-MS/MS analysis using a Waters NanoAquity 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

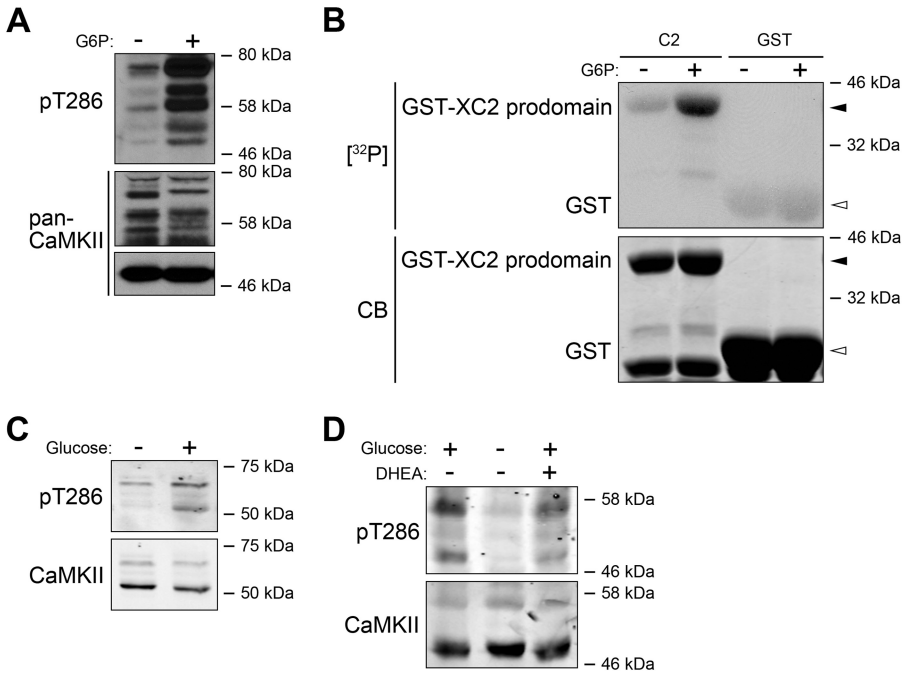

FIGURE 1. **CaMKII activation is sensitive to metabolic status.** *A*, *Xenopus* egg extracts were treated with or without G6P for 0. 5 h at room temperature and analyzed for CaMKII Thr^{286} autophosphorylation using a Thr(P)^{286} antibody. Note that the *middle* and *bottom panels* are from two different films as the CaMKII antibody recognized CaMKIIα (~50 kDa) much more strongly than the other isoforms, so detection of this isoform and the others required very different exposures. *B*, GST tagged *Xenopus* caspase-2 prodomain or GST bound to glutathione-Sepharose was incubated with *Xenopus* egg extracts supplemented with [γ-^{32}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 Thr^{286} autophosphorylation. *D*, glucose-starved 293T cells treated with or without glucose and with or without dehydroepiandrosterone (*DHEA*) were lysed and analyzed for CaMKII Thr^{286} autophosphorylation.

precursor ions were acquired either in the Orbitrap with r = 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 Da on Cys) was set as a fixed modification, whereas oxidation (+15.9949 Da on Met) and phosphorylation (+79.9663 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 phosphopeptides. 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 TiO_2 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 μl of glutathione-Sepharose containing 4 μg of recombinant GST tagged caspase-2 prodomain or GST-only fusion proteins were incubated in B55β-depleted or undepleted egg extracts together with 20 μCi of [γ-^{32}P]ATP, treated by 20 mM G6P or water, at 30 °C for 1 h. Samples were washed, eluted with SDS-PAGE sample buffer, and resolved by SDS-PAGE for autoradiography.

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 Thr^{286}, an indicator of CaMKII activation. By directly treating egg extracts with or without G6P, we discovered that G6P increased phosphorylation of Thr^{286} on CaMKII, a known site of CaMKII autophosphorylation (Fig. 1*A*; 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 treatment 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. 1*B*). 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. 1*C*). 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. 1*D*).

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. 2*A*, 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 CaMKIIγ 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 CaMKIIα) phosphorylation (Fig. 2*C*). 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. 2*A* 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. 2*B*). 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. 3*A*). 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. 3*D*; 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.

B55β 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′ (B56), B″ (PR72), and B‴ (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, B55β whose interaction with CaM-Sepharose was up-regulated upon G6P treatment. B56 isoforms were unaffected (Fig. 4*A*). In agreement, G6P could stimulate the interaction of recombinant GST-tagged B55β protein with multiple CaMKII isoforms (Fig. 4*B*). In addition, recombinant CaMKIIγ (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 B55β and the recombinant CaMKIIγ protein (Fig. 4*C*). Taken together, these data suggest that a complex containing CaMKII, PP2A C subunit, A subunit, and B55β, 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 B55β was required for the observed increased Thr^{286} phosphorylation of CaMKII following G6P treatment, we immunodepleted B55β from egg extracts and monitored G6P-induced alterations in CaMKII phosphorylation. As shown in Fig. 4*D*, B55β was largely depleted from egg

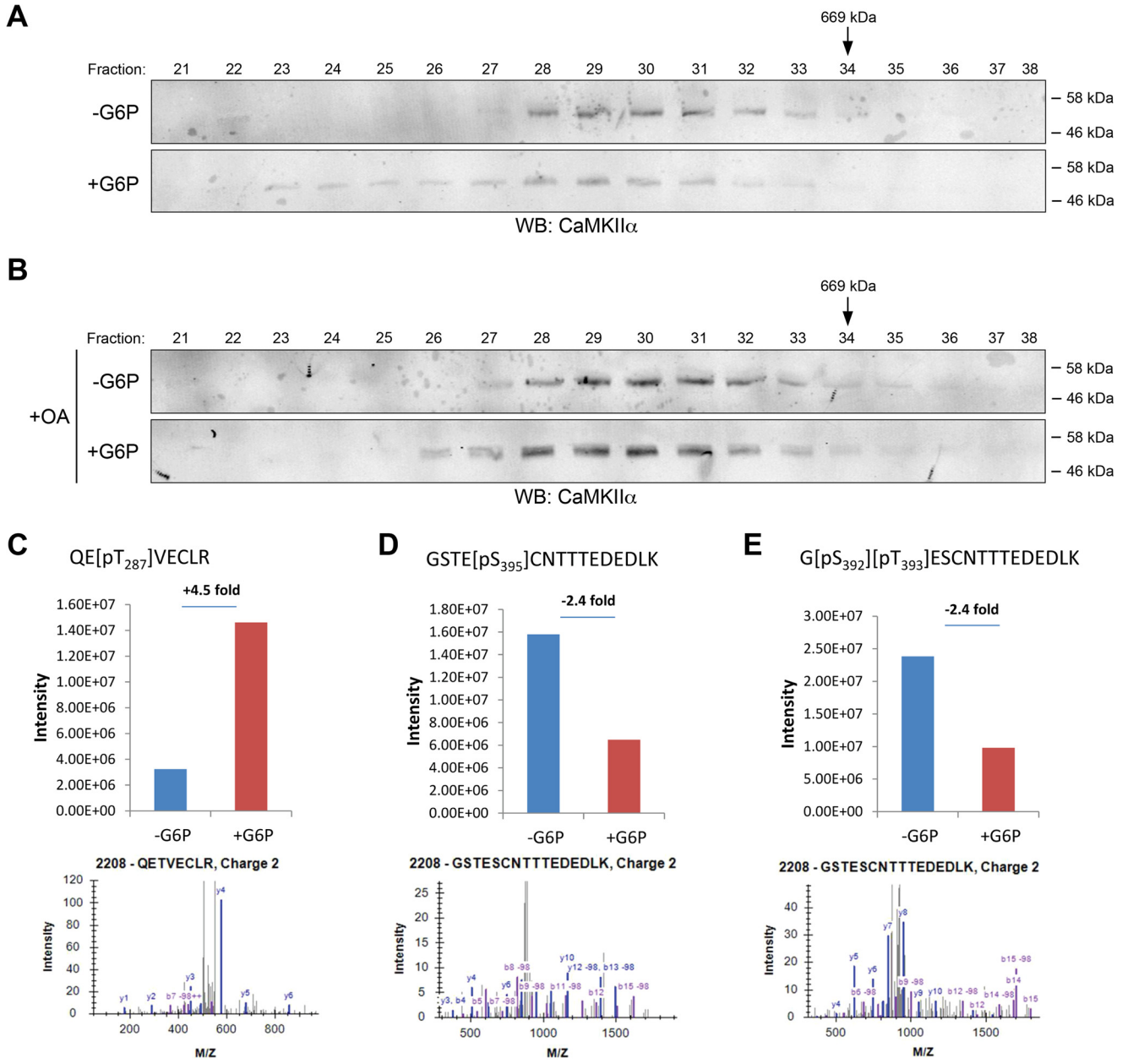

FIGURE 2. **Modification of CaMKII by metabolism.** *A*, *Xenopus* egg extracts were treated with or without G6P for 4 h at room temperature and centrifuged at 200,000 × *g*. Cytosolic extracts were fractionated by gel filtration chromatography, and fractions were immunoblotted for CaMKII. *B*, *Xenopus* 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^{287} phosphorylation levels with or without G6P. *D*, Ser^{395} phosphorylation levels with or without G6P. *E*, Thr^{393} phosphorylation levels with or without G6P. *WB*, Western blot.

extracts without significant depletion of the PP2A C subunit. The depleted and undepleted extracts were treated with or without G6P, and the Thr^{286} autophosphorylation of CaMKII was monitored. Compared with undepleted extracts, the depleted extracts showed significantly diminished Thr^{286} autophosphorylation in the presence of G6P (Fig. 4*E*). In addition, by using the GST-tagged *Xenopus* caspase-2 prodomain as a substrate, we were able to monitor the ability of B55β-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 B55β depletion (Fig. 4*F*). Because Thr^{286} autophosphorylation and caspase-2 phosphorylation were both indicators of CaMKII activation, both measures indicated diminished CaMKII activation in the B55β-depleted extracts, suggesting that B55β is critical for G6P-driven CaMKII activation in the *Xenopus* egg extract system. In addition, the fact that the immunodepletion of B55β diminished multiple Thr^{286} autophosphorylation bands suggests that B55β can regulate multiple CaMKII isoforms (Fig. 4*E*). Moreover, when endogenous B55β was knocked down by B55β siRNA in 293T cells, the glucose-driven stimulation of CaMKII Thr^{286} autophosphorylation was suppressed, suggesting that the role of B55β in regulating CaMKII is evolutionarily conserved (Fig. 4*G*).

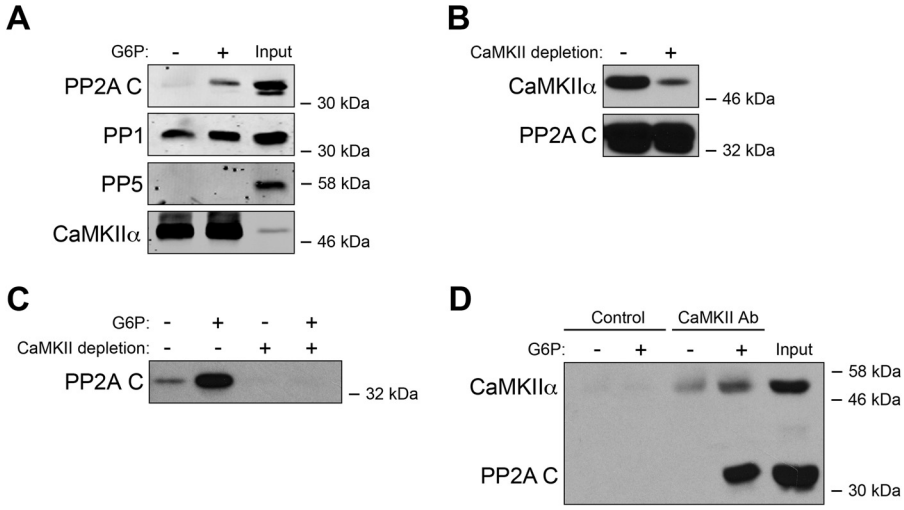

FIGURE 3. **CaMKII-PP2A interactions are regulated by G6P.** *A*, CaM-Sepharose was dipped into *Xenopus* egg extracts that had been treated with or without G6P for 0.5 h at room temperature and incubated for 1 h at 4 °C. Beads were retrieved by centrifugation and analyzed for the presence of candidate phosphatases by immunoblotting. *B*, CaM-Sepharose was incubated with *Xenopus* egg extracts for 1 h at 4 °C and then removed by centrifugation. This process was repeated three times. Depleted and undepleted extracts were analyzed by CaMKII or PP2A immunoblotting. *C*, CaM-Sepharose was incubated with either CaMKII-depleted or undepleted extracts described in *B* treated with or without G6P. Beads were retrieved by centrifugation and analyzed by PP2A C subunit immunoblotting. *D*, CaMKII antibody or control IgG coupled to protein G beads was dipped into *Xenopus* egg extracts treated with or without G6P, incubated for 1 h at 4 °C, and retrieved for CaMKII or PP2A C immunoblotting.

PP2A Dephosphorylates Ser^{395} on CaMKII in the Presence of G6P—Because the MS analysis showed that Thr^{393} and Ser^{395} of CaMKII were dephosphorylated in the presence of G6P, we postulated that these sites might be targeted by B55β-PP2A. To assess this, we generated phospho-antibodies recognizing each of these sites on the *Xenopus* CaMKIIγ isoform. Although we were unable to produce a high quality antibody specific for phosphorylated Thr^{393}, we were successful with Ser(P)^{395}. As shown in Fig. 5*A*, WT CaMKIIγ protein (SF9, baculovirus produced) exhibited basal phosphorylation of Ser^{395}. However, the S395A mutation greatly diminished antibody recognition, attesting to its phospho-specificity. To confirm that the phosphorylation level of Ser^{395} was down-regulated by G6P, we incubated *Xenopus* egg extract with or without G6P, precipitated endogenous CaMKII with CaM-Sepharose, and immunoblotted with the phospho-antibody. As shown in Fig. 5*B*, phosphorylation of Ser^{395} was down-regulated in the presence of G6P (note that the γ isoform is shown in the loading control as the Ser^{395} antibody was designed based on a γ phosphopeptide sequence). Additionally, when B55β was depleted from the *Xenopus* egg extract, the G6P effect was inhibited, strongly suggesting that Ser^{395} was dephosphorylated by the B55β-PP2A (Fig. 5*C*). Although the Ser^{395} antibody was unable to recognize the CaMKIIα isoform, because B55β immunodepletion could suppress the activation of all CaMKII isoforms, it is quite possible that Ser^{344} (the equivalent residue to Ser^{395} in the α isoform) is also dephosphorylated by B55β-PP2A.

The Phosphorylation Status of Thr^{393} and Ser^{395} Affects CaMKII Activation—To determine whether B55β-mediated dephosphorylation of Ser^{395} is critical for nutrient-driven activation of CaMKII, we first added CaMKIIγ WT, T393A/S395A mutant, and T393D/S395D mutant proteins into *Xenopus* egg extract, treated with or without G6P, and monitored Thr^{287} (Thr^{286} for CaMKIIα) autophosphorylation. As shown in Fig. 5*D*, after only 10 min of incubation with G6P, the T393A/S395A mutant exhibited higher phosphorylation of Thr^{287}, compared with WT CaMKII. Consistent with these data, G6P-induced stimulation of CaMKII Thr^{287} autophosphorylation was significantly dampened by the T393D/S395D mutations (which may not perfectly mimic phosphorylation) (Fig. 5E). In addition, an *in vitro* kinase assay was performed, incubating CaMKIIγ T393A/S395A or T393D/S395D with calmodulin and the *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^{393} and Ser^{395} is important for CaMKII activation (Fig. 5*F*). Finally, CaMKIIγ WT, T393A/S395A, and T393D/S395D were added into egg extracts, treated with or without G6P, and pulled down using GST-tagged B55β to monitor the interaction between B55β and CaMKII proteins. We found that the T393A/S395A mutant exhibited stronger interactions and that the T393D/S395D showed weaker interactions with B55β, compared with the WT CaMKII, suggesting that the dephosphorylation of Thr^{393} and Ser^{395} might stabilize the binding of B55β to CaMKII (Fig. 5, *G* and *H*). Collectively, these data suggest that nutrient status is communicated to CaMKII in part through the B55β/PP2A-mediated dephosphorylation of CaMKII, a novel locus for the control of CaMKII.

DISCUSSION

Although calcium is a central regulator of CaMKII, previous work from our lab has shown that the centrifugal removal of Ca^{2+} stores from *Xenopus* egg extracts does not impede G6P-mediated activation of CaMKII, suggesting that increased available Ca^{2+} does not underlie nutrient-dependent CaMKII activation. Studies of CaMKII regulation have largely focused on Thr^{286} autophosphorylation after Ca^{2+}/CaM binding. Indeed, G6P treatment of egg extract impairs PP1-mediated dephosphorylation of Thr^{286} (25). However, this is unlikely to drive

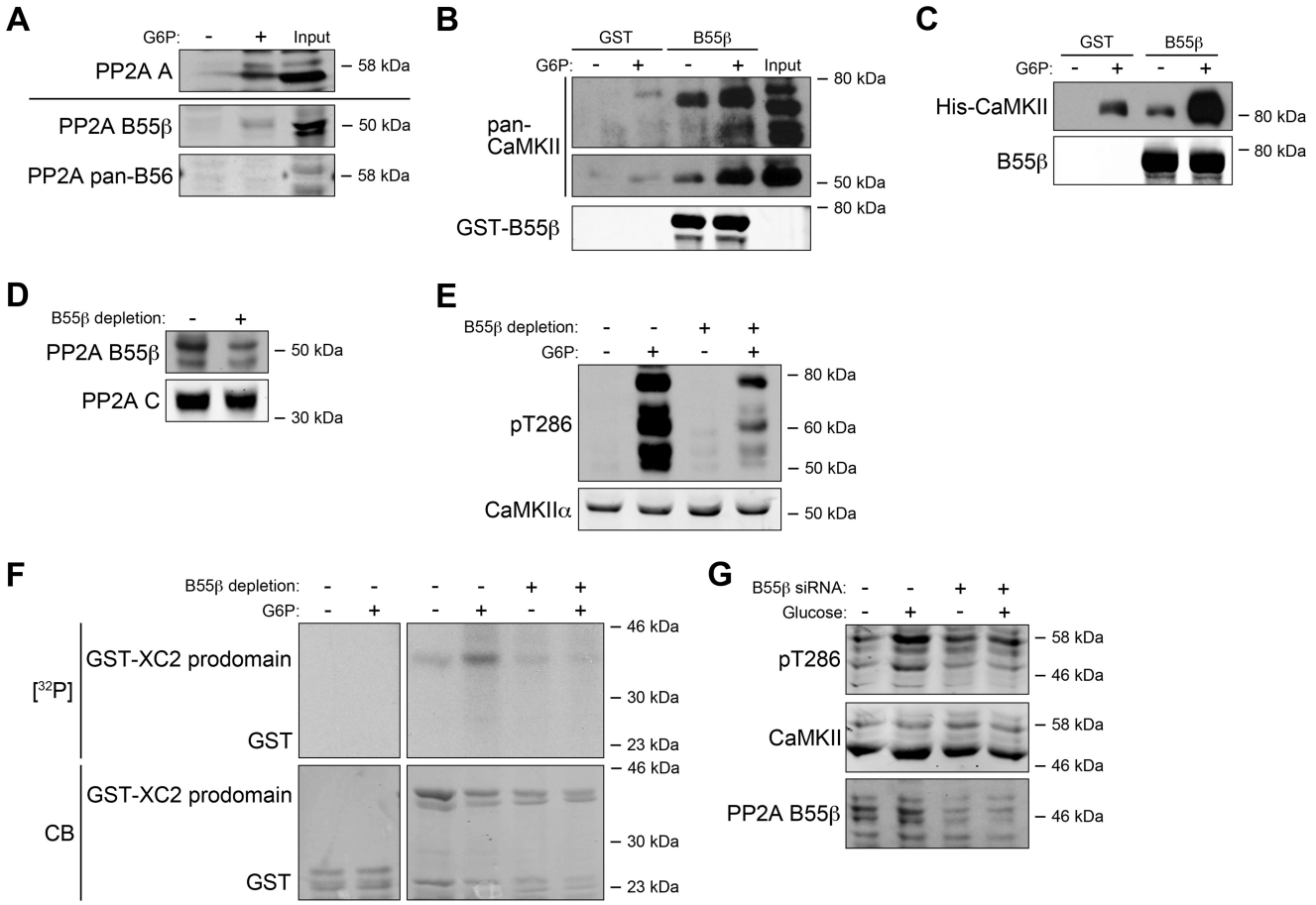

FIGURE 4. **B55β regulates CaMKII activation.** *A*, CaM-Sepharose was incubated with *Xenopus* egg extracts treated with or without G6P and incubated for 1 h at 4 °C. Beads were retrieved by centrifugation and analyzed by immunoblotting for PP2A regulatory subunits. *B*, GST-B55β or GST bound to glutathione-Sepharose was incubated for 1 h at 4 °C with *Xenopus* egg extracts treated with or without G6P. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. Note that the *top* and *middle panels* are from two different films with different exposures as the CaMKII antibody recognized CaMKIIα much more strongly than the other isoforms. *C*, *Xenopus* CaMKIIγ WT protein expressed from baculoviral vectors in SF9 cells was added into *Xenopus* egg extracts for 0.5 h at room temperature, and then the extracts were treated with or without G6P for 0.5 h at room temperature and incubated with GST-B55β or GST bound to glutathione-Sepharose for 1 h at 4 °C. The beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. *D*, B55β antibody or control rabbit IgG bound to Dynabead-linked protein A was incubated with *Xenopus* egg extracts for 1 h at 4 °C and removed with a magnet (repeated three times). Extracts were analyzed by B55β and PP2A C subunit immunoblotting. *E*, the B55β-depleted or undepleted extract was treated with or without G6P for 0.5 h and analyzed by pT286 immunoblotting. *F*, GST tagged *Xenopus* caspase-2 prodomain or GST bound to glutathione-Sepharose was incubated with B55β-depleted or undepleted *Xenopus* egg extracts supplemented with [γ-^{32}P]ATP and treated with or without G6P. The samples were resolved by SDS-PAGE and detected by autoradiography. *CB*, Coomassie Blue. *G*, B55β-specific or control siRNA-treated 293T cells were glucose-starved for 12 h and then incubated with or without 25 mM glucose. The lysates were analyzed by immunoblotting for pT286, CaMKII, and B55β.

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 Thr^{393}/Ser^{395}.

In addition to Thr^{393}/Ser^{395} phosphorylation, MS analysis also identified several previously uncharacterized sites with increased phosphorylation in the presence of G6P: Ser^{311}, Ser^{326}, Ser^{333}, and Thr^{421} (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 Thr^{393}/Ser^{395} might be required. It is attractive to speculate that Thr^{393}/Ser^{395} dephosphorylation might allow binding of additional regulatory factors to the CaMKII association domain. Interestingly, one variant of CaMKIIγ (CaMKIIγG-2) contains within its association domain a targeting sequence essential for ERK

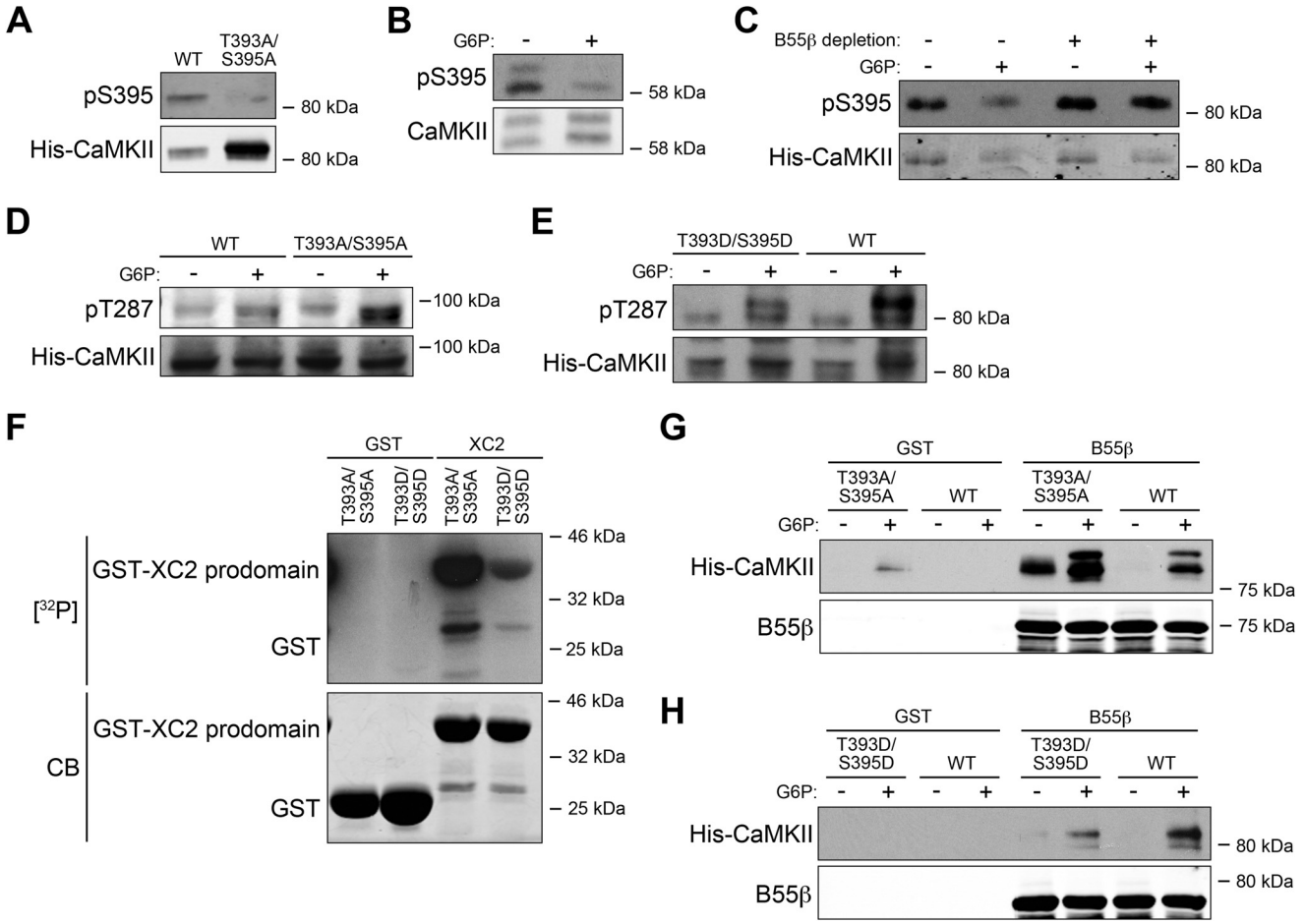

FIGURE 5. **B55β-mediated Thr^{393}/Ser^{395} dephosphorylation contributes to CaMKII activation.** *A*, *Xenopus* WT and T393A/S395A mutant CaMKIIγ expressed from baculoviral vectors in SF9 cells were analyzed by Ser(P)^{395} immunoblotting. *B*, CaM-Sepharose was incubated with *Xenopus* egg extracts treated with or without G6P, retrieved by centrifugation, and analyzed by Ser(P)^{395} immunoblotting. *C*, B55β-depleted or undepleted *Xenopus* egg extracts with the addition of recombinant CaMKIIγ were treated with or without G6P. Recombinant CaMKIIγ was precipitated with CaM-Sepharose. The beads were retrieved by centrifugation and analyzed for Ser(P)^{395}. *D*, CaMKIIγ WT or T393A/S395A mutant was added into *Xenopus* egg extracts for 0.5 h at room temperature, and then the extracts were treated with or without G6P and analyzed for pT287. *E*, CaMKIIγ WT or T393D/S395D mutant was treated and processed as in *D*. *F*, GST tagged *Xenopus* caspase-2 prodomain or GST bound to glutathione-Sepharose was incubated with *Xenopus* CaMKIIγ T393A/S395A or T393D/S395D mutants, calmodulin, and [γ-^{32}P] ATP at 30 °C for 0.5 h. Samples were resolved by SDS-PAGE and detected by autoradiography. *CB*, Coomassie Blue. *G*, *Xenopus* CaMKIIγ WT or T393A/S395A mutant proteins were added into *Xenopus* egg extracts for 0.5 h at room temperature, and then the extracts were treated with or without G6P for 0.5 h at room temperature and incubated with GST-B55β or GST bound to glutathione-Sepharose for 1 h at 4 °C. The beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. *H*, *Xenopus* CaMKIIγ WT or T393D/S395D mutant proteins were added into *Xenopus* egg extracts for 0.5 h at room temperature, and then the extracts were treated with or without G6P for 0.5 h at room temperature and incubated with GST-B55β or GST bound to glutathione-Sepharose for 1 h at 4 °C. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting.

activation and contractility of smooth muscle cells, suggesting that the association domain can perform roles other than self-association (31).

Regulation of Thr^{393}/Ser^{395} Phosphorylation—Our data demonstrate a previously unknown role for the B55β 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-B55β association is regulated at the level of CaMKII modification, we speculate that B55β 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.

Thr^{393} and Ser^{395} are phosphorylated in the egg extract before any treatment. Therefore, *Xenopus* egg extracts must contain a kinase(s) directed against these sites. Because Thr^{393}/Ser^{395} 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, CaMKIIγ 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 Ca^{2+} flux, metabolically controlled dephosphorylation of Thr^{393} and Ser^{395} might also control the activation of CaMKIIγ in smooth muscle cells. It will be interesting to determine whether these sites play a

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.

Acknowledgments—We thank Dr. M. Arthur Moseley for supporting the MS analysis and Dr. Christopher Freel for assistance with all aspects of figure preparation.

REFERENCES

1. Cheung, H. H., Lynn Kelly, N., Liston, P., and Korneluk, R. G. (2006) Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp. Cell Res.* **312,** 2347–2357
2. 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
3. Tu, S., McStay, G. P., Boucher, L. M., Mak, T., Beere, H. M., and Green, D. R. (2006) *In situ* trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. *Nat. Cell Biol.* **8,** 72–77
4. Ho, L. H., Taylor, R., Dorstyn, L., Cakouros, D., Bouillet, P., and Kumar, S. (2009) A tumor suppressor function for caspase-2. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 5336–5341
5. Parsons, M. J., McCormick, L., Janke, L., Howard, A., Bouchier-Hayes, L., and Green, D. R. (2013) Genetic deletion of caspase-2 accelerates MMTV/c-neu-driven mammary carcinogenesis in mice. *Cell Death Differ.* **20,** 1174–1182
6. Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* **297,** 1352–1354
7. 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
8. Bonzon, C., Bouchier-Hayes, L., Pagliari, L. J., Green, D. R., and Newmeyer, D. D. (2006) Caspase-2-induced apoptosis requires bid cleavage: a physiological role for bid in heat shock-induced death. *Mol. Biol. Cell* **17,** 2150–2157
9. Nutt, L. K., Margolis, S. S., Jensen, M., Herman, C. E., Dunphy, W. G., Rathmell, J. C., and Kornbluth, S. (2005) Metabolic regulation of oocyte cell death through the CaMKII-mediated phosphorylation of caspase-2. *Cell* **123,** 89–103
10. Nutt, L. K., Buchakjian, M. R., Gan, E., Darbandi, R., Yoon, S. Y., Wu, J. Q., Miyamoto, Y. J., Gibbons, J. A., Andersen, J. L., Freel, C. D., Tang, W., He, C., Kurokawa, M., Wang, Y., Margolis, S. S., Fissore, R. A., and Kornbluth, S. (2009) Metabolic control of oocyte apoptosis mediated by 14-3-3ζ-regulated dephosphorylation of caspase-2. *Dev. Cell* **16,** 856–866
11. Andersen, J. L., Thompson, J. W., Lindblom, K. R., Johnson, E. S., Yang, C. S., Lilley, L. R., Freel, C. D., Moseley, M. A., and Kornbluth, S. (2011) A biotin switch-based proteomics approach identifies 14-3-3ζ as a target of Sirt1 in the metabolic regulation of caspase-2. *Mol. Cell* **43,** 834–842
12. Waxham, M. N., Malenka, R. C., Kelly, P. T., and Mauk, M. D. (1993) Calcium/calmodulin-dependent protein kinase II regulates hippocampal synaptic transmission. *Brain Res.* **609,** 1–8
13. Silva, A. J., Stevens, C. F., Tonegawa, S., and Wang, Y. (1992) Deficient hippocampal long-term potentiation in α-calcium-calmodulin kinase II mutant mice. *Science* **257,** 201–206
14. Rokolya, A., and Singer, H. A. (2000) Inhibition of CaM kinase II activation and force maintenance by KN-93 in arterial smooth muscle. *Am. J. Physiol. Cell Physiol.* **278,** C537–C545
15. Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* **8,** 2527–2539
16. Kolodziej, S. J., Hudmon, A., Waxham, M. N., and Stoops, J. K. (2000) Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIα and truncated CaM kinase IIα reveal a unique organization for its structural core and functional domains. *J. Biol. Chem.* **275,** 14354–14359
17. Colbran, R. J., Smith, M. K., Schworer, C. M., Fong, Y. L., and Soderling, T. R. (1989) Regulatory domain of calcium/calmodulin-dependent protein kinase: II. mechanism of inhibition and regulation by phosphorylation. *J. Biol. Chem.* **264,** 4800–4804
18. Colbran, R. J., Fong, Y. L., Schworer, C. M., and Soderling, T. R. (1988) Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **263,** 18145–18151
19. Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. *Neuron* **12,** 943–956
20. Meyer, T., Hanson, P. I., Stryer, L., and Schulman, H. (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* **256,** 1199–1202
21. Patton, B. L., Miller, S. G., and Kennedy, M. B. (1990) Activation of type II calcium/calmodulin-dependent protein kinase by Ca^{2+}/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin-binding domain. *J. Biol. Chem.* **265,** 11204–11212
22. Hanson, P. I., and Schulman, H. (1992) Inhibitory autophosphorylation of multifunctional Ca^{2+}/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. *J. Biol. Chem.* **267,** 17216–17224
23. McCoy, F., Darbandi, R., Lee, H. C., Bharatham, K., Moldoveanu, T., Grace, C. R., Dodd, K., Lin, W., Chen, S. I., Tangallapally, R. P., Kurokawa, M., Lee, R. E., Shelat, A. A., Chen, T., Green, D. R., Harris, R. A., Lin, S. H., Fissore, R. A., Colbran, R. J., and Nutt, L. K. (2013) Metabolic activation of CaMKII by coenzyme A. *Mol. Cell* **52,** 325–339
24. 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
25. McCoy, F., Darbandi, R., Chen, S. I., Eckard, L., Dodd, K., Jones, K., Baucum, A. J., 2nd, Gibbons, J. A., Lin, S. H., Colbran, R. J., and Nutt, L. K. (2013) Metabolic regulation of CaMKII protein and caspases in *Xenopus laevis* egg extracts. *J. Biol. Chem.* **288,** 8838–8848
26. Shields, S. M., Ingebritsen, T. S., and Kelly, P. T. (1985) Identification of protein phosphatase 1 in synaptic junctions: dephosphorylation of endogenous calmodulin-dependent kinase II and synapse-enriched phosphoproteins. *J. Neurosci.* **5,** 3414–3422
27. Margolis, S. S., Perry, J. A., Forester, C. M., Nutt, L. K., Guo, Y., Jardim, M. J., Thomenius, M. J., Freel, C. D., Darbandi, R., Ahn, J. H., Arroyo, J. D., Wang, X. F., Shenolikar, S., Nairn, A. C., Dunphy, W. G., Hahn, W. C., Virshup, D. M., and Kornbluth, S. (2006) Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell* **127,** 759–773
28. Healy, A. M., Zolnierowicz, S., Stapleton, A. E., Goebl, M., DePaoli-Roach, A. A., and Pringle, J. R. (1991) CDC55, a *Saccharomyces cerevisiae* gene involved in cellular morphogenesis: identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. *Mol. Cell. Biol.* **11,** 5767–5780
29. Kolb, S. J., Hudmon, A., Ginsberg, T. R., and Waxham, M. N. (1998) Identification of domains essential for the assembly of calcium/calmodulin-dependent protein kinase II holoenzymes. *J. Biol. Chem.* **273,** 31555–31564
30. Shen, K., and Meyer, T. (1998) *In vivo* and *in vitro* characterization of the sequence requirement for oligomer formation of Ca^{2+}/calmodulin-dependent protein kinase IIα. *J. Neurochem.* **70,** 96–104
31. Marganski, W. A., Gangopadhyay, S. S., Je, H. D., Gallant, C., and Morgan, K. G. (2005) Targeting of a novel Ca^{2+}/calmodulin-dependent protein kinase II is essential for extracellular signal-regulated kinase-mediated signaling in differentiated smooth muscle cells. *Circ. Res.* **97,** 541–549