Stimulation of T lymphocytes results in a rapid increase in intracellular calcium concentration ([Ca^{2+}_i]) that parallels the activation of Ca^{2+}-calmodulin–dependent protein kinase IV (CaMKIV), a nuclear enzyme that can phosphorylate and activate the cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB). However, inactivation of CaMKIV occurs despite the sustained increase in [Ca^{2+}_i], that is required for T cell activation. A stable and stoichiometric complex of CaMKIV with protein serine-threonine phosphatase 2A (PP2A) was identified in which PP2A dephosphorylates and inactivates CaMKIV. This complex is essential for T cell activation, and its disruption results in impaired T cell function. These findings reveal an intracellular signaling mechanism whereby a protein serine-threonine kinase (CaMKIV) is regulated by a tightly associated protein serine-threonine phosphatase (PP2A).

REFERENCES AND NOTES

10. The plasmid pCMV/BJEGO was constructed by inserting the BJEGO fusion fragment [Xho I to Xba I] [G. Friedich et al., Genes Dev. 5 (no. 9), 1513 (1991)] into pCMV (Clonetech) between the Not I sites.
11. The transgene was detected in transfected cells, fusions, and tissue from adult animals by PCR using a 21-base sense primer (ACT4BEGO) 5’- GACCTGCGTACACCTCCTGTCGG-3’, and a 22-base antisense primer (ACT4BEGO) 5’-CACACCCAGTCCGACGAACTGCTG-3’ (Ampfot Biotech). Reactions were run for 35 cycles, with denaturation at 95°C for 30 s, annealing at 65°C for 1 min, followed by extension for 2 min at 72°C. A final extension for 10 min at 72°C was included after the last cycle. The amplified product was a 752-base pair (bp) fragment. The samples were analyzed by separating them by size in a 1% tris-borate EDTA agarose gel containing ethidium bromide.
12. For nuclear transfer, bovine oocytes were aspirated before a homozygous mutant is produced. The two rounds of selection will require at least two generations, and likely more, to obtain a production herd. This is a savings of 2 years for each generation. Finally, the somatic cell nuclear transfer approach could broaden the scope of use of transgenic cattle. With previous microinjection techniques, about 500 embryos would have to be injected and transferred to recipient cows to get one transgenic offspring (16). For the nuclear transfer technique with transgenic somatic cells, the transfer of nine embryos to four cows produced a transgenic offspring, greatly reducing the time and costs involved. With the nuclear transfer approach, an entire herd of the appropriate sex transgenic cattle could be produced in one generation, whereas the traditional microinjection approach would require at least two generations, and likely more, to obtain a production herd. This is a savings of 2 years for each generation. Finally, the somatic cell nuclear transfer approach could broaden the scope of use of transgenic cattle because it allows the targeting of DNA inserts to specific sites in the genome. This is important for deleting or replacing bovine genes that might interfere with human protein isolation or cause rejection of grafted tissues. Inserting genes into a selected site could be used to ensure tissue-specific and consistent expression levels of transgenes. Furthermore, insertion of genes into the same site in multiple lines of animals could be used to quickly generate homozygous lines of animals while avoiding inbreeding.
plexes containing active protein kinases and phosphatases provide an alternative and conceptually attractive mechanism by which the appropriate phosphorylation state of intracellular substrates is maintained. The α subunit of casein kinase II (CKII), but not the CKII holoenzyme (containing α and β subunits), associates with the catalytic (C) subunit of PP2A (3). This association was proposed as a mechanism for growth suppression in which CKII-stimulated phosphorylation of the C subunit of PP2A would increase the activity of the phosphatase. However, PP2A exists in cells as a heterotetrameric holoenzyme rather than as a free catalytic subunit (4), and phosphorylation of the C subunit of PP2A on Ser-Thr residues in vivo has not been shown.

CaMKIV is important for T cell activation (5). Stimulation of the T cell receptor (TCR) causes increases in [Ca\(^{2+}\)], and activation of CaMKIV. Because CaMKIV phosphorylates the nuclear protein CREB on Ser133, it appears to contribute to increased phosphorylation of the nuclear protein CREB on activation of CaMKIV. Because CaMKIV phosphorylates the nuclear protein CREB on Ser133, it appears to contribute to increased CREB phosphorylated on Ser 133 (CREB phosphorylated on Ser 133) in this cell type, which may be an important mechanism for growth suppression (6).

CaMKIV is phosphorylated by the catalytic subunit of PP2A (7). Because CaMKIV binds to the C subunit of PP2A, it appears to contribute to increased CREB phosphorylation on activation of CaMKIV. Because CaMKIV phosphorylates the nuclear protein CREB on Ser133, it appears to contribute to increased CREB phosphorylated on Ser 133 in vivo. In this cell type, which may be an important mechanism for growth suppression (6).

We tested whether PP2A holoenzyme bound calmodulin-Sepharose (an affinity resin for CaMKIV). A small fraction (less than 10%) of PP2A holoenzyme (ABaC) from rat brain soluble extracts bound calmodulin-Sepharose in a Ca\(^{2+}\)-dependent manner (Fig. 1C). We attempted to copurify CaMKIV and PP2A from rat brain soluble extract by sequential purification on phenyl-Sepharose, calmodulin-Sepharose, and Superdex-200 gel filtration columns (14). Immunoblot analysis of the gel filtration fractions demonstrated that CaMKIV remained associated with the PP2A holoenzyme (Fig. 1D). Calmodulin overlay of the peak gel filtration fraction revealed the presence of a single Ca\(^{2+}\)-calmodulin–binding protein that comigrated with CaMKIV (Fig. 1E). The CaMKIV-PP2A complex had an apparent molecular mass of 232 kD (from gel filtration chromatography), and both its size and protein immunoblot analysis (15) indicated that the PP2A heterotrimer and kinase were present in a 1:1 ratio. Furthermore, immune complexes obtained from the peak gel filtration fraction with an antibody specific for CaMKIV contained CaMKIV and PP2A in a 1:1 stoichiometry (7, 16).

To independently assess the presence of a complex, we tested whether PP2A holoenzyme bound calmodulin-Sepharose (an affinity resin for CaMKIV). A small fraction (less than 10%) of PP2A holoenzyme (ABaC) from rat brain soluble extracts bound calmodulin-Sepharose in a Ca\(^{2+}\)-dependent manner (Fig. 1C). We attempted to copurify CaMKIV and PP2A from rat brain soluble extract by sequential purification on phenyl-Sepharose, calmodulin-Sepharose, and Superdex-200 gel filtration columns (14). Immunoblot analysis of the gel filtration fractions demonstrated that CaMKIV remained associated with the PP2A holoenzyme (Fig. 1D). Calmodulin overlay of the peak gel filtration fraction revealed the presence of a single Ca\(^{2+}\)-calmodulin–binding protein that comigrated with CaMKIV (Fig. 1E). The CaMKIV-PP2A complex had an apparent molecular mass of 232 kD (from gel filtration chromatography), and both its size and protein immunoblot analysis (15) indicated that the PP2A heterotrimer and kinase were present in a 1:1 ratio. Furthermore, immune complexes obtained from the peak gel filtration fraction with an antibody specific for CaMKIV contained CaMKIV and PP2A in a 1:1 stoichiometry (7, 16).

To determine whether the kinase domain of CaMKIV is sufficient to interact with PP2A, and to test whether kinase activity was necessary for this interaction, we analyzed a series of GST-CaMKIV deletion and point mutants for their ability to interact with PP2A (9, 17). Wild-type and several mutant GST-CaMKIV fusion proteins associated with PP2A, but GST alone (Fig. 2A) or glutathione-Sepharose beads did not (18). As evidenced by the interaction of the deletion mutant that contained the entire catalytic domain (residues 1 to 317), association with PP2A did not require the autoinhibitory or calmodulin-binding domains of CaMKIV (Fig. 2A). In contrast, an NH\(_2\)-terminal deletion mutant of CaMKIV that contained amino acid residues 306 to 474 did not interact with PP2A. Catalytically inactive GST-CaMKIV mutants and a mutant GST-

**Fig. 1.** Identification of a CaMKIV-PP2A complex. (A) Coimmunoprecipitation of PP2A with CaMKIV. Immunoprecipitations from Jurkat T cell extracts were performed as described (7) with a rabbit polyclonal antibody raised against the COOH-terminal 17 amino acid residues of human CaMKIV (Imm.) or preimmune serum (Preimm.). Immune complexes were subjected to immunoblot analysis with a monoclonal antibody to the C subunit of PP2A (15). (B) Isolation of PP2A with a GST-CaMKIV fusion protein (9). Rat brain soluble extracts were incubated with buffer or wild-type GST-CaMKIV fusion protein (10 μg) for 3 hours at 4°C and then purified with glutathione-Sepharose (17). The resin was extensively washed and bound proteins were eluted with 20 mM glutathione, resolved by SDS-PAGE, and subjected to immunoblot analysis with antibodies to the C and B\(_\alpha\) regulatory subunits of PP2A. (C) Binding to calmodulin-Sepharose. Fractions containing proteins of large molecular mass (250 to 700 kD) from gel filtration of rat brain soluble extracts were incubated with calmodulin-Sepharose in the presence (--+Ca\(^{2+}\)) or absence (−Ca\(^{2+}\)) of 5 mM CaCl\(_2\) (36). Bound proteins were eluted with 15 mM EGTA, resolved by SDS-PAGE, and subjected to protein immunoblotting with antibodies to the indicated PP2A subunits. (D) Elution of CaMKIV and PP2A from a Superdex-200 gel filtration column after sequential fractionation of brain extracts on phenyl-Sepharose, calmodulin-Sepharose, and Mono Q columns (14). Presented is an immunoblot of fractions from the final gel filtration column showing CaMKIV and the C and B\(_\alpha\) subunits of PP2A. Although not shown, the A subunit of PP2A was also present. (E) Calmodulin overlay was performed on fraction 23 of the gel filtration column in the presence of 1 mM Ca\(^{2+}\) or 1 mM EGTA (15).
CaMKIV (T200A) not activated by CaMKK (9 associated with PP2A; this demonstrated that neither CaMKIV activity nor its phosphorylation by CaMKK is required for interaction with PP2A.

To test whether PP2A catalytic activity was required for association with CaMKIV, we used microcystin-Sepharose to purify PP2A from extracts prepared from rat brain and Jurkat T cells (19). Both PP2A and CaMKIV were isolated from the extracts with the resin (Fig. 2B). Because microcystin is an inhibitor of PP2A that binds to the substrate binding site of the phosphatase catalytic subunit (20), PP2A activity appeared not to be required for association with CaMKIV. Association of CaMKIV and PP2A with microcystin-Sepharose was attenuated by pretreatment of the extracts with free microcystin. Hence, the purification of the proteins by the affinity resin occurred in a microcystin-dependent fashion and was not a result of nonspecific association with the beads.

Although association of CaMKIV with PP2A did not require activity of either enzyme, we tested the potential activity of CaMKIV and PP2A toward each other in vitro. The highly enriched complex of CaMKIV-PP2A was incubated in the presence or absence of Ca\(^{2+}\)-calmodulin, CaMKK, and the PP2A inhibitor okadaic acid, and the proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE). The only phosphorylated protein detected by autoradiography appeared to be CaMKIV, as indicated by its size and increased phosphorylation after treatment with CaMKK (Fig. 3). Immunoblot analysis confirmed that the only okadaic acid–sensitive phosphatase present in the preparation was PP2A (18). Thus, the enhanced phosphorylation of CaMKIV observed in the presence of okadaic acid (Fig. 3) indicates that CaMKIV is a substrate for PP2A and suggests that PP2A may also regulate CaMKIV activity in vivo.

To determine whether PP2A regulates CaMKIV activity in intact cells, we examined the effects of SV40 small t antigen, a specific inhibitor of PP2A activity (21–24), on CaMKIV-mediated activation of CREB-dependent transcription in Jurkat T cells. We used a Gal4-CREB construct together with a 5×(Gal4)-luciferase reporter plasmid because this assay specifically requires phosphorylation of Ser\(^{133}\) of the Gal4 CREB molecule. This chimeric protein binds DNA as a monomer and is therefore not influenced by endogenous CREB or other proteins that can heterodimerize with CREB. Previous studies have established that CaMKIV activates transcription by direct phosphorylation of Gal4-CREB on Ser\(^{133}\) (25) and that this assay requires phosphorylation of CaMKIV by CaMKK (9, 25, 26). Expression of small t antigen augmented CaMKIV-mediated Gal4-CREB activity that had been elicited by the Ca\(^{2+}\) ionophore ionomycin (Fig. 4) or by the CD3 antibody (16). This effect appeared to be specific for Ca\(^{2+}\)–dependent, CaMKIV-mediated activation of Gal4-CREB, as expression of small t antigen had no effect on its own or on Gal4-CREB activity stimulated by constitutively active cAMP-dependent protein kinase, which also specifically and directly phosphorylates Gal4-CREB on Ser\(^{133}\) but in a Ca\(^{2+}\)–independent manner (25, 26). These results indicate that the CaMKIV-PP2A complex regulates CaMKIV activity and plays a key role in controlling CRE-mediated gene transcription in T cells.

In T cells, the Ca\(^{2+}\)–calmodulin–dependent dephosphorylation of nuclear factor of activated T cells (NFAT\(_{c}\)) by calcineurin allows its translocation from the cytosol to the nucleus, where it participates in the in-
duction of IL-2 gene transcription (27–29). Sustained increases in [Ca\textsuperscript{2+}], during T cell activation maintain NFAT, in the nucleus long enough to induce IL-2 transcription; once [Ca\textsuperscript{2+}], returns to basal amounts, NFAT is rapidly redistributed out of the nucleus to the cytosol (30, 31). Our experiments show how another Ca\textsuperscript{2+}-initiated regulatory event (CaMKIV-stimulated gene transcription) may be inhibited without a decrease in [Ca\textsuperscript{2+}]. The CaMKIV-PP2A complex would permit rapid dephosphorylation and inactivation of CaMKIV.

The complex of CaMKIV and PP2A exists in resting T cells and is not altered as a function of time after activation (16). How, then, would phosphorylation of CaMKIV and CREB happen at all? Several possible mechanisms may explain the inactivation of CaMKIV in the face of a sustained elevation of [Ca\textsuperscript{2+}]. First, it is plausible that PP2A is constitutively active toward CaMKIV but that CaMKIV transiently outpaces the phosphatase (before becoming inactive itself) to ensure that CaMKIV remains active long enough to phosphorylate CREB. After phosphorylation of CREB, PP2A would be overwhelmed by the extreme NH\textsubscript{2}-terminus of CaMKIV. Our experiments may reveal a way in which the extreme NH\textsubscript{2}-terminus (CaMKIV mutant L317) and the NH\textsubscript{2}-terminal Glu point mutation, which disrupts the CaMKIV-PP2A complex also occur in the brain suggest that similar regulatory mechanisms may contribute to synaptic plasticity and its role in the molecular basis for memory, where the phosphorylation state of CREB is regulated in a Ca\textsuperscript{2+}-dependent manner by CaMKIV (35).

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Regardless of the additional mechanisms involved, our results may reveal a way in which the duration of early gene expression can be regulated independently of [Ca\textsuperscript{2+}]. Our findings that CaMKIV-PP2A complexes also occur in the brain suggest that similar regulatory mechanisms may contribute to synaptic plasticity and its role in the molecular basis for memory, where the phosphorylation state of CREB is regulated in a Ca\textsuperscript{2+}-dependent manner by CaMKIV (35).
A Signaling Complex of Ca\textsuperscript{2+}-Calmodulin-Dependent Protein Kinase IV and Protein Phosphatase 2A

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