Nuclear Calcium/Calmodulin-dependent Protein Kinase IIδ Preferentially Transmits Signals to Histone Deacetylase 4 in Cardiac Cells

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Class II histone deacetylases (HDACs) act as repressors of cardiac hypertrophy, an adaptive response of the heart characterized by a reprogramming of fetal cardiac genes. Prolonged hypertrophy often leads to dilated cardiomyopathy and heart failure. Upstream endogenous regulators of class II HDACs that regulate hypertrophic growth are just beginning to emerge. Here we demonstrate that the δ1B isoform of calcium/calmodulin-dependent protein kinase II (CaMKIIδ1B), known to promote cardiac hypertrophy, transmits signals specifically to HDAC4 but not other class II HDACs. CaMKIIδ1B efficiently phosphorylates both a glutathione S-transferase (GST)-HDAC4 fragment spanning amino acids 207–311 and full-length FLAG-HDAC4 but not the equivalents in HDAC5. Although previous studies in skeletal muscle cells have shown that HDAC4 lacking serine 246 cannot be phosphorylated by CaMKI/IV, a similar mutant is still phosphorylated by CaMKIIδ1B. Importantly, mutation of serine 210 to alanine totally abolishes phosphorylation of the GST fragment and significantly reduces phosphorylation of full-length HDAC by CaMKIIδ1B. RNA interference knockdown of CaMKIIδ1B prevents the effects of hypertrophic stimuli. Overexpression of CaMKIIδ1B in primary neonatal cardiomyocytes increases the activity of the Mef2 transcription factor and completely rescues HDAC4-mediated repression of MEF2 but only partially rescues inhibition by HDAC5 or the HDAC4 S210A mutant. CaMKIIδ1B strongly interacts with HDAC4 in cells but not with HDAC5. These results demonstrate that CaMKIIδ1B preferentially targets HDAC4, and this involves serine 210. These findings identify HDAC4 as a specific downstream substrate of CaMKIIδ1B in cardiac cells and have broad applications for the signaling pathways leading to cardiac hypertrophy and heart failure.

Histone acetylation and deacetylation represent a central mechanism in the control of gene expression (1, 2). Histone acetyltransferases activate transcription by opening chromatin while histone deacetylases (HDACs)3 antagonize this effect by deacetylating histones and transcription factors, causing transcriptional repression. Three classes of HDACs have been isolated and differ according to their structure, complex formation, and expression pattern (3). Class II HDACs (−4, −5, −6, −7, −9, and −10) are expressed abundantly in heart, brain, and skeletal muscle, the same tissues that express high levels of the myocyte enhancer factor-2 (Mef2) family of transcriptional activators. In skeletal muscle cells, association of class II HDACs with Mef2 results in repression of Mef2 activity and inhibition of myogenesis (4). This effect is mediated by interaction of MEF2 with the N-terminal region of class II HDACs that contains two conserved calcium/calmodulin-dependent protein kinase (CaMK) phosphorylation sites. Phosphorylation of these sites by CaMKI or -IV recruits the chaperone protein 14-3-3, which results in the nuclear export of HDAC/14-3-3 complexes (5–8), and de-repression of HDAC target genes such as MEF2 (5, 9).

CaMKs are serine/threonine kinases regulated by calcium and calmodulin. Activation of CaMK signaling plays a role in cardiac diseases such as hypertrophic growth (see Ref. 10 for review). CaMKIV, although not expressed in the myocardium, can enter the nucleus, and mice expressing high levels of this kinase develop a profound hypertrophy (11). CaMKII is the major isoform expressed in heart muscle and exists as a homo- or heteromultimer of 8–12 subunits derived from four genes, α, β, γ, and δ (for reviews, see Refs. 12–14). The δ subunit of CaMKII predominates in the adult heart (15–17), and two splice variants, δB and δC, are expressed at the protein level in this organ (15, 16, 18). CaMKIIδ isoforms are highly homologous with the exception of a variable domain generated by alternative splicing. The δB isoform contains an 11-amino acid nuclear localization signal (19, 20), allowing expression of the kinase in the nucleus of cardiac cells. Thus, heteromultimers

The abbreviations used are: HDAC, histone deacetylase; Mef2, myocyte enhancer factor-2; CaMK, calcium/calmodulin-dependent protein kinase; siRNA, small interference RNA; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; CMV, cytomegalovirus; BioTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PE, phenylphosphine; TRITC, tetramethylrhodamine isothiocyanate; shRNA, short hairpin RNA; Ad, adenovirus; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; ANF, atrial natriuretic factor; GST, glutathione S-transferase.

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with a majority of 8b subunits localize to the nucleus and are expected to regulate transcriptional events in heart muscle. So far CaMKI110B has been shown to regulate transcription through one effect, the induction of the atrial natriuretic factor, which requires the nuclear localization signal of the kinase (21). Recent studies have linked the 8b subunit of CaMKII to cardiac hypertrophy, because transgenic mice expressing high levels of the kinase develop hypertrophy and dilated cardiomyopathy (22, 23). The details of the pathways involved remain to be determined.

In heart muscle class II HDACs act as repressors of cardiac hypertrophy (see Ref. 24 for review), an adaptive response of the heart to various pathological stimuli characterized by an increase in cell size and reprogramming of fetal cardiac genes (see Ref. 25 for review). Although initially beneficial, prolonged hypertrophy often leads to dilated cardiomyopathy and heart failure. Forced expression of HDAC5 and -9 prevents agonist-mediated hypertrophy in isolated cardiomyocytes (26), and mutant mice lacking HDAC5 and -9 are hypersensitive to hypertrophic stimuli and develop severe hypertrophic growth (26, 27). Hypertrophy mediated by phenylephrine treatment leads to an increase in Mef2 activity through a post-translational mechanism mediated by CaMK signaling (28). Activation of CaMKI and -IV promotes the phosphorylation of two conserved serines at the N-terminal of class II HDACs, Ser-246/467 in HDAC4, Ser-259/498 in HDAC5, and Ser-218/448 in HDAC9. Mutant HDACs lacking the conserved serines are resistant to hypertrophic signals and prevent myocardocyte hypertrophy (26). Protein kinases C and D1 also phosphorylate HDAC5 and regulate cardiac stress signaling (29, 30). The phosphorylation of class II HDACs provides a mechanism to connect extracellular stimuli to the genome. However, CaMKI and -IV are expressed at very low levels or not expressed at all in heart muscle, and the cardiac endogenous CaMK(s) responsible for these events has yet to be identified.

HDAC4, -5, and -9 are all substrates of CaMKI and -IV in non-cardiac cells. Here, we report the unsuspected finding that nuclear CaMKI110B transmits signals through phosphorylation specifically to HDAC4. A GST-HDAC4 fragment spanning amino acids 207–311 is heavily phosphorylated by CaMKI110B but not the equivalent fragment in HDAC5 or -9. Importantly, full-length HDAC4 but not HDAC5 is a substrate for CaMKI110B. Although an HDAC4 mutant lacking serine 246 cannot be phosphorylated by CaMKI and -IV, we find that such a mutant is still highly phosphorylated by the 8b subunit of CaMKII. We discovered a novel site in HDAC4 that confers responsiveness to CaMKI110B. Mutation of serine 210 to alanine in GST-HDAC4-(207–311) totally abolishes phosphorylation by CaMKI110B and substitution of Ser-210 with alanine in full-length HDAC4 (HDAC4-S210A) impairs phosphorylation by CaMKI110B. We also demonstrate that HDAC4, but not HDAC5, strongly interacts with CaMKI110B in cells. Furthermore, in the presence of the kinase, HDAC4 minimally affects Mef2 activity in primary cardiomyocytes, whereas HDAC4-S210A and HDAC5 have a strong repressive effect. We demonstrate that specific knockdown of CaMKI110B by siRNA inhibits Mef2 activity in primary cardiomyocytes and prevents the effect of hypertrophic stimuli. These findings demonstrate that HDAC4 is a specific substrate for CaMKI110B in cardiac cells and have broad applications for mechanisms of transcriptional repression and for pathways involved in the development of cardiac hypertrophy and heart failure.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Inhibitors, and Antibodies**—The 3×Mef2-luciferase reporter construct has been described previously (31). SRα empty plasmid and SRα-CaMKI110B were gifts from Joan Keller-Brown (University of California, San Diego, La Jolla, CA) and Howard Schulman (Stanford University). Constitutively active forms of CaMKI110B (CaMKI110B-T287D, CaMKI110B-T287D/S332A, and CaMKI110B-T287D/S332–335A) were generated by site-directed mutagenesis (Stratagene). Each mutation was confirmed by DNA sequencing. The construct Gal-Mef2C-(1–465), GST-HDAC fusion proteins, and GST-HDAC fusion proteins in which Ser-246 for HDAC4, Ser-259 for HDAC5, and Ser-218 for HDAC9 are mutated to Ala (26) were gifts from Eric Olson (University of Texas Southwestern Medical Center, Dallas, TX). GST-HDAC4 mutants with serines 209, 210, 219, 222, 223, 278, 288, 290, 293, 306, 307, 309, and 311 mutated to Ala were generated by site-directed mutagenesis and were expressed as recombinant proteins in DH5α bacterial cells. Mutagenesis was verified by DNA sequencing. Full-length HDAC4 and -5 plasmids carrying the FLAG epitope were generously provided by Stuart Schreiber (Harvard University, Cambridge, MA). Full-length HDAC4 with Ser-210 mutated to alanine was generated by site-directed mutagenesis. The presence of the mutation was verified by DNA sequencing. 14–3–3ε was a generous gift from Stuart Schreiber (Harvard University, Cambridge, MA). KN-93 CaMKII inhibitor and the KN-92 inactive analogue were purchased from Calbiochem. FLAG antibody (M2) and HA-affinity gel were from Sigma. HA (F-7 monoclonal or Y-11 polyclonal), GAPDH (V-18 goat polyclonal), GFP (FL polyclonal), GST (Z-5), and c-Myc (A-14) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-Actinin antibody (mouse monoclonal) was from Sigma, and ANF antibody (polyclonal) was from Bachem.

**Generation of Adeno-CaMKI110B and Adeno-CaMKI110B-T287D**—Adenoviruses expressing wild-type CaMKI110B and active CaMKI110B-T287D were generated using the AdEASY Vector System as described in a previous study (32). Briefly, each cDNA was subcloned into the EcoRI site of pAdTrack-CMV vector. The HA tag sequence was kept at the N terminus of both proteins. pAdTrack-CMV vectors bearing CaMKI110B or CaMKI110B-T287D cDNA were first linearized with PmeI, and then, together with adenoviral backbone vector pAdEasy, introduced into *Escherichia coli* strain BJ5183. Recombinant adenoviral plasmids were recovered and introduced into 293A cells by Lipofectamine (Invitrogen). The recombinant viruses (Ad-CaMKI110B and Ad-CaMKI110B-T287D) were propagated in 293A cells and recovered after several freezing/thawing cycles. After filtration through 45-μm filters, the virus titer was measured by serial dilution and counting the number of GFP-positive cells by fluorescence-activated cell sorting analysis.

**Expression of GST-HDACs in Bacteria**—GST and GST-HDACs, wild type and with serine to alanine mutations, were
expressed in bacteria as described previously (33). Equal amounts of fusion proteins were used in each phosphorylation assay.

In Vitro Phosphorylation of GST-HDACs by CaMKIIΔB—CaMKIIΔB-T287D was purified from HeLa cells infected with Ad-CaMKIIΔB-T287D using EZ-view Red HA-affinity gel following the manufacturer's instructions (Sigma). Phosphorylation of GST-HDAC recombinant proteins was performed in a kinase buffer (25 mM Hepes, 10 mM MgCl₂, 2 mM CaCl₂) in the presence of calmodulin (40 μg/ml) and [γ-32P]ATP (5 μCi/ml). GST-HDACs were added to 0.5 μg of CaMKIIΔB-T287D bound to HA-agarose. In vitro kinase assays were also performed with concentrations of CaMKIIΔB ranging from 0 to 10 ng and supplemented with 12.5 μM ATP. Concentration of the kinase was calculated by running aliquots on 4–12% NuPAGE BisTris gels (Invitrogen) alongside bovine serum albumin standards. Reactions were performed at room temperature for 20 min and stopped by addition of loading buffer. The reactions were then resolved on 4–12% NuPAGE BisTris gels. After Coomassie staining, the gels were dried onto filter paper and analyzed by autoradiography. Phosphorylation was quantified by densitometry using a Storm scanner and ImageQuant software. GST-HDAC fusion proteins were also detected by Western blot with an anti-GST antibody on aliquots of the substrate to demonstrate equal loading.

Phosphorylation of Full-length HDACs—COS7 cells were transfected with full-length FLAG-HDAC4, FLAG-HDAC4-S210A, FLAG-HDAC5, or HA-CaMKIIΔB-T287D using Lipopectamine 2000 (Invitrogen). Total cell extracts were prepared as previously described (31) in buffer containing 50 mM Tris, pH 7.6, 250 mM NaCl, and a protease inhibitor mixture (Sigma) for HDACs and in buffer containing 200 mM Heps, pH 7.6, 20% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% TX-100, 500 mM NaCl, and a protease inhibitor mixture for CaMKII. HDACs were purified using anti-FLAG M2 affinity gel (Sigma) and CaMKIIΔB-T287D using EZ-view Red anti-HA affinity gel (Sigma). HDACs were eluted using 100 mM glycine, pH 3.5, neutralized by addition of a one-tenth volume of 1 M Tris, pH 8.0, and added to 0.5 μg of CaMKIIΔB-T287D still bound to the HA affinity gel. Kinase reactions were also performed with limiting (5–100 ng) amounts of enzyme. Phosphorylation reactions were performed as described for GST-HDACs except that labeled proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences), and Western blot analysis was performed to verify equal loading of each HDAC and the kinase.

Immunoprecipitation and Western Blot Analysis—COS7 cells were co-transfected with full-length FLAG-HDAC4, FLAG-HDAC4-S210A, or FLAG-HDAC5 plus CaMKIIΔB or CaMKIIΔB-T287D carrying an HA epitope using Lipopectamine 2000 (Invitrogen). When indicated, the cells were transfected with Myc-tagged 14-3-3, or the atrial natriuretic factor (ANF, dilution 1:100). Secondary reactions were performed with a secondary antibody conjugated with TRITC or Cy3. The coverslides were mounted with Vectashield mounting media containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Signals were observed by confocal microscopy with a Zeiss confocal microscope (LSM 510) or an Olympus confocal microscope.
**Selective HDAC4 Phosphorylation by CaMKIIoB**

**siRNA Preparation and Transfection**—To design target-specific siRNA duplexes against the αB isoform of CaMKII, we selected sequences of the type AA(N19) (N, any nucleotide) from the open reading frame of rat CaMKIIoB mRNA. We obtained a 21-nucleotide sense and antisense strand with symmetric 2-nucleotide 3′-overhangs of identical sequences. The target sequence from rat CaMKIIoB (sense siRNA: 5′-GUUCGAGUGUUCAGAUGAUdTdT-3′) was utilized and was submitted to a BLAST search to ensure that only this isoform of CaMKII was targeted. A scramble oligoribonucleotide duplex that was not homologous to any mammalian genes was utilized as the control (sense siRNA: 5′-UUUCGCAAGCGU-CAGUdTdT-3′). siRNAs were obtained from Dharmacon Research (Lafayette, CO). Transient transfections of siRNAs in primary neonatal rat cardiomyocytes were carried out using Lipofectamine 2000 (Invitrogen). Primary cardiomyocytes were transfected with a 3′×Me2-luciferase reporter or the G5E1b-luciferase reporter and siRNA directed against CaMKII was targeted. A scramble oligoribonucleotide duplex that was not homologous to any mammalian genes was utilized as the control (sense siRNA: 5′-UUUCGCAAGCGU-CAGUdTdT-3′). siRNAs were obtained from Dharmacon Research (Lafayette, CO). Transient transfections of siRNAs in primary neonatal rat cardiomyocytes were carried out using Lipofectamine 2000 (Invitrogen). Primary cardiomyocytes were transfected with a 3′×Me2-luciferase reporter or the G5E1b-luciferase reporter and siRNA directed against CaMKII or control siRNA. Luciferase activity was measured 72 h later in cell extracts. Specific silencing was confirmed by Western blot analysis and by quantitative reverse transcription-PCR analysis.

**Reverse Transcription-PCR Analysis**—Primary cardiomyocytes were transfected with CaMKIIoB siRNA or control siRNA using Lipofectamine 2000 (Invitrogen). 48 h after transfection, total RNA was extracted with TRIzol reagent (Invitrogen) as previously described (31). After reverse transcription of total RNA using the RETROscript kit (Ambion), endogenous CaMKIIoB mRNA levels were measured by PCR with CaMKIIoB-specific primers in a reaction containing 

**RESULTS**

**CaMKIIoB Selectively Phosphorylates GST-HDAC4-(207–311) but Not the Equivalent Fragments in HDAC5 or HDAC9**—In skeletal muscle cells CaMKI and -IV phosphorylate and induce the nuclear export of HDAC4 and -5, which results in the activation of MEF2 and stimulation of myogenesis (9). Shuttling of these HDACs from the nucleus to the cytoplasm is mediated by the phosphorylation of two key serines conserved in HDAC4, -5, and -9 (Ser-246 and -467 in HDAC4, Ser-259 and -498 in HDAC5, and Ser-218 and -448 in HDAC9) (26). We hypothesized that the CaMK cardiac isoform IIoB would phosphorylate these serines of class II HDACs in cardiac cells. To test this, we performed an in vitro kinase assay with purified activated CaMKIIoB-T287D and similar amounts of substrate polypeptides of HDAC4, -5, and -9 (Fig. 1A, lower panel) encompassing the conserved serines fused to GST. Although GST-HDAC4 was strongly phosphorylated by the kinase, quite unexpectedly, the GST-HDAC5 and GST-HDAC9 fusion proteins were not (Fig. 1A, lanes 1, 3, and 5). Thus CaMKIIoB specifically phosphorylated a GST-HDAC4 fusion protein encompassing amino acids 207–311 but not the equivalent GST-HDAC5 or GST-HDAC9 fragments. Furthermore, a GST-HDAC4 fusion protein bearing the regulatory Ser-246 mutated to alanine was still phosphorylated by CaMKIIoB (Fig. 1A, lane 2). In fact, the phosphorylation of the
Selective HDAC4 Phosphorylation by CaMKII6B

Mutation of Ser-210 to Alanine abolishes HDAC4 phosphorylation by CaMKII6B—To characterize the novel site(s) in GST-HDAC4-(207–311), we performed an amino acid sequence alignment of GST-HDAC4, GST-HDAC5 and GST-HDAC9. A, 13 single amino acid substitutions were generated in GST-HDAC4 by site-directed mutagenesis. B, in vitro phosphorylation assays and GST Western blots were carried out as described in Fig. 1. Substitution of Ser-210 to alanine totally abolished phosphorylation of HDAC4. C, amount of GST-HDAC4 used in each in vitro phosphorylation assay measured by Western blot using anti-GST antibody. These experiments were repeated at least twice.

Mutated protein was slightly increased compared with the wild-type protein (Fig. 1, B and C). In vitro kinase assays with lesser amounts of GST-HDAC4 fusion proteins showed a similar result (Fig. 1, B (lanes 3 and 5) and C). We also performed in vitro kinase reactions with a constant amount of GST-HDAC substrates and increasing amounts of enzyme ranging from 5 to 100 ng. HDAC4, but not HDAC5 was phosphorylated by CaMKII6B in these conditions (supplemental Fig. S1). We also verified that, in our hands, activated CaMKIV phosphorylates GST-HDAC4 but not the GST-HDAC4 fusion protein bearing the regulatory Ser-246 mutated to alanine (data not shown). All together, these results show that the 6B isoform of CaMKII specifically phosphorylates HDAC4 but not the equivalent amino acids in HDAC5 or HDAC9. Furthermore the site of phosphorylation is not the regulatory serine 246 but, rather, one or more novel CaMKII6B phosphorylatable sites in HDAC4 that are not a target in other class II HDACs.

Mutation of Ser-210 to Alanine in HDAC4 Abolishes Phosphorylation by CaMKII6B—To characterize the novel site(s) in GST-HDAC4 phosphorylated by CaMKII6B, we performed an amino acid sequence alignment of GST-HDAC4-(207–311), GST-HDAC5-(218–329), and GST-HDAC9-(182–285) (Fig. 2A) and searched for serines and threonines in HDAC4 that are either not conserved in HDAC5 and HDAC9 or are conserved but have variations in the surrounding sequences. We engineered 13 individual GST-HDAC4 mutants with serine to alanine substitution and tested them in vitro for phosphorylation by CaMKII6B. The amount of fusion proteins used in each assay was measured by Western blot analysis using anti-GST antibody (Fig. 2C). Phosphorylation of GST-HDAC4 wild type was readily detected as expected (Fig. 2B, lane 2). Importantly, mutation of Ser-210 to alanine totally abolished phosphorylation by the kinase (Fig. 2B, lane 4). We also found that a subset of mutations in HDAC4 (Ser-219, Ser-222, Thr-223, and Thr-278) slightly decreased phosphorylation by CaMKII6B. This suggests that mutation at these sites is able to impair phosphorylation of Ser-210 or that these sites might be phosphorylated in an Ser-210-dependent manner. All together, our results suggest that Ser-210 in HDAC4 is a major site of phosphorylation by CaMKII6B or that this serine is important for the binding of the kinase to its substrate.

HDAC4 Interacts with CaMKII6B in Cells—Next, we determined whether class II HDACs associate with CaMKII6B in cells. To achieve high level expression of HDAC4 and -5, we introduced them into COS7 cells using a lipid-based delivery method. COS7 cells were co-transfected with HDAC4 or -5 expression vectors carrying a FLAG epitope and constitutively active CaMKII6B-T287D (14) carrying an HA epitope or SR α empty vector. HDACs were then immunoprecipitated using anti-FLAG affinity gel followed by Western blot analysis with an anti-HA antibody (Fig. 3A). When HDAC4 and CaMKII6B-T287D were simultaneously expressed in the cells, we found a strong association between them (Fig. 3B). The association was specific, because it was not observed in the presence of empty vector or when control mouse IgG was used for mock immunoprecipitation (not shown). HDAC4 is distributed between the nuclear and the cytoplasmic compartment in cells (34), including in unstimulated cardiomyocytes (data not shown). However, quite unexpectedly, we found that active CaMKII6B-T287D localized preferentially to the cytoplasm of cells (see Fig. 6D). To investigate whether the nuclear pool of HDAC4 interacts with CaMKII6B, we also tested HDAC4 interaction with wild-type CaMKII6B that has some low basal activity in cardiomyocytes and is expressed for the most part in cardiac nuclei. We found that the wild-type kinase, like active CaMKII6B-T287D, strongly associated with HDAC4 in cells (Fig. 3C). In contrast, we observed no binding or at best a very weak binding between HDAC5 and active CaMKII6B-T287D (Fig. 4A, compare lanes 1 and 4) or the wild-type CaMKII6B (not shown). These results demonstrate that CaMKII6B and HDAC4 bind to one another...
**Select HDAC4 Phosphorylation by CaMKIIβ**

**A**

Flag-HDAC4 or Flag-HDAC5

HA-CaMKIIβ

**Transfection**

IP with anti-Flag

Western blot with anti-Flag and anti-HA

**B**

Flag-HDAC4

HA-CaMKIIβ-T287D

**IP**: α-Flag

Western blot: α-Flag

**C**

Flag-HDAC4

HA-CaMKIIβ-T287D

**IP**: α-Flag

Western blot: α-Flag

**D**

Flag-HDAC4

HA-CaMKIIβ-T287D

**IP**: α-Flag

Western blot: α-Flag

**FIGURE 3. Interaction of HDAC4 and CaMKIIβ.** As shown in A and described under “Experimental Procedures” COS7 cells were co-transfected with expression vectors for FLAG-tagged HDACs and active HA-tagged CaMKIIβ-T287D (B), plus wild-type CaMKIIβ (C), or empty vector. FLAG-HDACs were immunoprecipitated from whole cell extracts with FLAG affinity gel and washed in lysis buffer containing 250 mM NaCl. HDACs were detected by FLAG antibody (upper panels) and CaMKIIβ using HA antibody (lower panels). Expression levels of the transfected proteins in whole cell extract are demonstrated by direct Western blot analysis of the extracts. These experiments were performed at least three times.

with a strong affinity in cells, whereas the kinase has at most a very low affinity for HDAC5. These findings together with the in vitro phosphorylation data suggest that HDAC4 is a preferred substrate of CaMKIIβ.

**CaMKIIβ Selectively Phosphorylates Full-length HDAC4, and Serine 210 to Alanine Substitution Impairs Phosphorylation by CaMKIIβ**—Our data showed that CaMKIIβ strongly phosphorylates GST-HDAC4-(207–311) but not the equivalent fragments in HDAC5 or HDAC9. Next, we investigated whether this selectivity for HDAC4 takes place in full-length HDAC4. COS7 cells were transfected with full-length FLAG-HDAC4 or FLAG-HDAC5 expression vectors, and tagged proteins were immunoprecipitated with FLAG affinity gel and incorporated in a kinase reaction with constitutively active CaMKIIβ-T287D. HDAC4, but not HDAC5, was strongly phosphorylated by CaMKIIβ (Fig. 4B). To confirm that serine 210 in HDAC4 is a relevant phosphorylation site, we transfected this mutant HDAC4 (HDAC4-S210A) in COS7 cells and incorporated immunoprecipitated proteins in a kinase reaction with purified CaMKIIβ-T287D. HDAC4 was strongly phosphorylated by CaMKIIβ, whereas HDAC4-S210A showed reduced phosphorylation by the kinase. A careful quantitation of the signal showed that phosphorylation of HDAC4-S210A by CaMKIIβ was decreased by one-third compared with wild-type HDAC4 (Fig. 4C). An in vitro kinase assay performed with limiting amounts of CaMKIIβ ranging from 5 to 50 ng showed similar results (supplemental Fig. S2). The reduced level of phosphorylation could be due to reduced binding of HDAC4-S210A to the kinase. To test this, we co-expressed HDAC4 or HDAC4-S210A with CaMKIIβ-T287D in COS7 cells and immunoprecipitated both HDACs with FLAG affinity gel. Western blot analysis revealed that the mutation did not affect binding of the kinase to its substrate (Fig. 4A, compare lanes 1 and 2). Collectively, these results demonstrate that CaMKIIβ selectively targets HDAC4 for phosphorylation and that serine 210 is a major residue phosphorylated by the kinase.

**CaMKIIβ Enhances MEF2C-dependent Transcription**—Me2 is activated by a variety of hypertrophic agents (28) and is required for proper cardiac development (35) and normal post-natal cardiac growth (36). In addition, Me2 controls various cardiac-specific genes and Is considered a point of convergence of various hypertrophic stimuli in the heart. Because the β subunit of CaMKII is the predominant nuclear isoform of CaMKII in cardiac muscle and is implicated in cardiac hypertrophy (22), we tested whether CaMKIIβ can regulate Me2-dependent transcription in primary neonatal rat cardiomyocytes. The 3×Me2-luciferase reporter was transiently transfected in cultured cardiomyocytes in the presence or absence of a CaMKIIβ expression vector. Forced expression of CaMKIIβ increased transcription of the reporter in a dose-dependent manner (Fig. 4B). We also tested the effect of constitutively active CaMKIIβ-T287D. Overexpression of CaMKIIβ-T287D resulted in a strong increase in the activity of the 3×Me2-luciferase reporter (Fig. 5B). We verified that both wild-type CaMKIIβ and active CaMKIIβ-T287D were expressed equally well in cardiac cells by Western blot analysis of protein extracts from transfected cardiac cells (Fig. 5C). We also transfected cardiac myocytes with CaMKIIβ expression vector and constructs in which the DNA-binding domain of Gal4 is fused to full-length Me2C (Gal-Me2C-(1–465)) or Me2C deletion constructs lacking either the MADS (Gal-Me2C-(247–327)) or the transactivation domains of Me2C (Gal-Me2C-(1–174)). When we measured transcriptional activity on a Gal-dependent reporter construct G5E1b-luciferase, we found that forced expression of CaMKIIβ indeed caused an increase in Me2C transcriptional activity (Fig. 5D). Interestingly, the kinase had strong transactivation effect on full-length Gal-Me2C as well as a partial effect on deletion constructs lacking either the MADS or the transactivation domains of Me2C.

**HDAC4 and -5 Differentially Inhibit CaMKIIβ-mediated Co-activation of Me2 in Primary Cardiomyocytes**—CaMKIIβIV activate Me2 by relieving repression by class II HDACs. To investigate the functional consequence of CaMKIIβ-mediated binding and phosphorylation of HDAC4, we tested whether activated CaMKIIβ-T287D in the presence of HDAC4 or -5 could release repression of Me2. If HDAC4 is a preferred substrate of the kinase, one would expect that CaMKIIβ would relieve repression of Me2 in the presence of HDAC4 but not HDAC5. Primary cardiomyocytes were transfected with CaMKIIβ-T287D along with the 3×Me2-luciferase reporter in the presence of small concentrations of HDAC4 or -5. In the presence...
Selective HDAC4 Phosphorylation by CaMKIIβ

FIGURE 4. CaMKIIβ selectively phosphorylates full-length HDAC4, and Ser-210-Ala mutation decreases HDAC4 phosphorylation. A, COS7 cells were co-transfected with full-length FLAG-HDAC4, FLAG-HDAC4-S210A, or FLAG-HDAC5 and HA-CaMKIIβ-T287D. 48 h after transfection, HDACs were immunoprecipitated with FLAG affinity gel, and after washing, eluted proteins were subjected to SDS-PAGE as described in Fig. 3. B, active CaMKIIβ-T287D was purified from COS7 cells transfected with HA-CaMKIIβ-T287D expression vector using HA-affinity gel. FLAG-HDAC4 or FLAG-HDAC5 was transfected in COS7 cells and immunoprecipitated with FLAG affinity gel. Immunoprecipitated HDACs were then incorporated in a reaction containing purified CaMKIIβ-T287D and [γ-32P]ATP. After SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes, HDAC levels were analyzed using anti-FLAG antibody, and the signal was revealed by autoradiography. C, COS7 cells were transfected with full-length FLAG-HDAC4, FLAG-HDAC4-S210A, or FLAG-HDAC5. After immunoprecipitation with FLAG affinity gel, the HDACs were incorporated in a kinase reaction as described in B. Quantitation analysis is from three separate experiments.

of active CaMKIIβ-T287D, HDAC4 slightly decreased Mef2 activity, whereas HDAC5 more strongly inhibited Mef2 activity (Fig. 6A). Constitutively active CaMKIIβ-T287D localized preferentially to the cytoplasm (Fig. 6D), and HDAC5 was expressed almost exclusively in the nuclei of cardiac cells under basal conditions (data not shown). Thus, the failure of CaMKIIβ to rescue HDAC5-mediated repression of Mef2 could be attributed to the different location of the kinase and HDAC5 in the cells. To test this hypothesis, we generated two additional forms of constitutive active CaMKIIβ enzymes expected to remain in the nuclear compartment (37). The first one was generated by substituting serine 332 with alanine (CaMKIIβ-T287D/S332A), and the second was made by mutating four serines (serines 333, 334, 335) to alanine (CaMKIIβ-T287D/S332–335A). Both enzymes were transfected in primary cardiomyocytes and were tested for their ability to prevent HDAC4 or -5 repression of Mef2. Similar to the results obtained with active CaMKIIβ-T287D, HDAC4 slightly decreased Mef2 activity, whereas HDAC5 strongly inhibited Mef2 activity in the presence of either CaMKIIβ-T287D/S332A or CaMKIIβ-T287D/S332–335A (Fig. 6, B and C). We verified that both enzymes were indeed expressed in the nuclei of cardiac cells. As expected, CaMKIIβ-T287D/S332A and CaMKIIβ-T287D/S332–335A were detected in cardiac nuclei. Interestingly, CaMKIIβ-T287D/S332–335A was also expressed in the cytoplasm of cardiac cells, although nuclear staining was stronger (Fig. 6D). To rule out the possibility that the differential effect of CaMKIIβ on HDAC4 and -5 could be due to differences in protein levels, we measured HDAC expression level in cardiac nuclei by Western blot analysis. HDAC4 and -5 were expressed at similar levels in transfected cardiac cells (Fig. 6E). These results demonstrate that HDAC4 and -5 have a differential effect on Mef2 activity. All together, our findings suggest that HDAC4 but not HDAC5 is a preferred substrate of the β isoform of CaMKII.

Mutation of Ser-210 to Alanine in HDAC4 Strongly Inhibits Mef2 Co-activation by CaMKIIβ—To investigate the functional consequence of Ser-210 mutation in HDAC4, we examined the effect of HDAC4-S210A on Mef2-mediated co-activation by CaMKIIβ. Primary neonatal cardiomyocytes were co-transfected with the 3′Mef2 reporter and increasing concentrations of HDAC4, HDAC4-S210A, or HDAC5 (ranging from 0.05 to 0.5 μg) in the presence or absence of CaMKIIβ-T287D. Without CaMKIIβ-T287D, all three HDACs
Selective HDAC4 Phosphorylation by CaMKII\&B

**FIGURE 5.** CaMKII\&B enhances cardiac transcription. A and B, primary cardiomyocytes were co-transfected with the 3xMEF2-luciferase reporter and a control empty SRx vector or with the reporter and expression vectors for CaMKII\&B or CaMKII\&B-T287D at the indicated concentrations. 48 h later, luciferase activity was measured in total cell lysates. In all transfections, the total amount of DNA was kept constant. Each value is the mean ± S.D. of three independent experiments carried out in triplicate. C, CaMKII\&B and CaMKII\&B-T287D protein levels from transfected cells were determined by Western blot analysis with an anti-HA antibody after SDS-PAGE of total cell lysates. Duplicates of cell lysates run on SDS-PAGE were stained with Coomassie Blue. D, primary cardiomyocytes were co-transfected with a Gal4-dependent reporter construct G5E1b-luciferase, a Gal4 construct fused to full-length MEF2C (Gal-MEF2C-(1–465)) or with deletion constructs containing the MADS domain (Gal-MEF2C-(1–174)) or lacking the MADS domain but retaining transcriptional activity (Gal-MEF2C-(247–327)) and with a CaMKII\&B expression vector SRx. Transcriptional activity was measured 48 h later from total cell lysates. Each value represents the mean ± S.D. of three independent experiments carried out in triplicate.

representative images are shown in Fig. 7B. Expression of HDAC4-S210A alone resulted in 68.8 ± 1.6% of the cells with a strict cytoplasmic staining. When 14-3-3\(\alpha\) was overexpressed with HDAC4-S210A, the fraction of cells with strict cytoplasmic staining increased to 92 ± 2.1%. When CaMKII\&B-T287D was overexpressed, HDAC4-S210A was strictly cytoplasmic in 98 ± 0.5% of the cells. Representative images are shown in Fig. 7C. These results suggest that CaMKII\&B-T287D promotes 14-3-3 binding to HDAC4 but not HDAC5. Furthermore, mutation of Ser-210 in HDAC4 is not sufficient to disrupt 14-3-3 binding. We also performed co-localization studies between HDAC4, HDAC4-S210A, or HDAC5, and wild-type CaMKII\&B, which is expressed at high levels in nuclei. Co-expression of CaMKII\&B with HDAC4, HDAC4-S210A, or HDAC5 did not affect the subcellular localization of these HDACs (supplemental Fig. S3). Next, we analyzed the effect of CaMKII\&B on 14-3-3 interaction with HDAC4 and HDAC4-S210A by co-immunoprecipitation assay. In agreement with our co-localization studies, CaMKII\&B-T287D induced increase binding of 14-3-3\(\alpha\) to HDAC4, and substitution of Ser-210 to alanine had no effect on HDAC4/14-3-3 interaction (supplemental Fig. S4). All together, our data suggest that CaMKII\&B transmits signals to HDAC4 but not to HDAC5. They also suggest that mutation of Ser-210 alone is not sufficient to abrogate 14-3-3 binding to HDAC4.

**Specific Silencing of CaMKII\&B Inhibits MeF2 Activity in Primary Cardiomyocytes and Prevents the Hypertrophic Response**—The \&B subunit of CaMKII regulates MeF2 activity (our data) and is implicated in cardiac hypertrophy (22). Next, we further investigated the physiological role of the kinase by blocking its activity using a specific inhibitor of CaMKII, KN-93, and by measuring the impact on MeF2-dependent transcription. Treatment of cardiomyocytes with KN-93 inhibited MeF2C transcription in primary cardiomyocytes by 42%, whereas the CaMK inactive analogue, KN-92, had no effect (Fig. 8A).

The predominant isoforms of CaMKII in heart muscle are the \&B and \&C subunits (15, 17). CaMKII\&B is defined by a nuclear localization signal, which is absent in the \&C isoform. Both enzymes are extremely homologous within their coding region, and CaMKII\&B inhibits the block of the activity of both isoforms. To specifically silence CaMKII\&B, we designed siRNA oligonucleotides within the nuclear localization region. The ability of CaMKII\&B siRNA to suppress expression of CaMKII\&B protein was validated by co-transfecting the kinase and the siRNA in cardiac cells. Western blot analysis revealed that the designed sequence specifically abolished CaMKII\&B expression in primary cardiac cells, whereas expression of GAPDH was not affected (Fig. 8B). Specific silencing of endogenous CaMKII\&B was also observed while equal concentrations of control siRNA had no effect (Fig. 8C). The G5E1b-luciferase or the 3xMeF2-luciferase reporters were then co-transfected with CaMKII\&B siRNA or control siRNA, and luciferase activity was measured. We observed that MeF2 activity was inhibited by CaMKII\&B siRNA while control siRNA had no effect (Fig. 8D and E). These results suggest that CaMKII\&B signaling is critical for MeF2 activity in cardiac cells.

Cardiac hypertrophy is characterized by increased cell size and reprogramming of fetal cardiac genes (25) such as those encoding the ANF and \(\alpha\)-actinin. We examined the effect of specific silencing of CaMKII\&B on hypertrophic growth induced by serum and agonist treatment in primary cardiac cells (28). Primary cardiomyocytes can be transfected with efficiencies reaching at best 20% using lipid-mediated delivery. To ensure silencing of the kinase in 100% of the cells, we developed an adenovirus expressing a small hairpin RNA against CaMKII\&B (Ad-CaMKII\&B shRNA). The ability of Ad-CaMKII\&B shRNA to suppress CaMKII\&B expression was validated in primary cardiomyocytes by infecting the cells with Ad-CaMKII\&B or a control adenovirus expressing GFP (Ad-
Selective HDAC4 Phosphorylation by CaMII\(\delta\)B

examined the impact on cardiac hypertrophy induced by serum and PE treatment using indirect immunofluorescence. Primary cardiomyocytes expressing GFP and treated with serum or PE developed organized sarcomeres (Fig. 9B). ANF showed a strong perinuclear staining in cells treated with PE (Fig. 9C). In contrast, suppression of CaMII\(\delta\)B expression reduced serum and PE-mediated assembly of sarcomeres as well as ANF expression (Fig. 9, B and C). The blockade of the PE response in cells expressing CaMII\(\delta\)B shRNA was also observed by Western blot analysis of \(\alpha\)-actinin levels (Fig. 9D). Taken together, these results demonstrate that CaMII\(\delta\)B is a potent regulator of Mef2-driven transcription and that CaMII\(\delta\)B-mediated signaling is critical to cardiac hypertrophy.

**DISCUSSION**

CaMK signaling activates transcriptional events associated with cardiac hypertrophy, an adaptative response characterized by the activation of fetal cardiac genes that leads to dilated cardiomyopathy and often heart failure. HDAC5 and -9 suppress cardiac growth, and HDAC9 mutant mice develop cardiac hypertrophy and are hypersensitive to hypertrophic stimuli (26, 27). Transgenic mice overexpressing CaMIV in the heart display a hypertrophic phenotype, as well as an increase in Mef2 transcriptional activity (11). Despite the fact that CaMKI and -IV are normally absent or at low abundance in the heart, these experiments have provided precious information on the pathways implicated. Subsequently Zhang and collaborators demonstrated the role of the endogenous CaMK in this process. The \(\delta\)B isoform of CaMII regulates the activity of the atrial natriuretic factor promoter (21), and transgenic mice expressing high levels of CaMII\(\delta\)B have an enlarged heart (22). However, the mechanisms leading to this effect and whether nuclear CaMII\(\delta\)B transmits signals to class II HDACs remain to be demonstrated. Our study identifies HDAC4 as a preferred substrate of CaMII\(\delta\)B. This result is substantiated by three separate observations. First, CaMII\(\delta\)B highly phosphorylates a GST-

GFP and increasing amounts of Ad-CaMII\(\delta\)B shRNA. CaMII\(\delta\)B protein levels were measured by Western blot analysis using an HA antibody. Specific silencing of the kinase was achieved because adenoviral overexpression of CaMII\(\delta\)B shRNA reduced levels of CaMII\(\delta\)B in cardiac cells but not levels of \(\beta\)-catenin (Fig. 9A). After establishing the ability of Ad-CaMII\(\delta\)B shRNA to reduce expression of CaMII\(\delta\)B, we
HDAC4 fragment but not an equivalent fragment in other class II HDACs. Secondly, HDAC4 and CaMKIIβ have a strong binding, whereas almost no interaction was detected between HDAC5 and the kinase. Finally, HDAC4 and -5 differentially modulate the activity of a Mef2 reporter in primary cardiomyocytes. The remarkable selectivity of the kinase for HDAC4 might represent a mechanism to regulate different sets of target genes in response to certain stimuli and has important implications for the pathogenesis of cardiac hypertrophy and failure.

Nuclear CaMKIIβ Phosphorylates a Novel Site in HDAC4—An elegant series of studies performed by the Olson group in skeletal muscle cells has shown that CaMKI and -IV phosphorylate two conserved serines at the N-terminal of the class II HDACs -4, -5, -7 and -9, leading to the disruption of HDAC-Mef2 complexes with the subsequent nuclear export of these HDACs and de-repression of MEF2 target genes (5–9). Our study shows that the cardiac enzyme CaMKIIβ has characteristics distinct from CaMKI and -IV, because it phosphorylates a GST-HDAC4 fusion protein encompassing the regulatory serines but not the corresponding HDAC5 and HDAC9 fusion proteins. Furthermore, nuclear CaMKIIβ can still phosphorylate an HDAC4 mutant with Ser-246→Ala substitution. To identify the new residues in HDAC4 phosphorylated by CaMKIIβ, we generated single mutants of HDAC4 with Ser/Ala substitution. Our data demonstrate that mutation of Ser-210 to alanine totally abolishes the phosphorylation of the GST-HDAC4 fragment. When the mutation was reconstituted in the full-length protein, we found a significant decrease in HDAC4 phosphorylation (33%). These results point toward Ser-210 in HDAC4 as a site of phosphorylation by CaMKIIβ. Surpris-
Selective HDAC4 Phosphorylation by CaMKIIδB

Our results also suggest that CaMKIIδB is different from the cardiac enzyme activated in response to hypertrophic stimuli reported by Zhang and collaborators (26). That study suggested the existence of a kinase present in cardiac extracts and activated by hypertrophic signals, that phosphorylates the CaMK sites present in all three class II HDACs, -4, -5, and 9. Our results show that the δB isoform transmits signals preferentially to HDAC4. This conclusion is further supported by the observation that CaMKIIδB can still phosphorylate an HDAC4 mutant with Ser-246 → Ala mutation, whereas heart extracts from thoracic aorta-banded mice cannot phosphorylate GST-HDAC proteins in which the CaMK target sites (Ser-246 in HDAC4, Ser-259 in HDAC5, and Ser-218 in HDAC9) were mutated to alanine. Another difference between the cardiac enzyme and CaMKI/IV comes from the finding that the CaMKI- and CaMKIV-responsive domain in Mef2C is located in the MADS/Mef2 domain of the protein (28). Our results show that CaMKIIδB stimulates the activity of Gal-Mef2C-(1-465) and Gal-Mef2C-(1-174), both of which contain the MADS and Mef2 domains. However, a deletion mutant lacking these regions Gal-Mef2C-(247-327) is still activated by the δB isoform. This suggests the existence of another region in Mef2C that confers responsiveness to CaMKIIδB. Studies aiming to determine the region(s) involved are also underway.

Selective Binding of CaMKIIδB to HDAC4 and Differential Repression of Mef2 by HDAC4 and -5 in Cardiac Cells—To understand the physiological relevance of HDAC4 phosphorylation by CaMKIIδB, and to validate the results of our in vitro study, we examined the ability of the kinase to bind HDAC4 and -5 in cells and to relieve Mef2 repression in cardiac cells. We found a preferential binding of the kinase to HDAC4 and a differential effect of HDAC4 and -5 on Mef2 activity in cardiomyocytes. These observations, together with our in vitro phosphorylation data, point toward HDAC4 as being a favorite substrate of the kinase.

The differential effect of the kinase on HDAC4 and -5 cannot be attributed to the different subcellular localization of the wild-type and the constitutive kinase. Although constitutively active CaMKIIδB-T287D is expressed at very high levels in the cytoplasmic compartment, our data showed an equally strong binding of wild-type CaMKIIδB to HDAC4. Furthermore, two

ngly, this serine is conserved in HDAC5 and -9, and with the exception of glutamine 207 and serine 209, surrounding amino acids are also conserved between all three class II HDACs. Because our data show that mutation of Ser-209 has no impact on HDAC4 phosphorylation by CaMKIIδB, glutamine 207 might be an important amino acid for the binding of the kinase to its substrate. This possibility is currently being investigated.

We also found that mutation of Ser-222, Thr-223, and Thr-278 to alanine slightly decreased phosphorylation by CaMKIIδB. This result supports the idea that secondary sites could also contribute to HDAC4 phosphorylation. The generation of double and triple mutants of the protein is on the way and should be valuable to understand the contribution of these residues in HDAC4 phosphorylation by CaMKIIδB.

Our results also suggest that CaMKIIδB is different from the cardiac enzyme activated in response to hypertrophic stimuli.
additional active forms of the enzyme, CaMKIIδB-T287D/S332A, which is strictly nuclear, and CaMKIIδB-T287D/S332–335A, which is both nuclear and cytoplasmic, mimic the effect of the endogenous kinase. These findings strongly suggest that CaMKIIδB transmits signals preferentially to HDAC4.

While this report was under consideration, Backs and collaborators also reported the selectivity of CaMKII for HDAC4 (38). Ser-467 and Ser-632 were identified as two amino acid residues targeted for phosphorylation by CaMKII. Phosphorylation of these amino acids allows binding of the chaperone protein 14-3-3 and the subsequent nuclear export of HDAC4. Our study provides clear evidence that Ser-210 is the third site targeted by nuclear CaMKIIδB. This is substantiated by the observation that amino acids 207–311 in HDAC4 are highly phosphorylated in vitro and that mutation of Ser-210 in this fragment or within full-length HDAC4 impairs phosphorylation. Furthermore, overexpression of this mutant HDAC4 in cells decreased Mef2 activity. Although Ser-210 does not overlap with motifs similar to those targeted by 14-3-3 (RXX-SXP) we investigated the binding of this HDAC4 mutant to 14-3-3 by co-localization and co-immunoprecipitation experiments (data not shown). We found that mutation of Ser-210 alone is not sufficient to disrupt HDAC4/14-3-3 interaction upon CaMKIIδB activation. Based on this, it is possible that other sites, like Ser-467 and Ser-632, might be required for HDAC4/14-3-3 interaction. Indeed, it has been reported that mutation of individual or even two 14-3-3 binding sites is not enough to abolish binding to HDAC4 (5). Based on this, binding and co-localization studies between 14-3-3 and HDAC4 where Ser-210, Ser-467, and Ser-632 are mutated in combination are in progress.

The study reported by Backs et al. shows interaction of HDAC4 with constitutively active CaMKIIδB but not with the wild-type kinase. However, our data showed a clear interaction between HDAC4 and wild-type CaMKIIδB. These discrepancies might be explained by different stringencies used for the immunoprecipitation experiments.

Our finding that HDAC4-S210A localizes in the cytoplasm when co-expressed with CaMKIIδB seems contradictory to our co-activation data, which clearly showed that forced expression of CaMKIIδB cannot rescue HDAC4-S210A-mediated repression of Mef2. A possible explanation is that phosphorylation of Ser-210 might alter the conformation of the molecule and subsequently alter the recruitment of other factor(s), which in turn lead to Mef2 silencing. Another possibility not necessarily exclusive is that the remaining nuclear HDAC4-S210A has stronger inhibitory potential than HDAC4.

CaMKIIδB and Cardiac Growth—CaMKII is a multimeric enzyme and after alternative splicing (39) assembles in homo- or heteromultimers. Among the many splice variants of the δ subunit identified so far the δB is the major nuclear cardiac isoform (20), (15). The existence of different isoforms of CaMK enzymes, as well as many splice variants that have different cellular localization and different ranges of expression in various tissues, suggests varying substrate specificity and a regulation of separate cellular functions. Our study supports the existence of such specificity with different HDAC kinases being activated during hypertrophic stimuli: CaMKIIδB targets HDAC4 while other kinases would transmit signals to other class II HDACs.

Prior studies have linked CaMKII signaling to cardiac hypertrophy. Ectopic expression of the δC isoform of CaMKII in mouse heart leads to dilated cardiomyopathy and heart failure (23). CaMKII inhibition in mice by expression of an inhibitory peptide prevents cardiac hypertrophy and myocardial infarction (40). Recently, CaMKII activation was shown to contribute to endothelin-1-mediated cardiac hypertrophy (41). Our experiments performed with siRNA and shRNA showed that specific reduction of the CaMKIIδB isoform decreases Mef2 activity and blocks hypertrophy induced by serum or agonist treatment in primary cardiac cells. Furthermore, overexpression of CaMKIIδB in mouse heart is associated with an increase in enzyme activity and triggers cardiac hypertrophy (22). These observations suggest that signaling mediated by the δB isoform of CaMKII is critical to cardiac growth.

There is substantial evidence that hypertrophic stimuli converge toward HDAC4 but also toward HDAC5. An expression screen has recently revealed a broad range of molecules, including Mark2, EDG, and Rho (34), which transmit signals to several class II HDACs. However, selectivity has not been identified so far in heart muscle. Our study suggests that CaMKIIδB has unique characteristics in that it preferentially targets HDAC4. Recently, several studies documenting the ability of CaMKII to transmit signals to both HDAC4 and -5 have been reported (41–43). However, none of these studies provide detailed phosphorylation and binding data. In addition, they were either performed in non-cardiac cells or involved the use of CaMKII inhibitors, which, although specific, do not distinguish between the different isoforms of CaMKII. Our study focused on phosphorylation events and brings compelling evidence for signals transmitted selectively through phosphorylation to HDAC4 by CaMKIIδB. In light of these reports (41–43), one might envisage the possibility that CaMKIIδB transmits signals to HDACs other than HDAC4 indirectly. Studies aiming to determine whether phosphorylation of class II HDACs by CaMKIIδB influences their subcellular localization in cardiac cells and whether this event is also selective for HDAC4 are underway and should prove useful to further understand signaling transmitted by CaMKII. The selective phosphorylation of HDAC4 by CaMKIIδB would allow the modulation of only a set of target genes and would result in the recruitment of different activators and repressors, resulting in high specificity of various signaling pathways. The existence of such specificity may prove useful to design therapeutic intervention for cardiac hypertrophy and failure.

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