δ Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II Isozyme-Specific Induction of Neurite Outgrowth in P19 Embryonal Carcinoma Cells

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Abstract: Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK-II) has been linked to the induction of differentiation in preneuronal cells. In these cells, δ isozymes represent the majority of CaMK-IIs expressed and are activated by differentiation stimuli. To determine whether δ CaMK-IIs are causative or coincident with in vitro differentiation, we overexpressed wild-type, constitutively active, and C-terminal domains of δ and γ CaMK-II isozymes in mouse P19 and NIH/3T3 cells using high-efficiency transfections. At 1–2 days after transfection, only constitutively active δ CaMK-II isozymes induced branched cellular extensions in both cell types. In P19 cells, retinoic acid induced neurite extensions after 3–4 days; these extensions were coincident with a fourfold increase in endogenous CaMK-II activity. Extensions induced by both retinoic acid and δ CaMK-IIs contained class III β-tubulin in a discontinuous or beaded pattern. C-terminal CaMK-II constructs disrupted the ability of endogenous CaMK-II to autophosphorylate and blocked retinoic acid-induced differentiation. δ CaMK-II was found along extensions, whereas γ CaMK-II exhibited a more diffuse, cytosolic localization. These data not only support an extranuclear role for CaMK-II in promoting neurite outgrowth, but also demonstrate CaMK-II isozyme specificity in these early steps of neuronal differentiation. Key Words: Ca\(^{2+}\)/calmodulin-dependent protein kinase II—Isozyme—Neurites—P19 embryonal carcinoma cells—Retinoic acid—Green fluorescent protein.


Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II (CaMK-II) is activated in cells induced to undergo differentiation (MacNicol et al., 1990; Tombes et al., 1999). Overexpression and inhibition studies have also linked CaMK-II with axon extension, guidance, and arborization (MacNicol et al., 1990; Kelly, 1991; Solem et al., 1995; VanBerkum and Goodman, 1995; Williams et al., 1995; Tashima et al., 1996; Massé and Kelly, 1997; Wang et al., 1997). It is also evident that the spectrum of CaMK-II isozymes is influenced by the state of cell growth and differentiation (Scholz et al., 1988; Brocke et al., 1995; Bayer et al., 1999; Tombes et al., 1999). Taken together, these findings suggest that neuronal differentiation is influenced by the enzymatic activity of specific CaMK-II isozymes.

We have directly tested this hypothesis by overexpressing only those CaMK-II isozymes that had previously been identified in preneuronal cells (Tombes et al., 1999). Almost 30 different isozymes of mammalian CaMK-II are produced from the expression of only four genes (α, β, γ, and δ). α CaMK-II is expressed exclusively in fully differentiated brain tissue (Tobimatsu and Fujisawa, 1989; Bayer et al., 1999), whereas β, γ, and δ CaMK-IIs are expressed in both neuronal and nonneuronal cell types (Nghiem et al., 1993; Edman and Schulman, 1994; Urquidi and Ashcroft, 1995; Singer et al., 1997). CaMK-II isozyme variability (50–65 kDa) is the result of alternatively spliced domains found in the central variable region (Karsl et al., 1992; Nghiem et al., 1993; Schworer et al., 1993; Edman and Schulman, 1994; Kwiatkowski and McGill, 1995; Tombes and Krystal, 1997). Isozymes do not differ dramatically in catalytic properties, including CaM binding (Braun and Schulman, 1995). Instead, alternatively spliced domains are capable of targeting CaMK-II to the nucleus (Srinivasan et al., 1994; Brocke et al., 1995), the plasma membrane (Urquidi and Ashcroft, 1995), or the sarcomere (Bayer et al., 1998). The domains in CaMK-II responsible for nonnuclear targeting have not yet been fully defined. Because CaMK-IIs can heterooligomerize (Braun and Schulman, 1995; Kolb et al., 1998; Shen et al., 1998), minority isozymes can direct an entire

Received July 4, 2000; revised manuscript received July 31, 2000; accepted August 1, 2000.

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Abbreviations used: ATRA, all-trans-retinoic acid; CaM, calmodulin; CaMK-II, Ca\(^{2+}\)/calmodulin-dependent protein kinase II; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; TBST, Tris-buffered saline (pH 7.4) and 0.1% Tween 20; βIII tubulin, class III β-tubulin.
CaMK-II complex to specific subcellular locations (Srinivasan et al., 1994; Shen et al., 1998).

Some of the previous studies that tested a role for CaMK-II in neuronal differentiation used α CaMK-II (Tashima et al., 1996; Massé and Kelly, 1997; Nomura et al., 1997), which is not naturally expressed in preneuronal cells. Cell growth studies have also been conducted using truncated CaMK-II (Plana-Silva and Means, 1992; Rasmussen and Rasmussen, 1994; Dayton et al., 1997). Truncated CaMK-II is composed primarily of the catalytic half of the protein and is constitutively active but no longer oligomerizes and lacks any specific targeting ability.

In this study, we overexpressed wild-type, constitutively active (full-length), and C-terminal constructs of two δ (δC and δc) and one γ (γc) CaMK-II isozymes into mouse embryonic NIH/3T3 cells and P19 embryonal carcinoma cells. These isozymes are expressed in pre-neuronal cells; δC CaMK-II was originally discovered in the LAN5 human neuroblastoma cell line (Tombes and Krystal, 1997). P19 cells were selected because (like NIH/3T3 cells) they could be routinely transfected at very high efficiency (>50%) and were morphologically ideal for localizations. P19 cells can also be induced to differentiate with retinoic acid (Bain et al., 1994). Both cell types could be used to localize CaMK-IIs indirectly using immunofluorescence and directly using green fluorescent protein (GFP). Finally, we compared the effectiveness and localization of transfected CaMK-IIs with known but limited amino acid differences in their variable domains. Our findings indicate that in both mouse embryonic NIH/3T3 and P19 cells, δ CaMK-IIs are preferentially capable of inducing neurite outgrowth through nonnuclear pathways. Certain amino acid domains found only in δ CaMK-IIs are implicated as responsible for this specific effect.

**MATERIALS AND METHODS**

**Cell lines and culture**

NIH/3T3 mouse embryoblasts and P19 mouse embryonal carcinoma cells were cultured on polystyrene dishes in Dulbecco’s modified Eagle’s medium (BioWhitaker, Walkersville, MD, U.S.A.) with 10% fetal bovine serum (GibcoBRL, Bethesda, MD, U.S.A.), supplemented with penicillin/streptomycin, in a 5% CO2 humidified chamber at 37°C. All-trans-retinoic acid (ATRA; Sigma, St. Louis, MO, U.S.A.) was added to P19 cells at 500 nM to induce differentiation.

**cDNA preparation**

Full-length human γ CaMK-II cDNAs (wild-type and constitutively active) were obtained from Dr. Howard Schulman (Stanford University, Palo Alto, CA, U.S.A.) in the eukaryotic expression vector pSRα (Nghiem et al., 1993). Full-length γc CaMK-II cDNAs were constructed by swapping in the 173-bp PvuII variable region fragment for the 242-bp γs fragment that flanks the variable domain. This fragment was obtained from a partial human γc CaMK-II cDNA, cloned as described (Tombes and Krystal, 1997). Full-length δc and δs CaMK-II cDNAs were prepared by swapping in their respective 535- or 502-bp BamI fragments, which also bracket the variable domain. BamI fragments were cut from 3′ CaMK-II clones that were prepared using rapid amplification of cDNA ends (3′), as described below. Resultant δ CaMK-II cDNAs are chimeric, i.e., the first 301 and the last 22 amino acids are encoded by γ CaMK-II. Both wild-type and constitutively active cDNAs were prepared in this fashion.

δc and δs CaMK-II 3′ ends were cloned using rapid amplification of cDNA ends (3′) from LAN5 human neuroblastoma cell total RNA (Tombes and Krystal, 1997). cDNA was synthesized using oligo(dT) as primer and Marathon cDNA adapters (Clontech, Palo Alto). “Hot-start” PCR procedures were conducted on this cDNA for 27 cycles at a melting temperature of 55°C using 10 μM sense primer (5′-GGG ACA CAG TGA CAC CTG AAC C-3′), 10 μM antisense primer AP1 (Clontech), and 2 U of a mixture of Taq/Per polymerases (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Nested PCR procedures were then conducted on a 1:100 dilution of the primary PCR products for 15 cycles at a melting temperature of 55°C using 10 μM sense primer (5′-GGT GCC ATC [C/T][T][C/G] AC[C/G/A] AC[C/T] ATG CT-3′), 10 μM antisense primer AP2 (Clontech), and 1 U of a mixture of Taq/Pwo. Distinct and prominent PCR products of 800–1,100 bp were then cloned directly into the pcR 2.1 cloning vector (Invitrogen, San Diego, CA, U.S.A.) and sequenced as described (Tombes and Krystal, 1997).

cDNAs chimeric with enhanced GFP (EGFP) were prepared using PCR-mediated directional cloning. Primers were synthesized that amplified wild-type cDNAs, prepared as described above, and restriction enzyme sites were introduced (BspEI at the 5′ end and BamHI at the 3′ end). These enabled the in-frame introduction of CaMK-II cDNAs with an N-terminal EGFP protein in the vector pEGFP-C1 (Clontech). In addition to wild-type and constitutively active CaMK-IIs, C-terminus vectors (from amino acid 311 to the stop codon) were prepared linked to EGFP.

**Transfection of DNA into mammalian cells**

cDNAs encoding full-length CaMK-II isozymes were transfected into NIH/3T3 and P19 cells using Lipofectamine PLUS (Life Technologies, Grand Island, NY, U.S.A.) for 3 h followed by culture for at least an additional 24 h. Transfection efficiencies in both cells routinely exceeded 50% and could be as high as 75%.

**Whole-cell lysate preparation**

To examine enzymatic activities and immunoreactive polypeptides, cells were grown in log phase, harvested with trypsin-EDTA, and then rinsed centrifugally (2,000 g for 5 min) with phosphate-buffered saline containing 2.5 mM EGTA. Pellets were resuspended in 3 volumes of ice-cold homogenization buffer, which consisted of 20 mM HEPES (pH 7.4), 2.6 mM EGTA, 20 mM MgCl2, 80 mM β-glycerophosphate, 50 mM NaF, 0.1 μM okadaic acid (GibcoBRL), 0.1 μM calyculin A (GibcoBRL), 0.1 mM dithiothreitol, and 0.01 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor (Sigma). Samples were then sonicated (two 5-s bursts on ice), centrifuged at 12,000 g for 15 min at 4°C, and either assayed immediately or frozen and stored at −80°C. Lysates prepared by sonication solubilized >90% of the total CaMK-II activity as measured by solution assays and immunoblots (data not shown).

**CaMK-II assays**

Cytosolic fractions were diluted to 0.1–0.2 mg/ml protein in homogenization buffer. Ten microliters of these samples was
then assayed under three conditions in triplicate: (a) without Ca\(^{2+}\) and without substrate, (b) without Ca\(^{2+}\) and with substrate, and (c) with Ca\(^{2+}\)/CaM and with substrate. Autonomous activity was determined from the difference in cpm incorporated between b and a, whereas total activity was determined from the difference in cpm incorporated between c and a.

Reactions were carried out in a total volume of 25 \(\mu\)l containing final concentrations of 20 mM HEPES (pH 7.4), 0.1 mM dithiothreitol, 15 mM magnesium acetate, 20 mM \(\beta\)-glycerophosphate, 10 mM NaF, 0.5 mM \(\mu\)M protein kinase A inhibitor peptide, 0.1 \(\mu\)M okadaic acid, 0.1 \(\mu\)M calyculin A, 40 \(\mu\)M sodium orthovanadate, 0.5 \(\mu\)Ci of \([\gamma-32P]ATP\), 20 \(\mu\)M total ATP, 35 \(\mu\)M autocamtide-2 (substrate), and either 1.04 mM EGTA (without Ca\(^{2+}\)) or 1 mM calmodulin plus 1 mM EGTA/3 mM CaCl\(_2\) (with Ca\(^{2+}\)). After 5–10 min at 32°C, 20 \(\mu\)l was pipetted onto P81 phosphocellulose (Whatman, Clifton, NJ, U.S.A.) paper squares that were air-dried for 1 min and then washed five times in 500 ml of 1% phosphoric acid. Dried paper squares were subjected to Cerenkov counting in a Beckman model LS 1801 scintillation counter. At these protein concentrations and assay conditions, this assay was linear for up to 15 min. Autocamtide-2 (KKALRRQETVDAL) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), calmodulin was from Boehringer Mannheim, \([\gamma-32P]ATP\) was from NEN-Du Pont (Wilmington, DE, U.S.A.), and all other reagents were from Sigma. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL, U.S.A.) in triplicate.

Samples for sodium dodecyl sulfate–polyacrylamide gel electrophoresis were phosphorylated for 30 s on ice in 30 mM HEPES (pH 7.4), 0.1 mM dithiothreitol, 15 mM magnesium acetate, 50 mM \(\beta\)-glycerophosphate, 0.5 mM cyclic AMP-dependent protein kinase inhibitor peptide, 0.5 \(\mu\)Ci of \([\gamma-32P]ATP\), 20 \(\mu\)M ATP, 1 \(\mu\)M calmodulin, and either 2 mM EGTA (without Ca\(^{2+}\)) or 1 mM EGTA/3 mM CaCl\(_2\) (with Ca\(^{2+}\)). The reaction was stopped with an equal volume of preheated 2X sample buffer, followed by boiling for 3 min. Discontinuous sodium dodecyl sulfate gels were prepared with 8% polyacrylamide, and transferred proteins were exposed to X-blue film (Kodak, Rochester, NY, U.S.A.) at −80°C.

**Immunoblots**

Sonicated whole-cell lysates were separated on 8 or 10% polyacrylamide gels using the Mini-Protein gel electrophoresis system (Bio-Rad, Richmond, CA, U.S.A.). Proteins were transferred to nitrocellulose sheets (pore size, 0.2 \(\mu\)m; Schleicher and Schuell, Keene, NH, U.S.A.) for 1 h at 100 V and blocked with Tris-buffered saline (pH 7.4) and 0.1% Tween 20 (TBST) containing 2.5% nonfat dry milk, 2.5% bovine serum albumin, and 2% normal goat serum for 1 h. A rabbit polyclonal antibody prepared against two conserved domains in all known mammalian CaMK-Is was routinely used for CaMK-II immunoblots (Upstate Biotechnology, Lake Placid, NY, U.S.A.). The anti-GFP antibody was a mouse monoclonal IgG (Clontech). Primary antibodies were typically diluted to 0.5 \(\mu\)g/ml in 2% bovine serum albumin in TBST with 2% normal goat serum and incubated between 2 and 12 h with the nitrocellulose blot. Blots were washed five times with TBST and incubated for 2 h with 0.5 \(\mu\)g/ml alkaline phosphatase-coupled goat anti-rabbit IgG (Kierkegaard Perry Labs, Gaithersburg, MD, U.S.A.) in 2% bovine serum albumin in TBST. Blots were developed with 0.25 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml nitro blue tetrazolium (Sigma) in 0.1 M Tris, 0.1 M NaCl, and 5 mM MgCl\(_2\), pH 9.4.

**Thymidine incorporation**

Thymidine incorporation was measured after transfecting 0.5 \(\mu\)g of DNA into cells at ~25% confluence in 12-well plates (22 mm in diameter). Cells were grown for 24 h and then incubated with 1–2 \(\mu\)Ci of \([^{3}H]^{3}H\)thymidine (NEN-Du Pont) for an additional 24 h. Culture medium was aspirated, and cells were lysed with 0.5 ml of fresh 0.5 M NaOH. DNA was subsequently precipitated with 12.5% trichloroacetic acid. After 30 min on ice, precipitated DNA was collected by filtration through glass fiber (GF/C) filters (Whatman). Filters were rinsed with 5% trichloroacetic acid and then 95% ethanol, and radioactivity was quantitated using liquid scintillation spectrometry.

**Immunohistochemistry and microscopy**

Transfected fibroblasts were grown on glass coverslips overnight. Coverslips were removed from culture, rinsed in phosphate-buffered saline, and then fixed in 100% methanol at −20°C for 3 min. Fixed cells were blocked with 5% bovine serum albumin and 5% rabbit serum in TBST for 30 min and then successively incubated with 2 \(\mu\)g/ml goat anti-\(\gamma\)CaMK-II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), 1 \(\mu\)g/ml biotinylated rabbit anti-goat IgG (Kierkegaard Perry Labs), and 1 \(\mu\)g/ml Texas Red–streptavidin (Molecular Probes, Eugene, OR, U.S.A.). This antibody is reactive with the C-terminal 21 amino acids found in all chimeric clones used in this study. For tubulin staining, mouse monoclonal antibodies specific for \(\alpha\)-tubulin (Sigma) or class III \(\beta\)-tubulin (BIII tubulin) were used. Coverslips were sealed and imaged using either a Photometrics (Tucson, AZ, U.S.A.) SenSys 12-bit digital camera on an Olympus Provis microscope or an Olympus Fastscan 2000 12-bit digital camera on an Olympus IX70 inverted fluorescent microscope (Olympus America, Melville, NY, U.S.A.). Image analysis was conducted using IP Labs (Vienna, VA, U.S.A.) Spectrum software or Adobe Photoshop Version 5.5. Statistical analysis was conducted using one-way ANOVA and SigmaStat version 2.0 software (Jandel Scientific Software). Adobe Photoshop was used to compile images.

**RESULTS**

Expression and characterization of CaMK-II isoforms

Wild-type, constitutively active, and C-terminal CaMK-II cDNAs were prepared in the eukaryotic expression vector pSR\(_{\alpha}\) (Takebe et al., 1988) or linked to EGFP in the vector pEGFP-C1, as described (see Materials and Methods). EGFP was found at the amino termini of the resulting proteins. Constitutively active mutants were prepared with a mutation of Thr\(^{287}\) to Asp\(^{287}\) (T287D), rendering the enzyme partially active in the absence of Ca\(^{2+}\)/CaM (Waldmann et al., 1990), while retaining its full length and thus its ability to oligomerize. C-terminal constructs were prepared by deleting the first 310 amino acids, which includes the catalytic and CaM-binding domains (see Materials and Methods). Splice variant terminology is used as previously described (Tombes and Krystal, 1997). We restricted these studies to \(\gamma\)_\(C\), \(\delta\)_\(C\), and \(\delta\)_\(B\) CaMK-II isoforms because they were expressed in these cells and contained minimal sequence differences. To simplify analysis and detection, we engineered \(\gamma\)_\(C\), \(\delta\)_\(C\), and \(\delta\)_\(B\) CaMK-II cDNAs to encode
common catalytic domains (first 301 amino acids) and C-terminal tails (final 22 amino acids).

In transient transfection assays, CaMK-IIs were expressed at high levels in NIH/3T3 mouse embryonic cells or P19 embryonal carcinoma cells after 1–2 days. Transfection efficiencies typically exceeded 50%. Extracts prepared from these cells were evaluated by CaMK-II solution assays and immunoblots. Figure 1A shows wild-type and constitutively active γ and δ CaMK-II splice variants that had been transfected into and expressed in NIH/3T3 cells and then reacted with a pan-specific CaMK-II antibody. All CaMK-II constructs migrated as expected between 52 (δC) and 62 kDa (γB) (Tombes et al., 1999), and wild-type isoforms were always expressed at greater mass levels than constitutively active CaMK-IIs for reasons that are not known. As indicated above, endogenous γ and δ CaMK-II isozymes are present in these cells (Tombes et al., 1999) but were below detection levels (lane –) when loaded at equivalent protein levels.

GFP-linked CaMK-IIs were detected on immunoblots with an anti-GFP monoclonal antibody (Fig. 1B). This antibody detected wild-type and constitutively active isoforms at 80–85 kDa and the C-terminal constructs at 50–55 kDa (GFP provides an additional 27-kDa mass). As with non-GFP isoforms, wild-type construct expression was typically twice that of constitutively active CaMK-II, whereas the truncated C-terminal constructs were expressed at four- to sevenfold higher levels than the constitutively active constructs, as determined by densitometry.

Oligomeric CaMK-II is characterized by its ability to autophosphorylate at Thr327 in a concentration-independent manner (Hanson et al., 1994). Oligomeric CaMK-II reaches maximal autophosphorylation in assays conducted for 30 s on ice. Both endogenous and transfected activities can be detected, and their catalytic interactions can be inferred through this assay. Only wild-type CaMK-II will autophosphorylate because the primary site of autophosphorylation (Thr327) is mutated to Asp in the constitutively active constructs and the C-terminal constructs begin at Thr311. When untransfected P19 cell lysates were subjected to this assay, at least three protein species representing endogenous CaMK-IIs were easily observed (Fig. 1C, lane Empty +). The lower two bands comigrate with δC and δB/CaMK-II at 52 and 57 kDa as seen in other cell lines (Tombes et al., 1999). The upper band (62 kDa) comigrates with δI or β CaMK-II. The level of autophosphorylation of these endogenous CaMK-IIs was approximately halved in cells transfected with GFP-linked constitutively active CaMK-II (lane δCcon +). This level was also dramatically reduced in cells transfected with the kinase-inactive C-terminal CaMK-II constructs (lane δCwt +), suggesting that this C-terminal domain can heterooligomerize with endogenous CaMK-II and act in a dominant-negative fashion. The effectiveness of these two constructs is proportional to their expression levels (Fig. 1B). GFP-linked wild-type δC CaMK-II autophosphorylates (at 80 kDa) but, as expected, does not disrupt endogenous CaMK-II autophosphorylation (lane δCwt +). These results strongly suggest that all three CaMK-II constructs used here can heterooligomerize with endogenous CaMK-IIs and either replace or influence endogenous CaMK-II.

CaMK-II solution assays confirmed the Ca2+/CaM dependence of CaMK-II constructs and their relative expression levels (Table 1). Wild-type CaMK-II isoform activity shows a 100-fold Ca2+/CaM dependence. As inferred from immunoblots, the Ca2+/CaM-dependent activity was ~10-fold greater than the endogenous levels of CaMK-II (empty vector, Table 1). GFP-linked wild-type CaMK-IIs were also highly Ca2+/CaM-dependent, indicating that the GFP domain does not disrupt catalytic activity. However, GFP-linked CaMK-IIs did not reach the same levels of expression as their non-GFP wild-type counterparts. Constitutively active isoforms never exceeded twice the endogenous Ca2+/CaM-dependent CaMK-II specific activity level, although their Ca2+/CaM-independent activity levels were up to 20-fold higher than endogenous levels. As expected, cells transfected with C-terminal CaMK-II constructs showed the same level of total CaMK-II activity as nontransfected

![Image of a diagram](https://example.com/diagram.png)

**FIG. 1.** Ectopic expression and autophosphorylation of CaMK-II constructs. A: Anti-CaMK-II immunoblot of whole-cell lysates (5 μg) from transfections with the indicated CaMK-II constructs. The antibody used was the pan-specific CaMK-II antibody (Upstate Biotechnology). CaMK-II cDNAs were wild-type (wt) or constitutively active (con; T287D point mutants) constructs of two γ and two δ isoforms expressed in NIH/3T3 cells. B: Anti-GFP immunoblot of GFP-linked wt, C-terminal (ct), and con CaMK-IIs expressed in P19 cells and reacted with the monoclonal anti-GFP antibody (Clontech). C: Autoradiogram of lysates from P19 cells transfected with GFP-linked con, ct, and wt mutants of δC CaMK-II and autophosphorylated for 30 s on ice with or without Ca2+/CaM. Although endogenous isozyme levels are undetectable by immunoblot relative to overexpressed variants, they show significant autophosphorylation (empty lanes).
cells. C-terminal constructs therefore can disrupt autophosphorylation but do not change the level of total endogenous CaMK-II. All constructs containing a catalytic domain were similarly dependent on Ca\(^{2+}\)/CaM, exogenous CaM, and substrate, indicating that they all expressed properly regulated and catalytically active enzyme.

**Effect of constitutively active CaMK-II isozymes on morphology and growth of NIH/3T3 cells**

As previously reported by other investigators (Massé and Kelly, 1997), we were unable to select stably transfected clones that were overexpressing certain CaMK-IIs. Within 1 or 2 days after transfection, it became clear that these same CaMK-IIs caused cells to grow more slowly and exhibit certain morphological changes. For example, within 2 days after transfection in NIH/3T3 cells, constitutively active \(\delta_E\) CaMK-II caused one to two branched extensions per cell (Fig. 2). \(\delta_E\) CaMK-II was enriched in the perinuclear region and found along the length of these occasionally branched extensions, which occurred in 70–80% of transfected cells (Fig. 2). We observed this for constitutively active \(\delta_E\) CaMK-II whether it was linked to GFP (Fig. 2) or not. Because these were transient transfections, untransfected cells (revealed by comparison with the phase images) demonstrate the normal morphology of these cells under these conditions as well as the high efficiency of transfections. \(\delta_C\) CaMK-II was also expressed in the perinuclear region but had a more subdued effect on the formation of cellular extensions in NIH/3T3 cells (see summary in Table 2). Neither wild-type isoforms nor constitutively active \(\gamma_C\) CaMK-II caused the long, branched extensions frequently seen with \(\delta_E\) CaMK-II. \(\gamma_C\) CaMK-II was also more uniformly expressed throughout the cells than the \(\delta\) CaMK-II isoforms.

Morphological changes in NIH/3T3 cells were quantitated from cells that expressed either wild-type or constitutively active constructs of non-GFP CaMK-II (Table 2). Transfected cells were identified by indirect immunofluorescence using a CaMK-II antibody, and images were acquired under identical exposure conditions. This analysis confirmed that constitutively active \(\delta_E\) CaMK-II was more effective than constitutively active \(\gamma_C\) CaMK-II in inducing lengthy extensions \((p < 0.001\) by one-way ANOVA), even though these isozymes were expressed at similar levels. Second, although wild-type \(\delta_E\) CaMK-II was expressed at mass levels more than twice that of its constitutively active counterpart, it did not cause significant increases in extension length or cell perimeter \((p < 0.001\) by one-way ANOVA). This result demonstrates that extensions are the result of increased (autonomous)

### TABLE 1. Specific activity levels of ectopically expressed CaMK-II isozymes

<table>
<thead>
<tr>
<th>Activity (nmol/min/mg)</th>
<th>(+)Ca(^{2+})</th>
<th>(-)Ca(^{2+})</th>
<th>% autonomy</th>
</tr>
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<tbody>
<tr>
<td>Empty vector</td>
<td>0.13</td>
<td>4.04</td>
<td>3.0</td>
</tr>
<tr>
<td>(\gamma_C)-wt</td>
<td>0.30</td>
<td>36.71</td>
<td>0.8</td>
</tr>
<tr>
<td>(\delta_C)-wt</td>
<td>0.51</td>
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<td>0.29</td>
<td>34.03</td>
<td>0.9</td>
</tr>
<tr>
<td>(\gamma_C)-wt-GFP</td>
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<td>19.97</td>
<td>0.8</td>
</tr>
<tr>
<td>(\delta_C)-wt-GFP</td>
<td>0.12</td>
<td>12.34</td>
<td>1.0</td>
</tr>
<tr>
<td>(\gamma_C)-wt-GFP</td>
<td>0.14</td>
<td>13.59</td>
<td>1.0</td>
</tr>
<tr>
<td>(\gamma_C)-con</td>
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<td>5.31</td>
<td>45.8</td>
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<td>(\delta_C)-con</td>
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<td>8.71</td>
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<tr>
<td>(\delta_C)-con</td>
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Whole-cell lysates from NIH/3T3 cells 2 days after transfection were assayed in triplicate for \(+\)Ca\(^{2+}\)/CaM-dependent \((\gamma_C\)-wt\) and \(-\)independent \((\gamma_C\)-wt-GFP\) activity using phosphocellulose paper assays and autocamtide-2. Values are averages of three experiments and are normalized per milligram of cellular protein. SD values were all around 10% between transfection experiments. Values were similar in P19 cells. wt, wild-type; con, constitutively active.

**FIG. 2.** Effect of CaMK-II on NIH/3T3 cells. NIH/3T3 cells were transfected with GFP-linked constitutively active isozymes of \(\gamma_C\), \(\delta_E\), and \(\delta_C\) CaMK-IIs. After 48 h, living cells were imaged directly using phase-contrast and fluorescence microscopy. Bar = 50 μm.
CaMK-II activity and not total expression (mass) levels. δE and δC CaMK-II isozymes also caused a statistically significant (p < 0.001 by one-way ANOVA) increase in total area and perimeter compared with untransfected cells or those transfected with wild-type isozymes. δC constitutively active cells did not exhibit a statistically significant increase in extensions in these cells. The isozyme-specific effect of δ CaMK-II isozymes in NIH/3T3 cells was further revealed by decreased cell number 2 days after transfection as quantitated by decreased cell counts and decreased thymidine incorporation (Fig. 3). None of the wild-type isozymes had a significant inhibitory effect on cell number. Constitutively active δC and δE CaMK-II decreased thymidine incorporation to 40% of the levels observed with empty vector transfectants. Decreased thymidine incorporation was not due to the induction of apoptosis as determined by nuclear fragmentation with DNA-binding dyes or flow cytometry (data not shown). Flow cytometry consistently showed no cell cycle arrest in any one particular phase of the cell cycle. There was also no decrease in extracellular signal-regulated kinase 1/2 activity or cyclin-dependent kinase 2 activity or an increase in the level of cyclin-dependent kinase inhibitor proteins, including p21cip1 and p27kip1, in these cells using assays previously described (Morris et al., 1998; Tombes et al., 1998).

Effect of constitutively active CaMK-II isozymes on P19 cells

P19 embryonal carcinoma cells can be induced to initiate steps toward neuronal differentiation, as assessed morphologically and biochemically, when treated with retinoic acid (Bain et al., 1994). We treated P19 cells with 0.5 μM ATRA or transfected them with the same CaMK-II constructs used in NIH/3T3 cells. Our results again revealed that constitutively active δ CaMK-II isozymes caused the formation of branched cellular extensions within 24 h of transfection. We photographed these cells after 48 h and observed both δC and δE constitutively active enzymes to have this effect (Fig. 4). Wild-type isozymes were completely ineffective, whereas constitutively active γC CaMK-II caused shape changes but did not cause the formation of extensions. The C-terminal constructs were also ineffective at inducing cellular extensions, even though they were expressed at much higher mass levels (see Fig. 1B). ATRA caused shape changes in cells throughout the population, including increased phase densities and rounding, but did not cause neurite extensions until 3–4 days after treatment (see Fig. 6). We quantitated the effect of δ CaMK-II expression on P19 cells by counting the number of cells that expressed outgrowths 48 h after transfection (Fig. 5). These numbers were averaged from four independent experiments and confirmed the effectiveness of constitutively active δ CaMK-II isozymes (p < 0.001 by one-way ANOVA). They also revealed that constitutively active constructs that are not linked to GFP were more effective in promoting outgrowths than GFP-linked vectors. This is most likely due to the higher expression levels of these constructs as confirmed by CaMK-II activity assays (see Table 1).

Outgrowths induced by constitutively active δ CaMK-II expression in P19 cells contained βIII tubulin. ATRA is known to induce increased βIII tubulin gene expression in P19 cells after 2–3 days (Laferrière and Brown, 1996).

### Table 2. Quantification of morphological changes induced by CaMK-II isozymes in NIH/3T3 cells

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>No. of cells</th>
<th>Intensity (arbitrary units)</th>
<th>Extension length (μm)</th>
<th>Area (μm²)</th>
<th>Perimeter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SREα</td>
<td>30</td>
<td>991 ± 625</td>
<td>40 ± 15</td>
<td>700 ± 224</td>
<td>250 ± 45</td>
</tr>
<tr>
<td>γC-wt</td>
<td>28</td>
<td>1,051 ± 518</td>
<td>38 ± 15</td>
<td>667 ± 385</td>
<td>162 ± 60</td>
</tr>
<tr>
<td>δC-wt</td>
<td>32</td>
<td>1,122 ± 670</td>
<td>54 ± 15</td>
<td>1,059 ± 641</td>
<td>210 ± 85</td>
</tr>
<tr>
<td>γC-con</td>
<td>27</td>
<td>440 ± 193</td>
<td>63 ± 15</td>
<td>847 ± 410</td>
<td>239 ± 81</td>
</tr>
<tr>
<td>δC-con</td>
<td>29</td>
<td>467 ± 228</td>
<td>132 ± 57*</td>
<td>1,366 ± 589*</td>
<td>434 ± 149*</td>
</tr>
<tr>
<td>δC-con</td>
<td>25</td>
<td>398 ± 231</td>
<td>87 ± 15</td>
<td>1,659 ± 844*</td>
<td>292 ± 95*</td>
</tr>
</tbody>
</table>

Cells expressing wild-type (wt) or constitutively active (con) isozymes were fixed, stained with a CaMK-II antibody, and imaged under identical conditions. Transfection efficiency was >50%. Images were evaluated for the following: (a) average overall cellular intensity of the fluorescent signal (after background subtraction), (b) average length of extensions starting at the nucleus, (c) cell area, and (d) cell perimeter. Data are average ± SD values.

*p < 0.001 by one-way ANOVA compared with all other isozymes.
We observed weak βIII tubulin expression throughout the tubulin array of many cells in culture after 2 days of ATRA (Fig. 6, ATRA 2d). After 4 days of ATRA, βIII tubulin-rich extensions or neurites were found in a subset (<10%) of these cells. Often, cells with neurites were found in clumps surrounded by cells that had not differentiated. The single cell shown here was used because it showed most clearly the discontinuous, beaded appearance of βIII tubulin along the neurite (ATRA 4d). α-Tubulin was uniformly distributed along these neurites (data not shown). This was observed whether cells were fixed with methanol or glutaraldehyde. The extensions induced by δC CaMK-II after 2 days also contained “beaded” βIII tubulin (δC-con 2d). βIII tubulin levels along these extensions became more intense and uniform by 4 days after transfection (δC-con 4d), suggesting that the discontinuity is a reflection of active growth of these extensions. Although extensions caused by both δC and δE CaMK-II expression temporally preceded neurites induced by retinoic acid, retinoic acid-induced extensions were longer and had a much higher level of βIII tubulin.

C-terminal constructs of CaMK-II did not induce neurite extension in P19 cells but did block the autophosphorylation of endogenous CaMK-IIs (see Fig. 1C). We therefore determined whether C-terminal constructs could block the effects of retinoic acid. This was tested by treating cells transfected with GFP-linked C-terminal constructs with retinoic acid for 48 h (Fig. 7). C-terminal constructs exhibited the same extranuclear localizations as the wild-type isozymes. Cells expressing the C-terminal constructs of both δC and δE CaMK-II (arrows) did not exhibit the differentiated morphology of cells that were not transfected (asterisks), as revealed by comparative phase-contrast and fluorescence (GFP) microscopy. None of the cells expressing these C-terminal constructs were induced to express βIII by ATRA.

Activation of CaMK-II by retinoic acid

The effectiveness of δ CaMK-II in inducing premature differentiation of P19 cells and the ability of C-terminal CaMK-II constructs to block retinoic acid-induced differentiation suggested that retinoic acid could induce CaMK-II expression and/or activation. We used both CaMK-II autophosphorylation assays (see Fig. 1) and solution assays (see Table 1) to measure CaMK-II activity in P19 cells up to 6 days after treatment with retinoic acid (Fig. 8). Immunoblots using tubulin-specific antibodies confirmed our immunofluorescence results that α-tubulin expression is stable over this time course, whereas βIII tubulin content increases between 2 and 4 days as previously described (Laferrière and Brown, 1996). CaMK-II autophosphorylation assays also revealed an increase in the intensity of the three endogenous CaMK-II bands between 2 and 4 days. This
assay was more specific and sensitive than any of the available CaMK-II antibodies. Solution assays also showed an increase in total CaMK-II activity at 3 days postretinoic acid. It is interesting that autophosphorylation capability dropped between day 4 and 6. Total CaMK-II activity dropped slightly at 6 days, but natural activation and thus autophosphorylation would also decrease incorporation of radiolabeled phosphate in this assay. These results indicate that retinoic acid causes total CaMK-II expression to increase about fourfold after 3 days to a new level coincident with the appearance of neurites. The overexpression of the correct (δ) CaMK-II isozyme accelerates this process.

DISCUSSION

This laboratory has previously reported that the principal CaMK-II isozymes in human and rodent embryonic cells are splice variants encoded by the δ CaMK-II gene (Tombes and Krystal, 1997). These δ CaMK-II isozymes are down-regulated upon oncogenic transformation, suggesting that they are growth-suppressive (Tombes et al., 1999). The present study has directly tested that hypothesis and has found results consistent with a specific role for δ CaMK-II activity in cellular differentiation. We report that constitutively active δC and δE CaMK-II overexpression in these cells slows cell growth and causes cell processes to form. γC CaMK-II, which has subtle structural differences, does not induce cellular extensions and is localized differently. C-terminal domains alone, which lack catalytic activity but which are targeted like full-length CaMK-II, do not induce extensions, even when expressed in great excess, but block the induction of neurites by retinoic acid. Extensions induced by both retinoic acid and δ CaMK-II isozymes in P19 cells were enriched in a neurite-specific marker, βIII tubulin, in a distinctive discontinuous pattern, although δ CaMK-II induced extensions more rapidly. Both the level and autonomy of the three principal CaMK-II species in these cells were increased by retinoic acid. These data support a model by which the phosphorylation of pro-

FIG. 6. Induction of βIII tubulin expression in P19 cells by δ CaMK-IIs and retinoic acid. P19 cells were either treated with 0.5 μM ATRA or transfected with constitutively active δC, CaMK-II (δC-con), then fixed after 2 or 4 days, and stained with a monoclonal antibody against βIII tubulin followed by phase-contrast and fluorescent microscopy. Bars = 50 μm.
CaMK-II. We also had difficulty propagating stable NIH/3T3 clones expressing constitutively active δ CaMK-IIs. Clones that can be propagated may be those that are unusually adept at remaining in the cell cycle.

It has also previously been reported that the overexpression of constitutively active CaMK-II in mammalian and fungal cells arrests cell growth at the G2/M transition (Planas-Silva and Means, 1992; Rasmussen and Rasmussen, 1994; Dayton et al., 1997). Those studies used truncated CaMK-II, which has access to the nucleus. In contrast, our studies were conducted with full-length CaMK-II isoforms that retain their association and targeting domains, are excluded from the nucleus, do not have a cell cycle phase-specific effect, and therefore most likely have a different locus of action.

We contrasted δ CaMK-II with γC CaMK-II because their amino acid differences were so slight and they were both naturally expressed in the cells studied here. Among the four mammalian CaMK-II genes (α, β, γ, and δ), amino acid differences are minimal in the catalytic (first 300 residues) and oligomerization domains (last 100 residues) and are greatest in the alternatively spliced central variable region (50–150 amino acids) (Tombes and Krystal, 1997). Nonetheless, to ensure that comparisons focused on this central variable domain, all isoforms prepared for this study were “chimeric,” i.e., they all used the same catalytic domain (amino acids 1–301) and 22-amino acid C-terminal tail. This excluded proteins by specific δ CaMK-II isoforms induces or stabilizes neurite outgrowth.

In previous studies, CaMK-II was directly implicated in promoting axon extension and neurite outgrowth (Kelly, 1991; Solem et al., 1995; VanBerkum and Goodman, 1995; Williams et al., 1995; Tashima et al., 1996; Massé and Kelly, 1997; Nomura et al., 1997). Isozyme specificity was implicated in one of these studies in which β CaMK-II was more capable of inducing neurite outgrowth than α CaMK-II in mouse Nb2a neuroblastoma cells (Nomura et al., 1997). Two of these studies (Tashima et al., 1996; Massé et al., 1997) concluded that overexpressed CaMK-II inhibits neurite outgrowth induced by either nerve growth factor or dibutyryl cyclic AMP. Both of these studies prepared stable cell lines overexpressing wild-type α CaMK-II, an isoform that is not normally expressed in these cells (Tobimatsu and Fujisawa, 1989; Tashima et al., 1996; Tombes and Krystal, 1997; Buyer et al., 1999). One interpretation of these potentially contradictory findings is that α CaMK-II, which is found in mature neurons, may phosphorylate proteins that stabilize neurites as opposed to δ CaMK-II, which may phosphorylate proteins that enable the dynamic growth and elongation of neurites. Alternatively, like the C-terminal constructs used in this study, α CaMK-II could heterooligomerize with endogenous (δ) CaMK-IIs and then act in a dominant-negative manner to misdirect endogenous CaMK-II and block neurite outgrowth. Finally, it may be inherently difficult to conduct CaMK-II overexpression studies using stable clones. One of these studies (Massé et al., 1997) reported difficulty in selecting clones expressing high levels of

FIG. 7. Inhibition of retinoic acid-induced differentiation by C-terminal (CT) CaMK-II constructs. P19 cells were transfected with the CT δc and δe CaMK-II constructs, then treated with 0.5 μM ATRA for 2 days, and imaged using phase-contrast and fluorescence microscopy. Cells that do not express the construct (*) show the typical protrusions associated with early stages of retinoic acid-induced morphological changes, whereas cells that express the construct (arrows) exhibit a control morphology.

FIG. 8. Retinoic acid induction of βII tubulin and CaMK-II. P19 cells were treated continuously with 0.5 μM ATRA and harvested at 24-h intervals. Protein lysates were analyzed by immunoblot for α- (1 μg) and βII- (5 μg) tubulin and by autophosphorylation (1 μg) and solution assays for CaMK-II activity. CaMK-II activity is presented as nmol/min/mg. Data are mean ± SD (bars) values.
γC, δE, and δC CaMK-IIs between variable domains II and VII (amino acids 317–362 for γC). This sequence begins with Ala and the nine underlined residues are not conserved between γC and δC CaMK-II.

a role for the divergent 21–22 amino acids found at the C terminus of some δ CaMK-IIs (Mayer et al., 1993; Tombes et al., 1999) in the association domain, there are only six nonconservative amino acid differences between γC and δE CaMK-II. In contrast, when the hypervariable region of alternative splicing is aligned (amino acids 317–362 in γC CaMK-II) and compared, nine nonconservative amino acid differences between γC and δE are observed (underlined in Fig. 9). The most striking differences are in the area of two proline-aspartate-glycine (PDG) domains. δE CaMK-II contains both of these domains, δC CaMK-II contains one, and γC CaMK-II has neither domain. In δE CaMK-II, a loose 10-amino acid domain repeat can be observed surrounding these two PDG domains. Both human and rodent α CaMK-IIs, which have been shown to be cytosolic (Shen et al., 1998) and have a domain structure identical to δC CaMK-II (Tombes and Krystal, 1997; Tombes et al., 1999), lack either PDG domain implicated from these studies. The PDG sequence has been identified as representing a special motif in various proteins and may be associated with the initiation of β hairpin turns (Bystroff and Baker, 1998). These PDG domains are present in human and rodent δ CaMK-II but not in β or γ CaMK-II, suggesting that these sequences are conserved between species and are thus functionally important and specific for δ CaMK-II. We do not yet know whether these domains confer specific binding characteristics.

The mechanism of action of δE and δC CaMK-II is likely to be through the direct induction and/or stabilization of cellular extensions rather than through the initiation of a program of differentiation. We believe this is true based on the following spatial and temporal evidence. First, δ CaMK-IIs were expressed along the entire length of these extensions and were completely excluded from the nucleus. Furthermore, they had no effect on either important cell cycle molecules or the cell cycle phase profile. Second, neurite outgrowth induced by constitutively active δ CaMK-IIs in P19 cells temporally preceded neurites induced by retinoic acid. δ CaMK-IIs typically caused extensions >100 μm in length to appear between 1 and 2 days after transfection. These extensions contained βIII tubulin in the discontinuous, beaded pattern that was also observed in cells treated with retinoic acid. Retinoic acid did not result in significant neurite outgrowth until day 4 but, as previously reported, induced an entire program of differentiation that is marked by the expression of βIII tubulin and other neuronal-specific proteins such as microtubule-associated protein 2 (MAP2) (Bain et al., 1994; Laferrière and Brown, 1996; Watanabe et al., 1999). CaMK-II levels increased fourfold at these same time points, but we do not yet know how expression is regulated. These neurites extended in length for several hundred micrometers and had a much higher level of βIII tubulin expression than transfected cells. Based on these findings, we propose that δ CaMK-IIs act downstream from the early effects of retinoic acid on gene expression and that transfected δ CaMK-II influences neurite outgrowth by modifying and recruiting preexisting protein pools.

All three isozymes used in this study were extranuclear isozymes. This is consistent with the immunolocalization pattern of endogenous δ and γ CaMK-IIs previously reported (Tombes et al., 1999) and of transfected δC CaMK-II (Srinivasan et al., 1994). γC typically showed a diffuse cytosolic pattern without any coassociation with cytoskeletal elements or with cellular extensions. In contrast, δE and, to a lesser extent, δC CaMK-II exhibited a more filamentous cytoskeletal pattern. The filamentous pattern implicated a cytoskeletal localization. It is possible that the relevant substrates of δ CaMK-II substrates are actin-associated because Ca2+ and CaMK-II have been implicated in the dynamics of actin-based systems in dendritic morphogenesis and neuronal plasticity (Wu and Cline, 1998; Lau et al., 1999; Maletic-Savatic et al., 1999). However, we do not see an exclusive colocalization of δ CaMK-IIs with actin, tubulin, or vimentin. Overexpressed β CaMK-II has been shown to exhibit a strong and distinct colocalization with actin (Shen et al., 1998), but β CaMK-II lacks the PDG domains implicated in these studies. It is possible that δ CaMK-II interacts with a subset of the cytoskeleton (Hall, 1998) at growth cones, sites of new extensions, or even branch points.

δC CaMK-II was more effective at inducing extensions in P19 cells, whereas δE CaMK-II was more effective in NIH/3T3 cells. Because the relative level of expression of these transfected CaMK-IIs was similar between the two cell types, we suspect that their effectiveness was influenced by the levels of endogenous CaMK-IIs, binding proteins, and/or substrates. For example, in P19 cells overexpressed δC CaMK-II may heterooligomerize with existing δE CaMK-II and be targeted appropriately. In contrast, NIH/3T3 cells have no preexisting δE CaMK-II and thus none for heterooligomerization with transfected δC CaMK-II.

In conclusion, we present evidence that δE and δC CaMK-IIs play a role in cellular differentiation through the induction of neurite-like outgrowths. These findings support the hypothesis that the multifunctionality of CaMK-II is due to variable domain targeting motifs and not catalytic domain differences. The role of specific amino acids in targeting CaMK-II isozymes to specific

FIG. 9. Variable domain sequence. Amino acid sequences are given for human γC, δE, and δC CaMK-IIs between variable domains II and VII (amino acids 317–362 for γC). The sequence begins with Ala and the nine underlined residues are not conserved between γC and δC CaMK-II.
intracellular locations and binding partners is the focus of current investigations.

Acknowledgment: This work was supported by grants from the A. D. Williams Committee at Virginia Commonwealth University, by the Kate and Thomas Jeffress Foundation Trust, and by grant 9904765 from the National Science Foundation. The authors are extremely grateful to J. Bradley Hullett, Abdul Rahman Kenyatta, Nicky Caran, Helen Han, and Nicolas Zollar for technical support, to Dr. Rita Shiang for providing the P19 cell line, to Dr. Don Porter for assistance with site-specific mutagenesis, and to Dr. Robert J. DeLorenzo for comments on the manuscript.

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CaMK-II IN DIFFERENTIATION