CaMK-II oligomerization potential determined using CFP/YFP FRET

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Received 1 June 2005; received in revised form 16 August 2005; accepted 16 August 2005
Available online 12 September 2005

Abstract

Members of the Ca²⁺/calmodulin-dependent protein kinase II (CaMK-II) family are encoded throughout the animal kingdom by up to four genes (α, β, γ, and δ). Over three dozen known CaMK-II splice variants assemble into ~12-subunit oligomers with catalytic domains facing out from a central core. In this study, the catalytic domain of α, β, and δ CaMK-IIs was replaced with cyan (CFP) or yellow fluorescent protein (YFP) for fluorescence resonance energy transfer (FRET) studies. FRET, when normalized to total CFP and YFP, reproducibly yielded values which reflected oligomerization preference, inter-subunit spacing, and localization. FRET occurred when individual CFP and YFP-linked CaMK-IIs were co-expressed, but not when they were expressed separately and then mixed. All hetero-oligomers exhibited FRET values that were averages of their homo-oligomeric parents, indicating no oligomeric preference or restriction. FRET for CaMK-II homo-oligomers was inversely proportional to the variable region length. FP s were monomerized (Leu²²¹ to Lys²²¹) for this study, thus eliminating any potential artifact caused by FP-CaMK-II aggregates. Our results indicate that α, β, and δ CaMK-IIs can freely hetero-oligomerize and that increased variable region lengths place amino termini further apart, potentially influencing the rate of inter-subunit autophosphorylation.

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Keywords: CaMK kinase II; FRET; CFP; YFP; Localization; Oligomerization

1. Introduction

CaMK-II (Ca²⁺/calmodulin(Ca)-dependent protein kinase II) is a highly conserved serine/threonine protein kinase encoded by 4 genes (α, β, γ, and δ) to produce at least 38 alternative splice variants [1,2], some of which are targeted to the cytoskeleton, membrane, or nucleus [3–5]. Like other kinases, CaMK-II location specifies function [6–9]. CaMK-IIs form uniquely shaped oligomers of approximately 12 subunits [10–13]. Oligomerization is necessary for the Ca²⁺/CaM-induced inter-subunit autophosphorylation, which renders the enzyme Ca²⁺-independent and thus active long after the initial Ca²⁺ pulse has subsided [2,14,15].

CaMK-II is expressed in all tissues in species as diverse as sea sponge and humans [1]. Although α and β are the most common CaMK-IIs in the central nervous system, and γ and δ CaMK-IIs are the most common in embryonic cells [16–24], most cells or tissues transcribe from two or more CaMK-II genes [16,25–28]. Hetero-oligomers exist naturally [29], raising the possibility of a dizzying potential array of mixed oligomers, each with its own potentially unique targeting or substrate specificity. For example, α and β CaMK-IIs are known to form mixed oligomers [29–32] and αKAP, a naturally truncated α CaMK-II, can hetero-oligomerize with β, γ, and δ CaMK-IIs [33]. However, quantitative assessments of the ability of one CaMK-II to hetero-oligomerize with another have been lacking.

The C-terminal oligomerization or association domain is sufficient for oligomer formation [30,34]. Throughout this ~132 amino acid domain (excluding the δ-specific C-terminal tail), there are 39 residue differences amongst all four human CaMK-IIs [1], raising the possibility that these sequences may confer an oligomeric preference, even if slight.

CaMK-II oligomers are formed by a core of oligomerization (O) domains linked to outwardly-facing N-terminal catalytic (C) domains via the variable (V) domain [10–12]. The 29 to 218 amino acid V domain is encoded by the alternative use of up to eight exons per CaMK-II gene. Variable domain exons encode targeting, not just linker sequences [1], but the roles of all alternative exons are not yet known. In purified CaMK-II holoenzymes, the inter-subunit spacing of C domains, as determined by electron microscopy, is ~7 nm [10,11].
FRET (fluorescence resonance energy transfer) between the fluorescent proteins, CFP (cyan) and YFP (yellow), has been used to demonstrate the interaction of proteins to which they are linked [35]. If two proteins stably interact, fluorescent excitation of CFP (donor) at 440 nm excites YFP (acceptor) at 480 nm to fluoresce at 535 nm. Such studies can be conducted on FP-linked proteins in living cells or in lysates. The Förster critical distance for CFP/YFP FRET pairs (50% efficiency) is 5 nm [36], making FRET possible if the 27 kDa CFP or YFP is placed at the N-terminus of CaMK-II. GFP has been successfully linked in this fashion to CaMK-II, as evaluated by normal location, oligomerization and Ca\(^{2+}/CaM\)-dependent activity [4,37].

There was therefore precedent and reason to use CFP/YFP FRET to systematically examine \(\alpha\), \(\beta\), and \(\delta\) CaMK-IIs for their propensity to oligomerize with each other and themselves. \(\gamma\) CaMK-II was omitted from this study, since as shown in Fig. 1, it is nearly identical to \(\beta\) CaMK-II in the oligomerization domain [1]. Most constructs used in this study were prepared by replacing the C domain with CFP or YFP. CFP and YFP naturally but weakly dimerize; they were monomerized to eliminate artificial aggregation of FP-linked CaMK-IIs. We limited our study to four cytosolic \(\alpha\), \(\beta\), and \(\delta\) CaMK-II variants chosen based on their comparable V domain structure [1]. NIH/3T3 cells were used since CaMK-II expression levels are low, minimizing complication by excess endogenous CaMK-II. This study demonstrates that CaMK-II assembly and structure can be quantitatively evaluated using CFP/YFP FRET.

### 2. Materials and methods

#### 2.1. CFP and YFP vector construction

Sequences encoding Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP) were linked to the 5' end of mouse or human CaMK-II cDNAs using the BamH I and BspEI restriction sites in the ECFP-C1 and EYFP-C1 vectors (Clontech Inc., Palo Alto CA), as previously described [37]. This limits the linker sequence between C/CYPF and CaMK-II to 1 glycine residue. Variable domain constructs begin with Thr\(^{311}\) (\(\beta\), \(\delta\)) or Thr\(^{310}\) (\(\alpha\)) and the \(\delta\) CaMK-II oligomerization domain construct begins with Thr\(^{351}\) to encode the last 142 amino acids, as previously described [37]. Monomerization of GFP, CFP and YFP by point mutagenesis (Leu\(^{221}\) to Lys\(^{221}\)) was accomplished using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with a complementary mutagenic primer pair [38]. The sense sequence for this primer pair was 5'-GATCACATGGCTTAAAGGAGTTGGTACC-3'. The reaction product was digested with DpnI for 30 min and then transformed into E. coli. Positive clones were screened for an introduced A/Ill restriction site and then sequenced through the FP and CaMK-II coding sequences, as previously described [16]. Once expressed, all mCFP and mYFP-linked CaMK-IIs were evaluated by GFP immunoblots and Superose-12 gel filtration, as described below.

#### 2.2. Transfection and preparation of lysates

CFP/YFP pairs were transfected into subconfluent NIH/3T3 cells using Lipofectamine 2000 (Invitrogen Inc., Carlsbad CA). After 1 to 2 days, cells...
were imaged, harvested by trypsinization, resuspended in homogenization buffer, briefly sonicated on ice and then centrifuged at 12,000 × g for 15 min, as previously described [37]. These supernatants were used for all subsequent FRET and immunoblot analyses. This homogenization buffer was previously optimized for solubilizing and maintaining maximum CaMK-II activity and consists of 30 mM Hepes, pH 7.4, 2.6 mM EGTA, 20 mM MgCl2, 80 mM β-glycerol phosphate, 0.1 mM okadaic acid, 0.01 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis MO).

2.3. FRET fluorometry

Lysates were assayed for CFP (donor) fluorescence (445nm/480nm), YFP (acceptor) fluorescence (485nm/535nm) and FRET (445nm/535nm) using a Wallac (Perkin Elmer) Victor2 Multilabel 96-well counter as previously described [39]. Each 0.1–0.2 ml sample was counted in replicate for 2 s each. After background (untransfected cells) subtraction, corrected FRET was calculated by subtracting CFP (17%) and YFP (3%) bleedthrough from raw FRET. Bleedthrough is the percentage of total CFP or YFP fluorescence detected in the FRET channel when CFP or YFP are transfected separately and is instrumentation-specific. Corrected FRET was then normalized by dividing by the square root of the product of the intensities of both fluorophores, as described [36], leaving a unitless value: NFRET = 100 × [FRET – (0.17×CFP) – (0.03 × YFP)]/[CFP × YFP] − 2.

2.4. FRET microscopy

For conventional microscopy, cells were imaged on an Olympus IX70 inverted epifluorescence microscope with a 40× dry objective. The Endow EGFP filter utilized a 470/40 nm excitation filter and a 525/40 nm emission filter with a 495 nm long pass dichroic filter (Chroma Technology, Rockingham VT) and was used for all 3 FPs. For FRET, filters were 440/21 nm excitation and 535/26 nm emission with a 455 nm long pass dichroic. Confocal images were acquired using an LSM-510 microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a Plan Apochromat 63× oil immersion objective (1.4 N.A.). Excitation was provided by a 30-mW argon laser. Excitation was tuned to 458 nm for CFP and 514 for YFP. Emission was set to 470–500 for CFP and 530–600 for the YFP and FRET channels using dichroic and band pass filters. Images were obtained sequentially in the CFP and YFP channels and then processed using the FRET version C macro, which subtracts bleedthrough from each channel and computes NFRET, as described [36]. Displayed images were acquired and then scaled using identical parameters within each channel for all samples.

2.5. Immunoblotting

5 μg protein of sonicated lysates from transfected cells per lane was separated on 7.5% polyacrylamide gels using the Mini-Protein II gel electrophoresis system (Bio-Rad). Proteins were transferred to 0.45 μm nitrocellulose for 1 h at 100 V and blocked with Tris-buffered saline with 0.1% Tween-20 (TBST), 5% BSA, and 2% normal goat serum for 1 h. The primary antibody was a mouse anti-GFP IgG (Clontech Inc.), which reacts equally well with CFP, YFP, and GFP. It was diluted to 1 μg/ml in 2% BSA/TBST and incubated overnight with the nitrocellulose. Blots were washed three times with TBST and incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG at 2 μg/ml in 2% BSA/TBST. Blots were washed three times with TBST and developed with 0.25 mg/ml each nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaCl, 5 mM MgCl2, pH 9.4.

2.6. Immunoprecipitation

100 μg of sonicated lysate protein from cells transfected with GFP-linked β and/or δ CaMK-II was incubated with either 1 μg of mouse anti-β CaMK-II (Zymed Laboratories, San Francisco CA) or goat anti-δ CaMK-II (Santa Cruz Biotechnology, Santa Cruz CA) overnight at 4 °C. This was immunoprecipitated with either biotinylated goat anti-mouse or biotinylated donkey anti-goat IgG (Kierkegaard Perry Labs, Gaithersburg MD) respectively, followed by streptavidin-magnespheres (Promega, Madison WI). Samples were washed three times with 0.5 ml TBST, resuspended in SDS sample buffer and processed for immunoblotting, as described above. Samples were probed with 1 μg/ml rabbit anti-GFP IgG (Clontech Inc.) and alkaline phosphatase conjugated goat anti-rabbit IgG at 2 μg/ml in 2% BSA/TBST as described above.

2.7. Native molecular weight determinations

CFP and YFP-linked CaMK-IIIs were co-expressed, from which whole cell lysates were prepared. Approximately 150 μg of lysate was loaded onto a 1.0 cm × 40 cm Superose-12 gel filtration column (Amersham Pharmacia Biotech, Piscataway, NJ) in 50 mM Tris, pH 7.4, 150 mM NaCl, 5% glycerol, and 0.001 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor. Superose-12 has a reported exclusion limit of 2 × 105 Da. Fractions were assessed for CFP and YFP fluorescence, which was then integrated and plotted as a function of KSV. KSV was determined from the elution volume (Vf), void volume (Vo) and total volume (Vt): KSV = (Vf − Vo)/(Vt − Vf), as previously described [37]. Standards included thyroglobulin (M, 669 kDa), apoferritin (M, 443 kDa), catalase (M, 232 kDa), aldolase (M, 158 kDa), and bovine serum albumin (M, 66 kDa). Peak retention times of each standard was determined from in-line absorbance at 280 nm.

3. Results

3.1. CaMK-II constructs

For this study, the 315-amino acid catalytic domain (C) was replaced by CFP or YFP (238 amino acids) to encode an FRET followed by variable (V) and oligomerization (O) domains (Fig. 1). In addition to VO constructs, one full length FP-linked construct was evaluated (CVO) as were VOT and OT constructs, which contain the δ CaMK-II specific alternative C-terminal tail (Fig. 1). The four FP-linked CaMK-II variants examined here included α, βo, δA (also known as δn), and δC (also known as δk). α and δc are comparable as they utilize no alternative variable domains, whereas δk and βo are both encoded by two gene-specific variable domain exons [1]. GFP linked at the amino terminus of VO CaMK-II exhibits native oligomeric size and location [7,37]. Since oligomerization is dependent on the O domain, its sequence in all four (α, β, γ, and δ) CaMK-IIIs is listed with gene-specific residues underlined (Fig. 1). There were 21 gene-specific residues in α, 14 in δ, 5 in β, and 4 in γ. Because γ has the least unique sequence (Fig. 1) and most identity to β [1], it was not included in this study.

3.2. Use of monomeric fluorescent proteins

In order to evaluate CaMK-II assembly, the tendency of FP-linked CaMK-IIs to form aggregates was addressed. Although the reported diameter of a CaMK-II dodecamer is ~20 nm [10] and naturally occurring CaMK-II aggregates of 100 nm have been reported in anoxic brain [40], overexpressed FP-linked CaMK-IIs form fluorescent aggregates up to 10 μm in diameter (Fig. 2). These intracellular fluorescent aggregates accumulated over time; small aggregates could be seen coalescing into larger ones during an imaging session. Aggregates were dependent on CaMK-II oligomerization and could be minimized if imaging occurred within the first 24 h [37]. Aggregates are demonstrated here with βo VO CaMK-II linked to CFP, GFP and YFP,
but were seen with all CaMK-IIs. YFP-CaMK-II had the highest proportion of aggregates and CFP-CaMK-II had the least. Although CaMK-II oligomerization is absolutely required for aggregate formation, CaMK-II sequence variations had no detectable effect on the degree of aggregate formation. Large aggregates were easily visible by phase contrast microscopy (Fig. 2 inset). Based on the dimensions of CaMK-II [10], these aggregates could contain as many as $10^9$ CaMK-II dodecamers.

GFP is known to dimerize [41] and the residues Ala$^{206}$, Leu$^{221}$, and Phe$^{223}$ are known to be responsible for this weak ($K_d=0.11$ mM) self-interaction [42]. Mutation of any of these residues (e.g., Leu$^{221}$ to Lys$^{221}$) disrupts this hydrophobic patch, thus yielding monomeric FPs [38]. Since the CaMK-II oligomerization domain is found at the C-terminus and the FP is at the N-terminus, we postulated that these aggregates reflected multiple weak FP dimerization domains juxtaposed by CaMKII oligomerization [10]. When FPs were monomerized by converting Leu$^{221}$ to Lys$^{221}$, aggregates were eliminated (Fig. 2). The cells shown here were imaged live exactly 24 h after transfection. All subsequent experiments utilized monomeric FPs (mFPs).

### 3.3. CaMKII oligomerization can be quantitatively evaluated by FRET

Each FRET analysis was conducted by co-transfecting equivalent masses of CFP and YFP bearing vectors into NIH/3T3 cells. Cleared cell lysates were measured in the CFP (donor), YFP (acceptor), and FRET channels. Bleedthrough (17% for CFP and 3% for YFP) was the average percentage of total CFP or YFP fluorescence detected in the FRET channel when CFP or YFP was transfected separately and was used in all subsequent calculations to obtain a corrected FRET value, Fig. 2. Monomerized GFP, CFP and YFP CaMK-IIs Do Not Aggregate. NIH/3T3 cells were transfected separately with original or “monomerized” GFP, CFP or YFP-linked to β,VO and imaged live after 24 h for 0.5 s each. Monomerized FPs were created through a point mutation, which converts Leu$^{221}$ to Lys$^{221}$. Scale bar = 50 μm.
which was then normalized by the amount of CFP and YFP present to yield NFRET (see Materials and methods).

As an example, FRET of α VO CaMK-II is shown (Table 1). Although quantum yields of CFP and YFP differ [43], CFP signals were similar to YFP signals when equivalent amounts of CFP and YFP-encoding vectors were transfected (rows 1, 8, and 11). NFRET was near zero when CFP and YFP alone were co-transfected (row 1), when either YFP or CFP was co-transfected with CFP-α CaMK-II or YFP-α CaMK-II, respectively (rows 4 and 5), or when CFP-α CaMK-II and YFP-α CaMK-II were transfected separately and then mixed (row 11). In contrast, when CFP-α CaMK-II and YFP-α CaMK-II were co-transfected, NFRET values were above 20.

When the ratio of CFP-α CaMK-II and YFP-α CaMK-II was intentionally varied (Table 1, rows 6–10), NFRET values peaked at D/A near 1 (log D/A = 0; Fig. 3). Curve fitting suggests relative stability of the NFRET signal within approximately 3-fold in either direction away from a D/A of 1. Beyond those ratios, NFRET decreased, as expected. We selected an arbitrary filter for all subsequent analyses to exclude the few samples in which the D/A ratio was less than 0.5 or greater than 2.0. The average D/A ratio was 0.98 ± 0.44 (S.D.) when averaged for all subsequent samples in which equivalent CFP and YFP vector masses were transfected.

3.4. Verification of construct integrity, relative mass, oligomerization and native size

Representative co-expressed CFP or YFP-linked constructs exhibited similar mass at the expected Mr, without degradation, when immunoblotted with an anti-FP antibody (Fig. 4A), demonstrating that all CaMK-IIs studied here had similar expression efficiency and stability.

The hetero-oligomerization of CaMK-IIs was also directly demonstrated using immunoblotting (Fig. 4B). CFP-linked β CVO CaMK-II and YFP-linked δα VOT CaMK-II were expressed separately (lanes 2 and 3, respectively) or together (lanes 1 and 4) and then immunoprecipitated with anti-β CaMK-II (lanes 1 and 2) or anti-δ CaMK-II (lanes 3 and 4). δα VOT CaMK-II was used because the anti-δ CaMK-II antibody reacts with the T domain. Samples were then probed with anti-FP to reveal both CaMK-IIs co-associating when reciprocally immunoprecipitated (lanes 1 and 4). This approach has previously demonstrated CaMK-II hetero-oligomerization [29], but cannot as easily be normalized or quantitated as NFRET and cannot distinguish co-migrating subunits.

The native sizes of all CFP-and YFP-linked CaMK-IIs were evaluated by Superose-12 gel filtration chromatography (Fig. 5). Shown here are α, βγ, and δα VO CaMK-IIs, each of which exhibited single peak fluorescence corresponding to ~700 kDa, as did all other constructs examined. The predicted size of FP-linked VO CaMK-II dodecamers is between 540 and 660 kDa. There is no indication that FP-linked CaMK-IIs partially or inefficiently oligomerize. Unlinked CFP or YFP alone migrated at 30 kDa. CFP and YFP α VO CaMK-IIs, which

Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Donor</th>
<th>Acceptor</th>
<th>FRET</th>
<th>D/A ratio</th>
<th>NFRET</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CFP and YFP</td>
<td>716,363</td>
<td>1,184,471</td>
<td>172,190</td>
<td>0.60</td>
<td>1.62</td>
</tr>
<tr>
<td>2. YFP-CaMKII alone</td>
<td>12,378</td>
<td>977,436</td>
<td>35,785</td>
<td>0.01</td>
<td>3.96</td>
</tr>
<tr>
<td>3. CFP-CaMKII alone</td>
<td>1,164,784</td>
<td>15,199</td>
<td>201,846</td>
<td>76.64</td>
<td>2.54</td>
</tr>
<tr>
<td>4. CFP-CaMKII and YFP</td>
<td>245,783</td>
<td>1,229,592</td>
<td>89,336</td>
<td>0.20</td>
<td>1.94</td>
</tr>
<tr>
<td>5. CFP and YFP-CaMKII</td>
<td>924,394</td>
<td>371,475</td>
<td>174,240</td>
<td>2.49</td>
<td>1.02</td>
</tr>
<tr>
<td>6. *CPF-CaMKII and YFP-CaMKII</td>
<td>52,622</td>
<td>940,385</td>
<td>72,276</td>
<td>0.06</td>
<td>15.79</td>
</tr>
<tr>
<td>7. *CPF-CaMKII and YFP-CaMKII</td>
<td>153,321</td>
<td>869,009</td>
<td>127,436</td>
<td>0.18</td>
<td>20.63</td>
</tr>
<tr>
<td>8. *CPF-CaMKII and YFP-CaMKII</td>
<td>358,839</td>
<td>553,762</td>
<td>183,376</td>
<td>0.65</td>
<td>23.72</td>
</tr>
<tr>
<td>9. *CPF-CaMKII and YFP-CaMKII</td>
<td>656,618</td>
<td>292,717</td>
<td>209,428</td>
<td>2.24</td>
<td>20.31</td>
</tr>
<tr>
<td>11. CFP-CaMKII and YFP-CaMKII</td>
<td>604,004</td>
<td>527,154</td>
<td>135,192</td>
<td>1.15</td>
<td>2.96</td>
</tr>
</tbody>
</table>

*Fluorometry values (donor: 445nm/480nm; acceptor: 485nm/535nm and FRET: 445nm/535nm) after background subtraction (untransfected cells) are shown with donor/acceptor ratios and calculated NFRET. Unless indicated (*), equivalent masses of CFP and YFP-based constructs were transfected into NIH3T3 cells. After 24 h, lysates were prepared and 2-s fluorometry measurements taken. Constructs used here included CFP alone, YFP alone, CFP-linked α VO CaMK-II and YFP-linked α VO CaMK-II in the combinations indicated. CFP/YFP vector mass ratios were intentionally varied in rows 6–10 (*) while maintaining a constant total amount of vector DNA.
were expressed separately and then mixed, behave as oligomers, even though they do not FRET.

3.5. CaMK-IIs freely hetero-oligomerize

NFRET values were averaged from 5 to 12 separate transfections for each condition and included reciprocal pairs for hetero-oligomers (Fig. 6). Only samples that (a) exceeded 10-fold over background and (b) exhibited D/A ratios between 0.5 and 2.0, as described above, were included. NFRET values for homo-oligomers (Fig. 6, solid bars) ranged from 8.3 (β CVO) to 24.4 (δ OT). As confirmed by immunoblot, β CVO encoded the largest FP-linked CaMK-II and δ OT the smallest. NFRET values for α, β, and δ mixed oligomers ranged from 12.2 to 20.3. All samples were significantly higher than unlinked CFP and YFP, as determined by t-test (P = 0.01). Such robust NFRET values for hetero-oligomers do not support preferential homo-oligomer formation, since the resultant CFP or YFP-only homo-oligomers would not FRET (Table 1). If subtle oligomerization preferences existed, NFRET values of hetero-oligomers should differ significantly from their parental homo-oligomers (Table 2). NFRET in all 6 possible VO hetero-oligomeric pairs was not significantly different (t-test) from the NFRET average of their homo-oligomeric parents (Table 2).

3.6. NFRET is influenced by variable domains, not gene of origin

The inferred proportionality of NFRET to CaMK-II size was examined more closely by plotting NFRET for homo-oligomers versus variable domain length (Fig. 7). The variable domain for this study spans from Thr311 in βδ or Thr310 in α to a common residue (Arg146 in δC CaMK-II) at the beginning of the oligomerization domain (RKQEI, Fig. 1). NFRET values for βC VO, δA VO, and δA VOT, which have 69–75 residue variable domains, were all low (11.9, 11.7 and 11.9). δA VOT and δA VO had indistinguishable NFRET, demonstrating that the T domain had no influence on NFRET. NFRET for α and δC VO, which have 35–36 residue variable domains, was higher (21.6 and 19.7). NFRET for δ OT, which is composed of only 8 linker residues, exhibited the highest NFRET (24.4). These 6 constructs all exhibited NFRET that was inversely proportional to their variable domain length (Fig. 7).

3.7. FRET microscopy

With conventional fluorescence microscopy, CaMK-II FRET intensity levels varied in parallel to fluorometry values, but neither the spectral separation nor quantitative capability matched that of confocal microscopy. For confocal microscopy, cells were transfected with combinations of CFP or YFP linked to α, βC, or δC VO constructs. Cells were then fixed after 24 h and imaged (Fig. 8). Spectral separation was excellent as bleedthrough was minimal in the samples transfected with CFP and YFP alone (images not shown). FRET and NFRET were low in cells transfected with unlinked CFP and YFP and high with CaMK-IIs. α CaMK-II was more uniformly distributed within the cytoplasm than β and δ, which were cytoskeletal and perinuclear, as previously described [4,37]. The perinuclear pattern of δ and the actin fiber pattern of β were dominant over
Fig. 6. FRET Fluorometry. One CFP and one YFP-linked construct were co-expressed in NIH/3T3 cells. NFRET values were determined in lysates prepared from 1- to 2-day transfectants. Homo-oligomers are graphed in solid bars and hetero-oligomers in striped bars. Unless otherwise indicated, all constructs were VO. Each bar represents 3–8 replicate and separate transfections including reciprocal pairs and are plotted as means with standard deviations. “α MIX” was α-CFP and α-YFP expressed separately and then mixed.

α in hetero-oligomers. β CaMK-II also exhibited focal adhesion-like intensities. In general, for these constructs and all other combinations tested, FRET localization matched the pattern in each separate channel. NFRET values across all cells varied in the same range (10–25; green to yellow) as the NFRET range determined in solution, but revealed no new targeting of CaMK-II hetero-oligomers.

4. Discussion

This study has demonstrated that fluorescence resonance energy transfer (FRET) can be used to quantitatively characterize the assembly and structure of oligomeric CaMK-II in solution and in cells. Although previous studies have demonstrated that CaMK-II can form hetero-oligomers when co-expressed [30,33], this is the first report that has demonstrated that FRET can be used to evaluate the propensity of CaMK-IIs to hetero-oligomerize. Our findings have also demonstrated that CaMK-II variable domains influence amino terminal spacing.

We have found that all co-expressed CaMK-IIs freely hetero-oligomerize. In contrast, when unlinked (C or Y)FP was cotransfected with (Y or C)FP-linked CaMK-II, FRET was not observed. This demonstrated that FRET was dependent on CaMK-II interaction, not FP interaction. It also demonstrated that homo-oligomers bearing a single fluorophore do not exhibit FRET. As importantly, FP-linked CaMK-IIs that were expressed separately and then mixed at high concentrations also lacked

### Table 2

<table>
<thead>
<tr>
<th>PAIR</th>
<th>n</th>
<th>NFRET (S.D.)</th>
<th>Predicted NFRET</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ_α and β_e</td>
<td>8</td>
<td>12.19 (1.02)</td>
<td>11.80</td>
<td>0.45</td>
</tr>
<tr>
<td>δ_C and β_e</td>
<td>6</td>
<td>15.50 (0.71)</td>
<td>15.84</td>
<td>0.43</td>
</tr>
<tr>
<td>δ_C and δ_A</td>
<td>5</td>
<td>15.95 (1.05)</td>
<td>15.69</td>
<td>0.73</td>
</tr>
<tr>
<td>α and β_e</td>
<td>5</td>
<td>15.87 (0.56)</td>
<td>16.75</td>
<td>0.07</td>
</tr>
<tr>
<td>α and δ_A</td>
<td>5</td>
<td>16.07 (0.80)</td>
<td>16.62</td>
<td>0.32</td>
</tr>
<tr>
<td>α and δ_C</td>
<td>7</td>
<td>20.33 (1.14)</td>
<td>20.66</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Determined NFRET values for VO hetero-oligomers are listed with standard deviations (SD) and are compared to the predicted average of the NFRET values of the parental homo-oligomers. Two-tailed t-test P values are listed for each comparison and indicate no significant difference between predicted and measured NFRET.

Fig. 7. NFRET Depends on Linker Distance. NFRET values of homo-oligomers were plotted as a function of number of amino acids in the V (variable) domain.
FRET, even though they efficiently formed oligomers. NFRET values for hetero-oligomers was indistinguishable from the average of their homo-oligomeric parents, indicating no oligomeric preference. If CaMK-II assembly had favored the formation of homo-oligomers, then NFRET levels would have decreased in co-expression studies relative to the average of the parents. It has been suggested that when two different CaMK-IIs are co-expressed, the resulting oligomers are composed of a mixture of homo-oligomers and hetero-oligomers [29]. Although a small percentage of homo-oligomers would form by random chance (<1%), our findings indicate that there is no significant formation of homo-oligomers in co-expression studies. The natural existence of CaMK-II homo-oligomers [29] must therefore be the result of expression of a single CaMK-II gene product at one time or place.

NFRET levels increased as CaMK-II variable domain length decreased, regardless of gene of origin. Since all CaMK-IIs tested here freely hetero-oligomerize, this can be interpreted as the result of either decreasing the distance between donor and acceptor, removing restrictions on their interaction imposed by

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**Fig. 8. FRET Confocal Imaging.** Selected VO constructs were transfected into NIH 3T3 cells, fixed 24 h after transfection and then imaged in the following channels: donor-CFP (column 1), acceptor-YFP (column 2) and FRET (column 3). NFRET (column 4) across the image was calculated from the first three channels as indicated in the methods. Shown are α, β, and δ, homo-oligomers or hetero-oligomers as indicated. Scale bar = 50 μm. NFRET color lookup bar values range from black (0) to red (30).
variable domain secondary structure or optimizing their relative angle for FRET. Since catalytic domains are the amino terminal domains of full length CaMK-IIs, our findings can be extrapolated to reflect an effect on the ability of native CaMK-IIs to undergo inter-subunit autophosphorylation in CaMK-IIs bearing shorter variable domains. Previous studies have shown that recombinant purified α and β CaMK-II homooligomers differ in their ability to bind CaM and autophosphorylate [11,29]. Integrated with our findings, the observed differences in autophosphorylation [29] could therefore be due to inter-subunit restriction caused by variable domain sequences. Such a dependence of autophosphorylation rates on linker length is exactly what was recently suggested in a study of β CaMK-II isoforms [44]. This may have developmental significance to CaMK-II Ca\(^{2+}\) sensitivity in general since β, γ, and δ CaMK-II variants with longer variable domains replace shorter ones as organisms proceed through development [1]. Interestingly, in spite of their structural similarity, α is expressed only in the postnatal central nervous system and δ is exclusively embryonic. The δ-specific C-terminal tail had no effect on FRET, oligomeric size, or localization; its role remains unknown.

Subunit spacing can be estimated from the FRET efficiency in each experiment and from R₀, the Förster critical distance. R₀ is 5 nm for CFP/YFP pairs [36]. Efficiency values can be determined from the corrected FRET signal and the donor fluorescence in the presence of acceptor [45] and for our experiments, varied from 8 to 37% depending on the pair tested and the ratio of donor to acceptor. At donor (CFP) to acceptor (YFP) ratios near unity, R ranged from 5.5 (α–δ heterooligomers or α–α homo-oligomers) to 7.5 nm (full length β homo-oligomers). This is consistent with the inter-subunit distance of ~7 nm as determined from electron microscopy for full length CaMK-II [11] and supports a role for variable domain length in inter-subunit spacing. Although we sought samples in which the molar ratios of CFP and YFP-linked CaMK-IIs were near unity, the dependence of NFRET on donor and acceptor ratios provides some insight into the oligomeric structure. Even in samples where the molar ratio, as determined by immunoblot, was closer to 10 than 1, the NFRET signal was still 50% of maximum. This suggests that regardless of the oligomeric model [10–13], amino terminal domains of all individual subunits must be near the minimum FRET distance (~10 nm) within the oligomer. Even with full length β CVO, where amino terminal domains would be even farther from each other, NFRET was still significantly above background, although about one third of the δ OT homo-oligomer.

We conducted confocal NFRET imaging to test whether any heterooligomeric combinations exhibited new targeting domains created by the juxtaposition of adjacent, but different monomers. Quantitative spatial analysis of FRET can define any such unique localization of hetero-oligomers, but must be normalized to account for uneven distribution of fluorophores. Using confocal microscopy, we were able to localize CFP/YFP CaMK-II hetero-oligomers and obtained NFRET values throughout the cell that were in the same range as the values obtained in cell lysates. Although individual fluorophore-linked CaMK-IIs localized as expected and β and δ localization dominated over α, hetero-oligomers exhibited no dramatically different or unique localization. NFRET did reveal some subtle variations throughout the cell; whether hetero-oligomers have altered substrate or binding partner specificity is a topic of current study.

FRET localization required that we eliminate fluorescent aggregates. These aggregates were an artifact of FPs but were dependent on CaMK-II oligomerization. In our constructs, the FP and CaMK-II binding domains are at each end of the resulting chimeric proteins. In the known CaMK-II oligomeric structure, multiple amino termini (FPs) are placed extending outward from a central core [10–13]. A single point mutation in the FP-dimerization domain (Leu\(^{221}\) to Lys\(^{221}\)) dramatically overcame this artifact in all FPs without altering their fluorescent properties, as previously described [38]. Any study using FP-linked to any oligomeric protein (particularly if the FP is at the opposite face from the oligomerization domain) is, in principal, susceptible to this artifact.

In conclusion, we have not only demonstrated the practicality of CFP/YFP FRET in evaluating dynamic CaMK-II assembly and structure, but have shown that α, β, and δ CaMK-IIIs freely hetero-oligomerize, when co-expressed, in spite of up to 21 unique amino acid residues (out of a total of 132 residues) per gene in the oligomerization domain. We have also shown that FRET can be used to localize hetero-oligomers in cells. Our findings indicate that the influence of variable domains on the CaMK-II holoenzyme is not always through specific targeting motifs, but through the introduction of variable domain spacers, which can alter the relative position of catalytic domains, potentially influencing Ca\(^{2+}\) sensitivity.

Acknowledgements

The authors are grateful to Nicole Caran Barat and Therese Kirsch-Krueger for expert assistance with construct preparation and transfections, Dr. Jan Chlebowski for discussions on fluorescence resonance energy transfer, Dr. Eric Snapp for discussions on the dimerization domain sequences in GFP and Dr. Andy Hudmon for discussions on CaMK-II aggregates. Matthew E. Seward and Charles A. Easley assisted in the preparation and analysis of FP-linked CaMK-IIIs. This research was supported by National Science Foundation grant IBN-0238821 and by NIH grant P30 CA16059.

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