

Characterization of the binding and phosphorylation of cardiac calsequestrin by ϵ protein kinase C¹

Manuel M. Rodriguez, Che-Hong Chen, Bradly L. Smith², Daria Mochly-Rosen^{2,*}

Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5332, USA

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Abstract In this study, we report the cloning of the rat cardiac isoform of calsequestrin on the basis of its interaction with an ϵ protein kinase C-unique sequence (ϵ V1) derived from the ϵ protein kinase C regulatory domain. Calsequestrin binds activated ϵ protein kinase C holoenzyme better than the inactive enzyme and nearly three times better than other protein kinase C isozymes. The interaction between ϵ protein kinase C and calsequestrin is mediated by sequences in both the regulatory and kinase domains of the ϵ protein kinase C. Finally, we show that calsequestrin is an ϵ protein kinase C substrate *in vitro* and protein kinase C phosphorylation of calsequestrin leads to a decreased binding of ϵ protein kinase C to calsequestrin.

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Key words: Calsequestrin; Protein kinase C; Protein-protein interaction; Enzyme activation

1. Introduction

The sarcoplasmic reticulum (SR) of muscle cells is central to the excitation-contraction process as it stores calcium and therefore contributes to the regulation of intracellular calcium levels. However, the mechanisms responsible for mediating calcium release from the SR are not fully resolved. Calsequestrin (CS) is a moderate affinity ($K_D \sim 1$ mM; [1]), high capacity (~ 40 mol Ca^{2+} /mol CS, [2]) calcium binding protein present in the lumen of the cardiac SR. CS is thought to be a key component in the binding, storage and release of intracellular calcium (reviewed in [3]), not only due to its high calcium buffering but also due to its apparent association with the ryanodine receptor and other intra-SR proteins such as junctin and triadin [4,5]. Transgenic mice overexpressing cardiac CS exhibit cardiac hypertrophy and a lowered chronotropy, indicating that CS is important for the normal cardiac function at the level of the intact animal [6]. CS is also a substrate of caesin kinases I and II ([7,8]). Whereas phosphorylation of skeletal muscle CS appears to influence the ryanodine receptor channel activity [9], the physiological effects of caesin kinase phosphorylation upon cardiac CS remain unclear.

*Corresponding author. Fax: (1) (415) 725 2952.
E-mail: mochly@leland.stanford.edu

¹ Deposited as GenBank accession number AF001334.

² Current Address: Santa Cruz Biotechnologies, 2161 Delaware Ave., Santa Cruz, CA 95060, USA.

Abbreviations: CS, calsequestrin; PKC, protein kinase C; RACK, receptor for activated C-kinase; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum

The protein kinase C (PKC) family is a homologous group of at least 11 isozymes that regulate a wide array of cellular functions [10,11]. In cardiac tissue, these effects include both positive and negative inotropy [12,13], chronotropy [14] and other functions (reviewed in [15]). PKC phosphorylates multiple proteins in cardiac tissue at different cellular sites such as the myofibrillar elements [16,17] and the SR [18,19]. PKC activation in myocardial cells decreases calcium accumulation in the SR, which in part accounts for the negative inotropic response to PKC activation [20]. Despite current knowledge, PKC isozyme-specific activities have yet to be attributed to phosphorylation of specific cardiac substrates.

Previous work has suggested that the regulatory domain of ϵ PKC confers the specificity for this PKC isozyme in binding to both substrates [21] and non-substrate proteins [22,23]. Because unique sequences for ϵ PKC lie within amino acids 1–142, the V1 region of ϵ PKC (ϵ V1), we and others [24] reasoned that at least a partial specificity for substrates is likely to reside within this region of the regulatory domain. We initially identified CS as a putative ϵ PKC binding protein via interaction cloning using recombinant ϵ V1 as a probe of a rat cardiac myocyte cDNA expression library. We present here biochemical evidence that CS specifically interacts with PKC, preferentially with the ϵ isozyme of PKC. We also demonstrate that CS is a substrate for ϵ PKC *in vitro* and that phosphorylation of CS by ϵ PKC inhibits its binding to CS.

2. Materials and methods

2.1. Interaction cloning of CS and isolation of CS cDNA

A Sprague-Dawley rat heart cDNA library was screened as described previously [22]. A detailed description of this method is provided elsewhere [25]. The CS cDNA clone was excised *in vivo* with r408 helper phage from the pBluescript (Stratagene) phagemid cloning vector, sequenced and identified as the cardiac form of CS by 90.8% identity in a 360 amino acid overlap (SIM program on ExPASy WWW site, [26]) to the prototypical cardiac canine form of CS. This clone expresses all but the 51 N-terminal amino acids of rat cardiac CS according to a previously deposited sequence (accession number U33287).

2.2. Expression of recombinant proteins

The CS clone was amplified using PCR (with forward primer ATAGGCTTTGTGATGGTG and reverse primer TACCGAGCA-CAGTGCTGCTT), digested with *EcoRI/BamHI* and inserted behind the *EcoRI/BamHI* restriction sites on the maltose binding protein (MBP) expression vector pMAL-c2 (New England Biolabs), generating a MBP fusion to CS (MBP-CS). This expression vector was transformed into an *Escherichia coli* TB1 host. The DNA fragment encoding ϵ V1-FLAG was generated as previously described [22]. Overexpressed MBP fusion proteins were, where necessary, purified on an amylose affinity column (New England Biolabs), digested with bovine factor Xa (New England Biolabs) and, when necessary, further purified on a DEAE-cellulose column using a linear salt gradient.

2.3. PKC isozymes

The baculovirus expression vector encoding ϵ PKC was obtained as a gift from Dr Robert Bell, Duke University. Insect Sf9 cells were infected with baculovirus and harvested according to kit procedures provided by Stratagene. Where noted, purified human recombinant PKC isozymes produced in insect cells were purchased from Panvera. Mammalian PKC was partially purified from rat brain as previously described [27] to yield a mixed isozyme preparation of $\sim 4.5 \mu\text{g/ml}$ total PKC with 5–9 U activity/ml relative to histone III-S (Sigma).

2.4. Amylose column affinity assay

For each experimental sample, 300 μl (settled volume) of amylose-agarose resin (New England Biolabs) was washed in a Bio-Rad Poly-Prep chromatography column with 4 ml of a column wash buffer (20 mM Tris-HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA, 10 mM β -mercaptoethanol). 50–500 μg of total protein from the 14000 \times g supernatant of the appropriate MBP extract was loaded onto the column and washed with 20 ml column wash buffer. Columns were then plugged at the base, brought to a 1 ml total incubation volume with overlay buffer (0.2 M NaCl, 50 mM Tris-HCl pH 7.5, 12 mM β -mercaptoethanol, 0.1% (w/v) BSA, 1% polyethylene glycol (7000 MW average), 10 μg each of soybean trypsin inhibitor and leupeptin, 20 $\mu\text{g/ml}$ aprotinin and 10 mM phenylmethylsulfonyl fluoride) and incubated with partially purified rat brain PKC or Sf9-expressed recombinant PKC isozymes in the presence or absence of activating co-factors (60 $\mu\text{g/ml}$ phosphatidylserine, 2 $\mu\text{g/ml}$ diacylglycerol, 1 mM Ca^{2+}) or other reagents when noted. Incubations were carried out for 30 min at room temperature with gentle agitation of the incubation slurry. Columns were then washed with 20 ml of wash buffer and the bound complex was eluted with 10 mM maltose in column wash buffer. Eluates were run on SDS-PAGE gels, transferred to nitrocellulose and subjected to Western blotting. Antibodies to δ - and ϵ PKC (Gibco BRL) were used at a 1:300 dilution and antibodies to β PKC (Seikagaku) were used at 1:500. Differences in binding were quantitated by assessing densitometric data obtained using a Galai ScanArray II (Galai Instruments, Galai, Israel) imaging system from films exposed to either enhanced chemiluminescent light emission or autoradiography. Quantitation was also obtained or verified by phosphor-imaging radioactivity or detection of enhanced chemifluorescent imaging of Western blots (Molecular Dynamics STORM 850, utilizing ImageQuant v 1.1).

2.5. Kinase assay

The ability of various PKC preparations to phosphorylate CS and histone III-S was assayed by following the incorporation of the [γ - ^{32}P]phosphate from [γ - ^{32}P]ATP into these substrates according to a method modified from that of Kikkawa et al. [28]. Specific protein phosphorylation was quantitated as described above (phosphorimaging) or the total protein phosphorylation was quantitated with liquid scintillation.

2.6. Immunoprecipitation of ϵ PKC

ϵ PKC was isolated from the partially purified rat brain PKC preparation (see above) by incubating 5 μg anti- ϵ PKC polyclonal rabbit IgG (Santa Cruz Biotech) with 0.5 ml of a 1:4 dilution (to $\sim 1 \mu\text{g/ml}$) of the PKC prep for 2 h at 4°C. IgG was then precipitated with 50 μl agarose-conjugated Protein G (Pharmacia) for 30 min at 4°C.

3. Results

3.1. Interaction cloning of CS

We have postulated that the specificity of subcellular localization and function for each activated PKC isozyme is at least partly mediated by the binding of an isozyme to its specific receptor for activated C-kinase (RACK) or RACKs [29,30]. We previously demonstrated that the V1 region of ϵ PKC (ϵ V1) at least partly mediates the *in vivo* interaction between ϵ PKC and proteins (including RACKs) important for the ϵ PKC function at RACK sites in cardiac myocytes [31,32]. We therefore chose to screen a rat cardiac cDNA expression library for proteins that interact with ϵ V1. This effort revealed

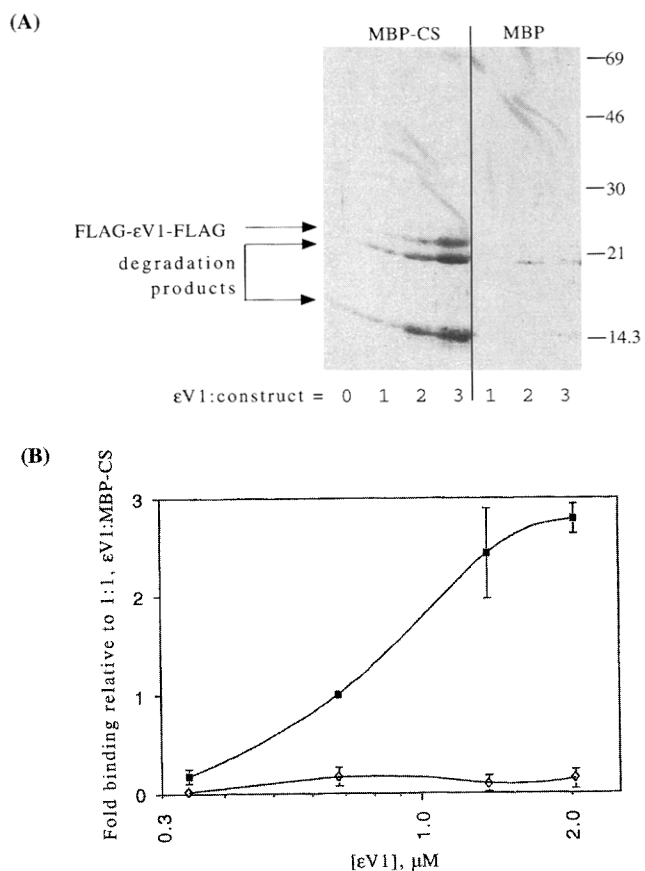


Fig. 1. The V1-domain of ϵ PKC (ϵ V1) binds CS in an affinity assay. Equimolar amounts of either recombinant MBP-CS or MBP were immobilized on amylose affinity columns. Immobilized proteins were then incubated with increasing amounts of FLAG- ϵ V1-FLAG (0–3:1, molar ratio FLAG- ϵ V1-FLAG:MBP-CS or MBP). After extensive washing, bound material was eluted with 10 mM maltose and the resultant eluates were subjected to Western blot analysis (anti-FLAG monoclonal antibodies). (A) A gel image that represents one of three independent experiments. (B) Graph data represent the mean \pm S.E.M. ($n \geq 3$) of binding of FLAG- ϵ V1-FLAG to MBP-CS (\blacksquare) or MBP (\diamond) relative to binding of FLAG- ϵ V1-FLAG to MBP-CS at a 1:1 molar ratio.

two independent clones, CS² and an ϵ PKC-specific RACK [22].

The CS clone exhibits two putative PKC phosphorylation sites (MacVector analysis, Kodak-IBI) using a R/KXS/TXR/K consensus and six putative PKC phosphorylation sites (PROSITE, ExpASY WWW site) using a S/TXR/K consensus search (Table 1). Of interest is the identity between the sequences flanking two of these sites and the sequences flanking phosphorylated serines and threonines on the PKC substrates MARCKS [33,34] and troponin I [35] (Table 1). Our clone (AF001334) differs from a previously deposited sequence of rat cardiac CS (U33287) in two places. The first begins at residue 50 of AF001334 and differs in the first three amino acids most likely due to a cloning artifact. The second is at residues 208–211. AF001334 encodes S-208 and flanking sequences identify this serine as a consensus PKC phosphorylation site. This residue is F-208 in U33287. However, the dog canine CS sequence (P12637) is identical to AF001334 in 208–211 (Table 1).

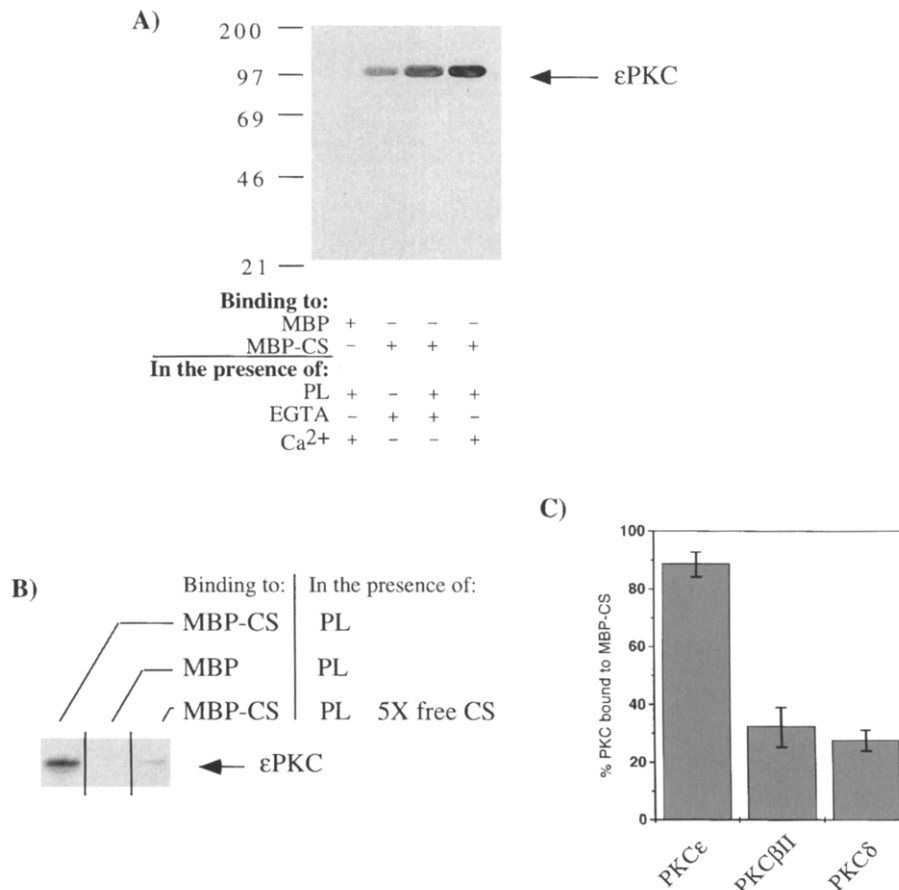


Fig. 2. ϵ PKC binds CS in an affinity assay. Assays were carried out as in Fig. 1, except that incubations were performed in the presence of PKC with or without phospholipid (PL, 60 μ g/ml phosphatidylserine, 2 μ g/ml diacylglycerol) activators, 1 mM CaCl and free purified recombinant CS. (A) Recombinant ϵ PKC (Panvera) binds to MBP-CS. Binding is specific to the CS portion of the MBP-CS fusion protein and binding is dependent upon PL activation of ϵ PKC. (B) Rat brain ϵ PKC binds MBP-CS. This binding is inhibited with a 5-fold molar excess of free (non-immobilized) CS. Data are representative of three independent experiments. (C) Recombinant ϵ PKC exhibits a higher binding activity to MBP-CS than β IIPKC and δ PKC (all isozyms from Panvera). 150 ng of each PKC isozyme was incubated separately with constant (excess) amounts of immobilized MBP-CS (see Section 2) with 1 mM Ca²⁺ and PL in duplicate over three independent experiments. Percentages of isozyme bound were determined based on quantitation of Western blots using a linear fit to densitometry of four internal standards of 25, 50, 100 and 200 ng of each isozyme on the same blot as the eluates (values represent mean \pm S.E.M.).

3.2. ϵ V1 binds CS in column affinity assays

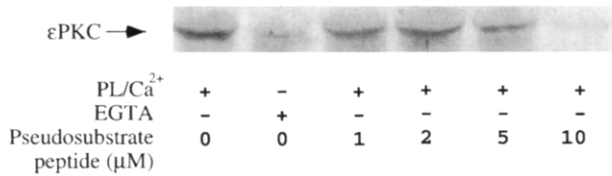
We first confirmed the interaction of the ϵ V1 region (amino acids 2–142 of ϵ PKC, lacking the pseudosubstrate site) with CS, employing an affinity binding assay independent of the original expression cloning method. CS was expressed and partially purified as a fusion protein to maltose binding protein (MBP-CS). MBP-CS bound recombinant ϵ V1 in a dose-dependent manner (Fig. 1). Binding was specific to the CS portion of the MBP-CS fusion protein, as ϵ V1 did not interact significantly with MBP alone under the same molar dose-response range tested (Fig. 1B). MBP-CS bound as well or better to two ϵ V1 degradation products of 14 and 20 kDa (Fig. 1), since Coomassie blue staining of the ϵ V1 sample revealed that these fragments were about five and two times less abundant than the full-length product, respectively (data not shown). N-terminal sequence analysis revealed that the 14 kDa form comprises residues 27–142 of ϵ PKC (Fig. 1). However, no information is available on the 20 kDa fragment. The 14 kDa fragment provides a direction for future studies to further map the CS binding site within ϵ V1.

3.3. ϵ PKC binds CS in column affinity assays

We next determined whether recombinant CS could bind ϵ PKC holoenzyme in vitro and ascertained the conditions under which this binding occurs with respect to PKC activation. MBP-CS bound ϵ PKC and this binding was enhanced upon phospholipid-induced activation of PKC (Figs. 2A, 3, 5). The apparent affinity of rat brain ϵ PKC for CS was further enhanced with 1 mM Ca²⁺ (Fig. 2A). Since ϵ PKC is a novel (Ca²⁺-independent) PKC isozyme, this result suggests that the calcium-loaded state of CS may influence its interaction with ϵ PKC, as the K_D of CS for Ca²⁺ is about 1 mM [1]. Binding of ϵ PKC to MBP-CS was specific to CS, since ϵ PKC did not bind to MBP alone (Fig. 2A, B) and since free purified recombinant CS competed with MBP-CS for PKC binding (Fig. 2B).

Phospholipid-activated recombinant ϵ PKC bound better to MBP-CS than either β IIPKC or δ PKC in vitro (Fig. 2C), which further supports the proposed preference of ϵ PKC for interacting with CS. The observation that isozyms other than ϵ PKC exhibit a binding activity to CS may indicate that part

A)



B)

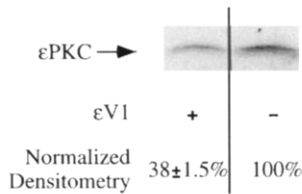


Fig. 3. The pseudosubstrate peptide and εV1 compete with rat brain εPKC for binding to CS. Assays were carried out as in Fig. 2. (A) The pseudosubstrate peptide (Gibco BRL) inhibits binding of εPKC to immobilized MBP-CS. Increasing amounts of peptide were co-incubated with PKC. Representative of three independent experiments. (B) εV1 competes with εPKC for binding to MBP-CS. The εV1 domain was co-incubated at a 2:1 molar ratio (εV1:MBP-CS) with PL-activated PKC. Densitometry data, below, are the mean ± S.E.M. for three independent experiments normalized to binding in the absence of εV1.

of the CS binding site on εPKC may lie within domains common to the PKC isozymes. We also found that the binding of εPKC to MBP-CS decreased by 62 ± 1.5% (mean ± S.E.M., n=3) in the presence of a 2-fold molar excess of εV1 (2:1 εV1:MBP-CS) and a saturating amount of εPKC (Fig. 3B). Thus, at least part of the CS binding site on εPKC lies within the V1 region of the enzyme.

To determine whether the interaction between CS and εPKC was also mediated via the active site of PKC, we measured the ability of the PKC pseudosubstrate peptide to inhibit CS binding to PKC. This peptide binds to the active site of the enzyme and is derived from the autoregulatory pseudosubstrate sequence (RFARKGALRQKNVHEVKN, amino acid 19–36) of εPKC [36]. Pseudosubstrate peptide exhibited a dose-dependent inhibition on the interaction between CS and εPKC, with 50% inhibition occurring around 5 μM peptide (Fig. 3A), suggesting that this interaction is partly mediated by the active site of the enzyme.

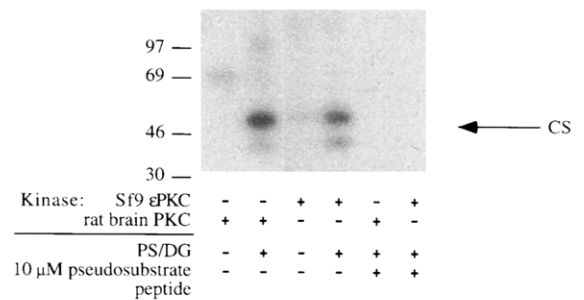
Although εV1 binding to CS occurs in the absence of lipids (suggestive of a direct protein-protein interaction), the requirement of lipid activators for εPKC holoenzyme binding to CS could reflect the presence of a lipid bridge between the proteins. However, the binding of PL-activated εPKC to MBP-CS was not attenuated when columns were washed with buffer containing 0.1% Triton X-100 prior to elution (data not shown).

3.4. εPKC phosphorylates CS

Upon identifying potential consensus phosphorylation sites on CS (Table 1), we determined whether CS is a PKC substrate. Recombinant εPKC phosphorylated CS in a phospholipid-dependent manner which was completely inhibited in the presence of 10 μM pseudosubstrate peptide (Fig. 4A). CS was phosphorylated by rat brain PKC at a ratio of 3.01 ± 0.18 mol Pi/mol CS (mean ± S.E.M., n=3). Thus, of the six potential PKC phosphorylation sites on CS identified by consensus alignment (Table 1), at least three appear to be phosphorylated by PKC in vitro.

We next investigated the specificity of CS phosphorylation by εPKC since it has been suggested that εPKC has a substrate-specificity distinct from other PKC isozymes [37,38]. Recombinant PKC (ε, βI and βII isoforms overexpressed in Sf9 cells) exhibited little isozyme-specificity towards CS phosphorylation (data not shown). While isozyme-specific phosphorylation of PKC substrates in vitro has been difficult to establish [39], we nonetheless tested an alternative hypothesis. If CS is a superior substrate relative to other PKC substrates (e.g. histone), then, the εPKC activity toward CS should be greater than its activity toward histone when compared to other isozymes or group of isozymes. We thus purified εPKC from a rat brain mixed isozyme preparation by immunoprecipitation and determined the ratios of specific activity

A)



B)

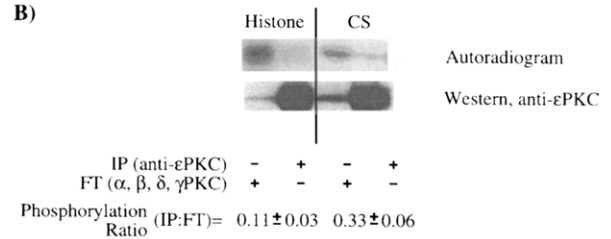


Fig. 4. εPKC phosphorylates CS. (A) Both Sf9-expressed εPKC and partially purified rat brain PKC phosphorylate CS. Recombinantly expressed and purified CS was subjected to phosphorylation by these PKC sources for 10 min in the presence or absence of 240 μg/ml phosphatidylserine (PS) and 8 μg/ml diacylglycerol (DG) and 10 μM pseudosubstrate peptide. Autoradiographs follow the incorporation of the γ-phosphate of [γ-³²P]ATP. (B) Immunoprecipitated (IP) εPKC phosphorylates CS. Anti-εPKC polyclonal IgG bound to Protein G was used to isolate εPKC from a mixed isozyme rat brain PKC preparation. The resultant IP or the immunodepleted (FT) PKC sample (containing α, β, δ, γPKC) were then used as kinase sources for phosphorylation of 1 μM histone III-S and purified recombinant CS. The ratio of phosphorylation between the IP and the FT (IP:FT) is given as the mean ± S.E.M. of three experiments.

Table 1

Amino acid sequence alignment of rat cardiac CS (AF001334) cloned in this study with the prototypical canine cardiac muscle sequence (P12637) and the full sequence of rat cardiac CS (U33287). Alignment was performed with the aid of the MultiAlign sequence alignment program [49], utilizing the Blossum62 symbol comparison table with a gap weight of 12, a gap length weight of 2 and high and low consensus levels of 90 and 50%, respectively.

	1				50
AF001334A
U33287	MKRIYLLVVG	LYLLSFSRAE	EGLNFPPTYDG	KDRVVSLSEK	NLKQVLKRYD
P12637	MKRTHLFIAG	LYLLASCRAE	EGLNFPPTYDG	KDRVVSLTEK	NFKQVLKRYD
Consensus	mkr..l...g	lyll...rae	eglnfptydg	kdrvvs1.ek	n.kqvlk.yd
	51				100
AF001334	RVCLYYHEPV	SSDKVAQKQF	QLKEIVLELV	AQVLEHKNIG	FVMVDSRKEA
U33287	LLCLYYHEPV	SSDKVAQKQF	QLKEIVLELV	AQVLEHKNIG	FVMVDSRKEA
P12637	VLCLYYHESV	SSDKVAQKQF	QLKEIVLELV	AQVLEHKDIG	FVMVDAKKEA
Consensus	.lCLYYHEpV	SSDKVAQKQF	QLKEIVLELV	AQVLEHK#IG	FVMVDSrKEA
	101				150
AF001334	KLAKRLGFSE	EGSLYVLKGG	RTIEFDGEFA	ADLVEFLLD	LIEDPVEIVN
U33287	KLAKRLGFSE	EGSLYVLKGG	RTIEFDGEFA	ADLVEFLLD	LIEDPVEIVN
P12637	KLAKKLGfDE	EGSLYVLKGD	RTIEFDGEFA	ADLVEFLLD	LIEDPVEIIN
Consensus	KLAKrLGFsE	EGSLYVLKGG	RTIEFDGEFA	ADLVEFLLD	LIEDPVEI!N
	151				200
AF001334	NKLEVQAFER	IEDQIKLLGF	FKNEDSEYYK	AFQEAAEHFQ	PYIKFFATFD
U33287	NKLEVQAFER	IEDQIKLLGF	FKNEDSEYYK	AFQEAAEHFQ	PYIKFFATFD
P12637	SKLEVQAFER	IEDQIKLlGF	FKSESEYYK	AFEEAAEHFQ	PYIKFFATFD
Consensus	nKLEVQAFER	IEDQIKLlGF	FKnE#SEYYK	AF#EAAEHFQ	PYIKFFATFD
	201				250
AF001334	KGVAKKLSLK	MNEVGFYEPF	MDEPSVIPNK	PYTEELVEF	VKEHQRPtLR
U33287	KGVAKKLFLE	VNEVGFYEPF	MDEPSVIPNK	PYTEELVEF	VKEHQRPtLR
P12637	KGVAKKLSLK	MNEVDFYEPF	MDEPIAIPDK	PYTEELVEF	VKEHQRPtLR
Consensus	KGVAKKLsLk	mNEVgFYEPF	MDEPsvIP#K	PYTEELVEF	VKEHQRPtLR
	251				300
AF001334	PLRPEDMFET	WEDDLNGIHI	VAFAEKSDPD	GYEFLEILKQ	VARDNTDNDP
U33287	PLRPEDMFET	WEDDLNGIHI	VAFAEKSDPD	GYEFLEILKQ	VARDNTDNDP
P12637	RLRPEDMFET	WEDDLNGIHI	VAFAEKSDPD	GYEFLEILKQ	VARDNTDNDP
Consensus	rLRPEDMFET	WEDDLNGIHI	VAFAEKSDPD	GYEFLEILKQ	VARDNTDNDP
	301				350
AF001334	LSILWIDPDD	FLLLVAYWEK	TFKIDLFKPQ	IGVVNVTAD	SVWMEIPDDD
U33287	LSILWIDPDD	FLLLVAYWEK	TFKIDLFKPQ	IGVVNVTAD	SVWMEIPDDD
P12637	LSIVWIDPDD	FLLLVAYWEK	TFKIDLFKPQ	IGVVNVTAD	SVWMEIPDDD
Consensus	LSIlWIDPDD	FLLLVAYWEK	TFKIDLFKPQ	IGVVNVTAD	SVWMEIPDDD
	351				400
AF001334	DLPTAEELD	WIEDVLSGKI	NTEDDDNEDE	DDGDNDND	DDDDDDNSDE
U33287	DLPTAEELD	WIEDVLSGKI	NTEDDDNEDE	DDGDNDND	DDDDDDNSDE
P12637	DLPTAEELD	WIEDVLSGKI	NTEDDDNE.E	GDDGDDDED	DDDDGNSDE
Consensus	DLPTAEELD	WIEDVLSGKI	NTEDDDNEde	dDDGD#D#DD	DDDDd#NSDE
	401	413			
AF001334	DNDDSDDDDD	DDE			
U33287	DNDDSDDDDD	DDE			
P12637	ESNDDSDDDDD	E..			
Consensus	#n#DsdDDDD	#de			
Consensus PKC phosphorylation site alignment:					
AF001334	246-251	RPTLRr			
AF001334	320-323		KTFK		
	MARCKS		KSFK		
	troponin I	RPTLRr			

Consensus symbols are as follows: ! is any one of (I, V); \$ is any one of (L, M); % is any one of (F, Y); # is any one of (N, D, Q, E, B, Z). PROSITE consensus phosphorylation residues in AF001334 are noted in bold underscore (S, T).

of the ϵ PKC immunoprecipitate (IP) to the total remaining PKC in the flow-through (FT, containing α , β I, β II, δ and γ PKC isozymes) relative to both CS and histone III-S. We found that this ratio (IP:FT) was 3-fold higher for purified CS than for histone III-S (Fig. 4B), suggesting that ϵ PKC phosphorylates CS three times better than histone III-S. This is noteworthy since the mixed isozyme PKC preparation was originally purified on the basis of activity toward histone III-S. These data indicate that CS may be a preferred ϵ PKC substrate *in vitro*.

3.5. Phosphorylation of CS by PKC disrupts their association

If CS is a PKC substrate, then, PKC should not bind as

well to PKC-phosphorylated CS as the unphosphorylated form of CS, as seen for other PKC binding proteins which are substrates [40]. Binding assays were carried out on immobilized MBP-CS in the presence of 20–100 μ M ATP and 20 mM Mg^{2+} . After phosphorylation, the amount of bound ϵ PKC decreased by $72 \pm 6.4\%$ (average \pm S.E.M., $n=4$) (Fig. 5, lanes 1 versus 4, 5). PKC in this eluate (incubated with ATP) also underwent autophosphorylation as evidenced by an upward band shift. These columns were then washed and the phosphorylated CS immobilized on the column was incubated with fresh PKC in the absence of ATP/ Mg^{2+} . We observed that MBP-CS, once phosphorylated by PKC, lost binding for additional ϵ PKC by $62 \pm 10\%$ ($n=4$) in ATP-free

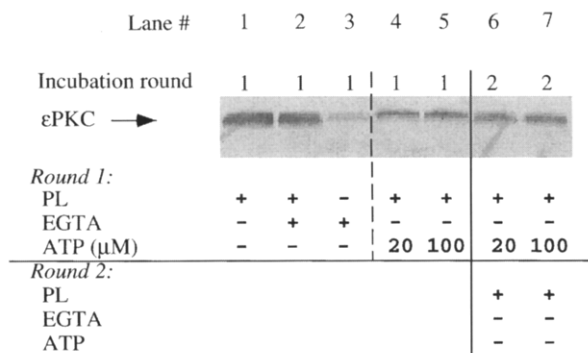


Fig. 5. Phosphorylation of CS inhibits binding of rat brain εPKC and attenuates subsequent re-association of εPKC in the absence of ATP. Equimolar amounts of MBP-CS were immobilized as in Fig. 1 and subjected to PKC binding and/or phosphorylation. PKC activation was performed with a phospholipid (PL) preparation of 60 μg/ml phosphatidylserine (PS) and 2 μg/ml diacylglycerol (DG). Lanes 1, 2 and 3 represent εPKC eluted with the MBP-CS fusion after incubation with PL, PL+10 mM EGTA and 10 mM EGTA, respectively. Lanes 4 and 5 represent εPKC eluted with the MBP-CS fusion protein after PKC phosphorylation (incubation with PL and 20 and 100 μM ATP, respectively). Lanes 6 and 7 represent columns prepared identically to those in lanes 4 and 5, respectively, followed by a buffer wash and re-incubation with fresh PKC and PL under non-phosphorylating conditions.

buffer (Fig. 5, lanes 1 versus 6, 7). Thus, the decrease in εPKC binding to phosphorylated CS was mainly due to the phosphorylation on CS as opposed to PKC autophosphorylation. Taken together with the kinase assays, these data suggest that CS is a PKC substrate *in vitro* and that the degree of εPKC binding to CS is a function of the phosphorylated state of CS.

4. Discussion

We have demonstrated that CS is an εPKC binding protein and substrate. Many other PKC binding proteins have also been identified as PKC substrates (e.g. [41–43]). Like CS, PKC phosphorylation of many of these substrates also mediates their association with PKC. CS may represent an additional class of PKC binding proteins whose interaction with PKC is mediated via both isozyme-unique regions and the catalytic site of the enzyme, thus providing determinants for both isozyme-specificity and kinase-specific activity. Similar proteins have recently been described (e.g. [44,45]), which, like CS, would fall into the broader category of PKC substrates termed STICKS, or substrates that interact with C-kinase [46].

Subcellular fractionation, immunofluorescence and immunoelectron microscopy have shown that CS aggregates within the lumen of the SR (reviewed in [5]). On the other hand, PKC has not been reported to reside within the endoplasmic reticulum (ER)/SR. How, then, can PKC regulate CS in the cellular setting? (i) It is conceivable that some CS translocates to the cytosol, as has been shown for the typically ER/SR resident protein calreticulin (reviewed in [47]). However, no reports have yet indicated such a finding. (ii) Nascent chain CS may be phosphorylated by PKC as the polypeptide exits the ribosomal complex but before vectorial discharge into the ER/SR lumen is completed. This mechanism may occur through pause translocation sequences which have been shown to flank up to 10 kDa of polypeptide sequence, allow-

ing for transient exposure (and thus possibly phosphorylation) of the nascent polypeptide chain to the cytosol [48]. In either case, phosphorylation of CS is likely to alter the calcium homeostasis regulated by this major calcium binding protein in the heart [9].

We have described what will likely be a useful approach toward identifying PKC isozyme-specific binding proteins which also act as PKC substrates. Utilizing this method, we find that the cardiac isoform of CS appears to be an εPKC-selective binding protein and substrate. The *in vitro* characterization of the interaction between εPKC and CS suggests that CS may be a member of a distinct class of PKC substrates which bind to PKC in an isozyme-selective manner by interaction with both variable (isozyme-unique) domain(s) and the catalytic site of the enzyme. Thus, *in vivo*, CS may be phosphorylated preferentially by εPKC over other PKC isozymes due to binding properties contributed by the εPKC-unique region, εVI.

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