FEBS Letters 454 (1999) 240–246 FEBS 22153

Characterization of the binding and phosphorylation of cardiac calsequestrin by ε protein kinase C[1]

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Received 18 April 1999

Abstract In this study, we report the cloning of the rat cardiac isoform of calsequestrin on the basis of its interaction with an εprotein kinase C-unique sequence (εV1) derived form the εprotein kinase C regulatory domain. Calsequestrin binds activated εprotein kinase C holoenzyme better than the inactive enzyme and nearly three times better than other protein kinase C isozymes. The interaction between εprotein kinase C and calsequestrin is mediated by sequences in both the regulatory and kinase domains of the εprotein kinase C. Finally, we show that calsequestrin is an εprotein kinase C substrate in vitro and protein kinase C phosphorylation of calsequestrin leads to a decreased binding of εprotein kinase C to calsequestrin.

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.

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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 εPKC confers the specificity for this PKC isozyme in binding to both substrates [21] and non-substrate proteins [22,23]. Because unique sequences for εPKC lie within amino acids 1–142, the V1 region of εPKC (εV1), 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 εPKC binding protein via interaction cloning using recombinant εV1 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 εPKC in vitro and that phosphorylation of CS by εPKC 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 TACCGAGCACAGTGCTGCTT), digested with *Eco*RI/*Bam*HI and inserted behind the *Eco*RI/*Bam*HI 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 εV1-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.

PII: S0014-5793(99)00697-3

2.3. PKC isozymes

The bacculovirus expression vector encoding εPKC was obtained as a gift from Dr Robert Bell, Duke University. Insect Sf9 cells were infected with bacculovirus 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 ~4.5 μg/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×*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 μg/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 cofactors (60 μg/ml phosphatidlyserine, 2 μg/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 εPKC (Gibco BRL) were used at a 1:300 dilution and antibodies to βPKC (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 phosphorimaging 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}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 εPKC

εPKC was isolated from the partially purified rat brain PKC preparation (see above) by incubating 5 μg anti-εPKC polyclonal rabbit IgG (Santa Cruz Biotech) with 0.5 ml of a 1:4 dilution (to ~1 μg/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 εPKC (εV1) at least partly mediates the in vivo interaction between εPKC and proteins (including RACKs) important for the εPKC 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 εV1. This effort revealed two independent clones, CS[2] and an εPKC-specific RACK [22].

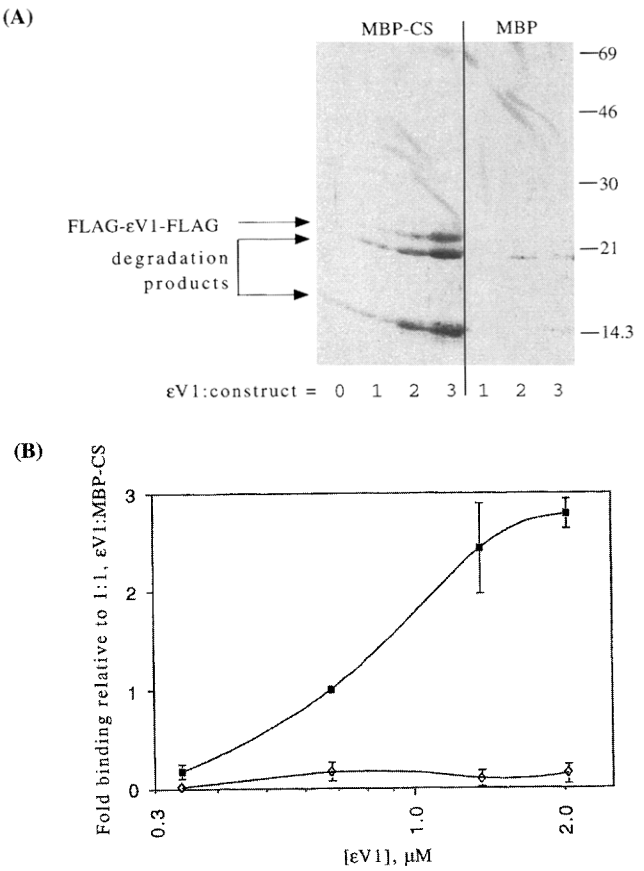

Fig. 1. The V1-domain of εPKC (εV1) binds CS in an affinity assay. Equimolar amounts of either recombinant MBP-CS or MBP were immobilized on amylose affinity columns. Immobilized proteins were then incubated with increasing amounts of FLAG-εV1-FLAG (0–3:1, molar ratio FLAG-εV1-FLAG:MBP-CS or MBP). After 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 ± S.E.M. ($n \geq 3$) of binding of FLAG-εV1-FLAG to MBP-CS (■) or MBP (◇) relative to binding of FLAG-εV1-FLAG to MBP-CS at a 1:1 molar ratio.

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).

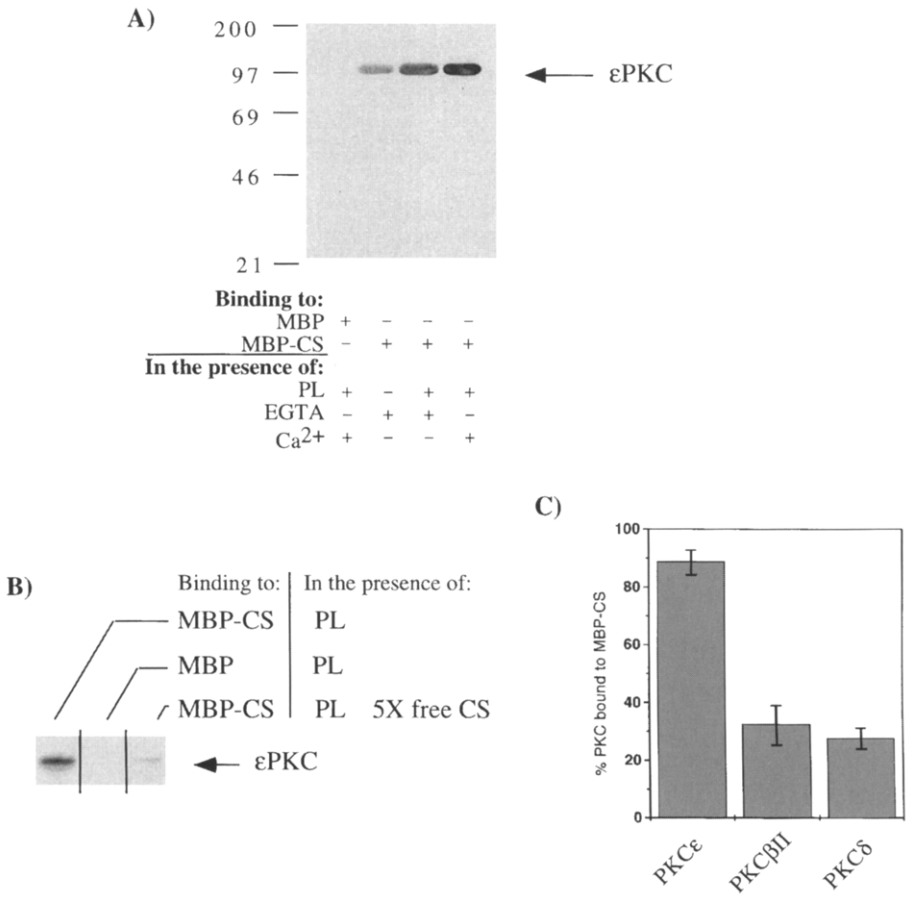

Fig. 2. εPKC binds CS in an affinity assay. Assays were carried out as in Fig. 1, except that incubations were performed in the presence of PKC with or without phospholipid (PL, 60 μg/ml phosphatidylserine, 2 μg/ml diacylglycerol) activators, 1 mM CaCl and free purified recombinant CS. (A) Recombinant εPKC (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 εPKC. (B) Rat brain εPKC 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 εPKC exhibits a higher binding activity to MBP-CS than βIIPKC and δPKC (all isozymes 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 Ca^{2+} 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 ± S.E.M.).

3.2. εV1 binds CS in column affinity assays

We first confirmed the interaction of the εV1 region (amino acids 2–142 of εPKC, 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 εV1 in a dose-dependent manner (Fig. 1). Binding was specific to the CS portion of the MBP-CS fusion protein, as εV1 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 εV1 degradation products of 14 and 20 kDa (Fig. 1), since Coomassie blue staining of the εV1 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 εPKC (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 εV1.

3.3. εPKC binds CS in column affinity assays

We next determined whether recombinant CS could bind εPKC holoenzyme in vitro and ascertained the conditions under which this binding occurs with respect to PKC activation. MBP-CS bound εPKC and this binding was enhanced upon phospholipid-induced activation of PKC (Figs. 2A, 3, 5). The apparent affinity of rat brain εPKC for CS was further enhanced with 1 mM Ca^{2+} (Fig. 2A). Since εPKC is a novel (Ca^{2+}-independent) PKC isozyme, this result suggests that the calcium-loaded state of CS may influence its interaction with εPKC, as the K_D of CS for Ca^{2+} is about 1 mM [1]. Binding of εPKC to MBP-CS was specific to CS, since εPKC 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 εPKC bound better to MBP-CS than either βIIPKC or δPKC in vitro (Fig. 2C), which further supports the proposed preference of εPKC for interacting with CS. The observation that isozymes other than εPKC exhibit a binding activity to CS may indicate that part

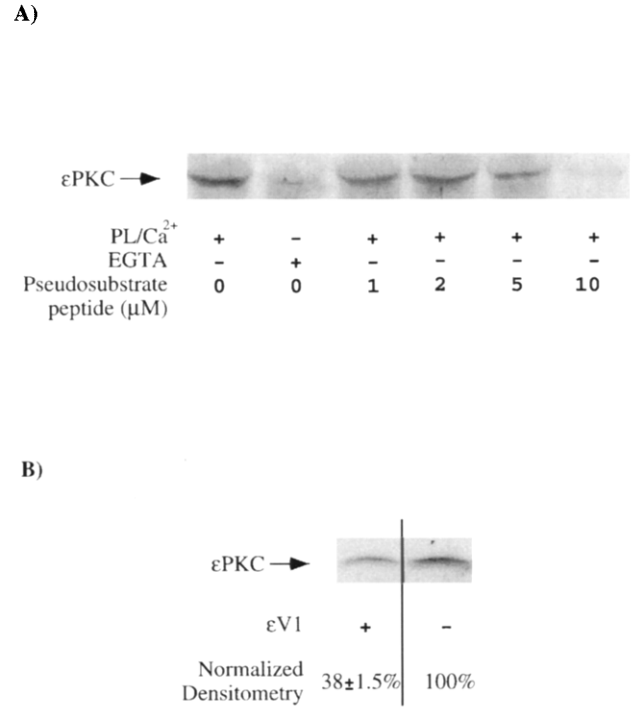

Fig. 3. The pseudosubstrate peptide and εV1 compete with rat brain εPKC for binding to CS. Assays were carried out as in Fig. 2. (A) The pseudosubstrate peptide (Gibco BRL) inhibits binding of εPKC to immobilized MBP-CS. Increasing amounts of peptide were co-incubated with PKC. Representative of three independent experiments. (B) εV1 competes with εPKC for binding to MPB-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

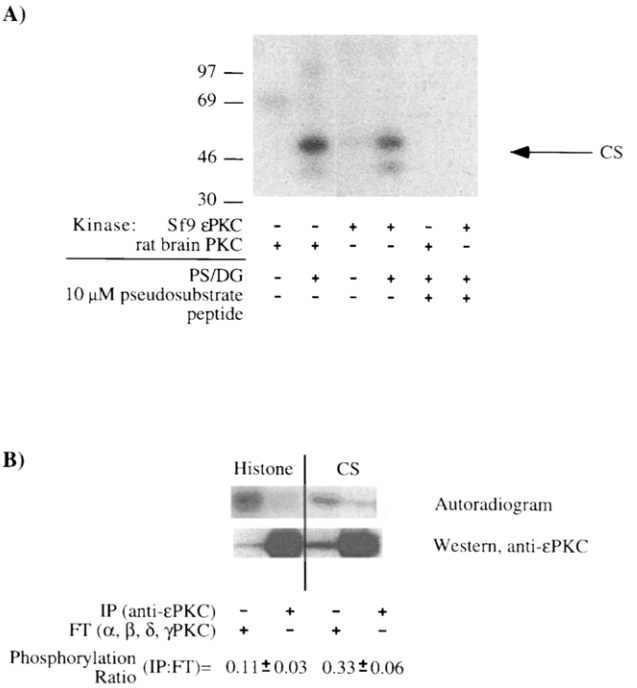

Fig. 4. εPKC phosphorylates CS. (A) Both Sf9-expressed εPKC and partially purified rat brain PKC phosphorylate CS. Recombinantly expressed and purified CS was subjected to phosphorylation by these PKC sources for 10 min in the presence or absence of 240 μg/ml phosphatidylserine (PS) and 8 μg/ml diacylglycerol (DG) and 10 μM pseudosubstrate peptide. Autoradiographs follow the incorporation of the γ-phosphate of [γ-^{32}P]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 Blosum62 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.

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          1                                                            50
AF001334  .......... .......... .......... .......... .........A
  U33287  MKRIYLLVVG LYLLSFSRAE EGLNFPTYDG KDRVVSLSEK NLKQVLKRYD
  P12637  MKRTHLFIAG LYLLASCRAE EGLNFPTYDG KDRVVSLTEK NFKQVLKKYD
Consensus mkr..l...g lyll...rae eglnfptydg kdrvvsl.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 ADVLVEFLLD LIEDPVEIVN
  U33287  KLAKRLGFSE EGSLYVLKGG RTIEFDGEFA ADVLVEFLLD LIEDPVEIVN
  P12637  KLAKKLGFDE EGSLYVLKGD RTIEFDGEFA ADVLVEFLLD LIEDPVEIIN
Consensus KLAKrLGFsE EGSLYVLKGg RTIEFDGEFA ADVLVEFLLD LIEDPVEI!N

          151                                                         200
AF001334  NKLEVQAFER IEDQIKLLGF FKNEDSEYYK AFQEAAEHFQ PYIKFFATFD
  U33287  NKLEVQAFER IEDQIKLLGF FKNEDSEYYK AFQEAAEHFQ PYIKFFATFD
  P12637  SKLEVQAFER IEDQIKLIGF FKSEESEYYK AFEEAAEHFQ PYIKFFATFD
Consensus nKLEVQAFER IEDQIKLlGF FKnE#SEYYK AF#EAAEHFQ PYIKFFATFD

          201                                                         250
AF001334  KGVAKKLSLK MNEVGFYEPF MDEPSVIPNK PYTEEELVEF VKEHQRPTLR
  U33287  KGVAKKLFLE VNEVGFYEPF MDEPSVIPNK PYTEEELVEF VKEHQRPTLR
  P12637  KGVAKKLSLK MNEVDFYEPF MDEPIAIPDK PYTEEELVEF VKEHQRPTLR
Consensus KGVAKKLsLk mNEVgFYEPF MDEPsvIP#K PYTEEELVEF VKEHQRPTLR

          251                                                         300
AF001334  PLRPEDMFET WEDDLNGIHI VAFAEKSDPD GYEFLEILKQ VARDNTDNPD
  U33287  PLRPEDMFET WEDDLNGIHI VAFAEKSDPD GYEFLEILKQ VARDNTDNPD
  P12637  RLRPEDMFET WEDDLNGIHI VAFAERSDPD GYEFLEILKQ VARDNTDNPD
Consensus rLRPEDMFET WEDDLNGIHI VAFAEkSDPD GYEFLEILKQ VARDNTDNPD

          301                                                         350
AF001334  LSILWIDPDD FPLLVAYWEK TFKIDLFKPQ IGVVNVTDAD SVWMEIPDDD
  U33287  LSILWIDPDD FPLLVAYWEK TFKIDLFKPQ IGVVNVTDAD SVWMEIPDDD
  P12637  LSIVWIDPDD FPLLVAYWEK TFKIDLFKPQ IGVVNVTDAD SVWMEIPDDD
Consensus LSIlWIDPDD FPLLVA%WEK TFKIDLFKPQ IGVVNVTDAD SVWMEIPDDD

          351                                                         400
AF001334  DLPTAEELED WIEDVLSGKI NTEDDDNEDE DDDGDNDNDD DDDDDDNSDE
  U33287  DLPTAEELED WIEDVLSGKI NTEDDDNEDE DDDGDNDNDD DDDDDDNSDE
  P12637  DLPTAEELED WIEDVLSGKI NTEDDDNE.E GDDGDDDEDD DDDDGNNSDE
Consensus DLPTAEELED WIEDVLSGKI NTEDDDNEdE dDDGD#D#DD DDDDd#NSDE

          401        413
AF001334  DNDDSDDDDD DDE
  U33287  DNDDSDDDDD DDE
  P12637  ESNDDSDDDD E..
Consensus #n#DsdDDDD #de

Consensus PKC phosphorylation site alignment:
AF001334 246-251    RPTLRR
AF001334 320-323              KTFK
          MARCKS              KSFK
      troponin I    RPTLRR
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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 εPKC immunoprecipitate (IP) to the total remaining PKC in the flow-through (FT, containing α, βI, βII, δ and γPKC 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 εPKC 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 εPKC 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 μM ATP and 20 mM Mg^{2+}. After phosphorylation, the amount of bound εPKC decreased by 72 ± 6.4% (average ± 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 εPKC by 62 ± 10% ($n=4$) in ATP-free

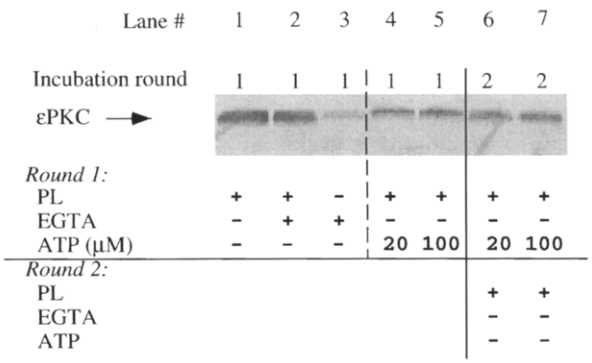

Fig. 5. Phosphorylation of CS inhibits binding of rat brain εPKC and attenuates subsequent re-association of εPKC in the absence of ATP. Equimolar amounts of MBP-CS were immobilized as in Fig. 1 and subjected to PKC binding and/or phosphorylation. PKC activation was performed with a phospholipid (PL) 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 vectoral 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, allowing 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, εV1.

Acknowledgements: This work was supported in part by NIH AAA11147 to D.M.-R. and a NSF pre-doctoral fellowship to M.M.R. The authors wish to thank V. Lingappa for helpful discussions and M. Csukai and C. Pearson for critical reading.

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