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Phosphorylation of calmodulin by Ca^{2+}/calmodulin-dependent protein kinase IV

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Abstract

Calmodulin-dependent protein kinase IV (CaM-kinase IV) phosphorylated calmodulin (CaM), which is its own activator, in a poly-L-Lys (poly(Lys)) -dependent manner. Although CaM-kinase II weakly phosphorylated CaM under the same conditions, CaM-kinase I, CaM-kinase kinase α, and cAMP-dependent protein kinase did not phosphorylate CaM. Polycations such as poly(Lys) were required for the phosphorylation. The optimum concentration of poly(Lys) for the phosphorylation of 1 μM CaM was about 10 μg/ml, but poly(Lys) strongly inhibited CaM-kinase IV activity toward syntide-2 at this concentration, suggesting that the phosphorylation of CaM is not due to simple activation of the catalytic activity. Poly-L-Arg could partially substitute for poly(Lys), but protamine, spermine, and poly-L-Glu/Lys/Tyr (6/3/1) could not. When phosphorylation was carried out in the presence of poly(Lys) having various molecular weights, poly(Lys) with a higher molecular weight resulted in a higher degree of phosphorylation. Binding experiments using fluorescence polarization suggested that poly(Lys) mediates interaction between the CaM-kinase IV/CaM complex and another CaM. The ^{32}P-labeled CaM was digested with BrCN and *Achromobacter* protease I, and the resulting peptides were purified by reversed-phase HPLC. Automated Edman sequence analysis of the peptides, together with phosphoamino acid analysis, indicated that the major phosphorylation site was Thr^{44}. Activation of CaM-kinase II by the phosphorylated CaM was significantly lower than that by the non-phosphorylated CaM. Thus, CaM-kinase IV activated by binding Ca^{2+}/CaM can bind and phosphorylate another CaM with the aid of poly(Lys), leading to a decrease in the activity of CaM.

Key words: calmodulin, CaM-kinase, Ca^{2+}-signaling, phosphorylation, poly-L-lysine.

Calcium ions are known to play important roles in the regulation of a variety of cellular functions. It is well known that calmodulin $(CaM)^3$ is the central protein mediator of intracellular Ca^{2+}-signaling. Following an increase in intracellular Ca^{2+}, CaM binds Ca^{2+} to alter its conformation, and the resulting Ca^{2+}/CaM complex activates a number of target enzymes including Ca^{2+}/CaM-dependent protein kinases (CaM-kinases) [1]. Therefore, regulation of the CaM function by post-translational modification may play a pivotal role in the Ca^{2+}/CaM-mediated intracellular Ca^{2+}-signaling system.

Instances of CaM phosphorylation by several protein kinases *in vitro* have been described; such as by the insulin receptor kinase [2-4], casein kinase II [5, 6], *src* kinase [7], the epidermal growth factor receptor kinase [8], spleen tyrosine kinase [5], phosphorylase kinase [9], and myosin light chain kinase [10]. Several investigators have also demonstrated that phosphorylation of CaM differentially modifies the activation of a number of CaM-dependent enzymes, including cyclic nucleotide phosphodiesterase [11-13], myosin light chain kinase [12, 13], CaM-kinase II [13, 14], the plasma membrane Ca^{2+}-ATPase [13, 15], and nitric oxide synthase [13]. Except for *src* kinase and phosphorylase kinase, polycations such as poly-L-lysine (poly(Lys)) are reported to be required for phosphorylation reactions; but physiological activators which can substitute for poly(Lys) are still obscure. Several lines of evidence indicate that CaM is phosphorylated *in vivo*. Phosphorylated CaM has been detected in chicken fibroblast [7], rat and chicken brain [9, 16], rat liver [15], rat hepatocytes [6, 17], rat adipocytes [18], and insulin-treated CHO/IR cells [19]. Thus, the phosphorylation of CaM may participate in some regulatory mechanisms in intact cells.

On the other hand, CaM-kinase IV, a Ca^{2+}-responsive multifunctional protein kinase that occurs abundantly in the brain and thymus, requires Ca^{2+}/CaM for activity [20-22]. CaM-kinase IV is strongly activated upon phosphorylation by another CaM-kinase, CaM-kinase kinase [20-22], and phosphorylates a number of proteins including synapsin I, microtubule associated protein 2 (MAP2), tau protein, myosin light chain, tyrosine hydroxylase, and CREB [23-26]. CaM-kinase IV is thought to play a role in mediating Ca^{2+}-regulated transcription through phosphorylation of transcription factors such as CREB, ATF-1, and SRF [20, 21, 24-28].

Here we report that CaM-kinase IV also phosphorylates CaM, its own activator, in the presence of polycations such as poly(Lys). We also determined the major phosphorylation site of CaM by CaM-kinase IV, and found that the phosphorylation was accompanied by a reduction in its activity to activate CaM-kinase II. Drawing on several lines of evidence, a possible role for poly(Lys) in the phosphorylation reaction is also discussed.

Materials and methods

Materials. ATP, poly(Lys) (poly(Lys)(128K), poly(Lys)(87K), poly(Lys)(23K), and poly(Lys)(9.6K), with average molecular weights of 128,000, 87,000, 23,000, and 9,600, respectively), poly-DL-lysine (poly(DL-Lys), average molecular weight 23,000), poly-L-arginine (poly(Arg), average molecular weight 40,000), poly-L-glutamic acid/lysine/tyrosine (6/3/1) (poly(Glu, Lys, Tyr), average molecular weight 23,000), and spermine were purchased from Sigma. Unless otherwise specified, poly(Lys) represents poly(Lys)(128K). Dansyl chloride, BrCN, *Achromobacte*r protease I (API), and Quick-CBB were obtained from Wako Pure Chemical Industries. [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham Pharmacia Biotech. Syntide-2 (PLARTLSVAGLPGKK) was synthesized by American Peptide Company Inc. Poly(Lys)(10mer) (KKKKKKKKKK), poly(Lys)(20mer) (KKKKKKKKKKKKKKKKKKKK), and PKIV peptide (KKKKEHQVLMKTVCGTPGY-NH_2) [29] were synthesized by a Shimadzu PSSM8 automated peptide synthesizer and purified by reversed-phase HPLC. Avidin-agarose and NHS-LC-biotin were purchased from Pierce.

Protein preparations. CaM-kinase II and its constitutively active 30-kDa catalytic fragment were prepared as described [30, 31]. CaM was purified from rat testis, essentially as described by Gopalakrishna and Anderson [32]. Unless otherwise specified, rat testis CaM was used in this study. The catalytic subunit of cAMP-dependent protein kinase (PKA) was purified from bovine heart as described previously [33]. Recombinant rat CaM-kinase I [34] and recombinant rat CaM-kinase IV [35], both expressed in Sf9 cells, were purified as described. Recombinant rat CaM-kinase IV(K71R), with Lys^{71} (ATP-binding

site) replaced with Arg, expressed in Sf9 cells was purified as described previously [36]. Recombinant rat CaM-kinase kinase α expressed in *E. coli* [37] was purified as described previously [36]. Dansyl-CaM was prepared from recombinant chicken CaM according to the method of Kincaid et al. [38]. The incorporation of dansyl to CaM was 0.19 mol/mol. Recombinant chicken CaM expressed in *E. coli* was prepared as described previously [35]. Biotinylated CaM was prepared from recombinant chicken CaM as described [39, 40]. MAP2 was purified from porcine brain as described previously [41]. Protamine sulfate was purchased from nacalai tesque. S100a was from Sigma.

Phosphorylation of CaM. The purified recombinant rat CaM-kinase I (226 μg/ml) and CaM-kinase IV (94 μg/ml) were activated by incubation with CaM-kinase kinase α (3 μg/ml) at 30 ˚C for 5 min in a reaction mixture comprising 50 mM 3-Morpholinopropanesulfonic acid (Mops)-NaOH (pH 7.0), 5 mM $Mg(CH_3COO)_2$, 0.4-0.5 mM EGTA, 5 μM CaM, 0.7 mM $CaCl_2$, 2 mM DTT, and 50 μM ATP. Phosphorylation reactions were terminated by adding excess EDTA (10 mM) and enzymes were stored at -80 ˚C until use. Rat testis CaM (1 μM) and other substrates were phosphorylated at 30 ˚C in a reaction mixture containing 40 mM Hepes-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 0.5 mM $CaCl_2$, 0.01% Tween 20, 50 μM [γ-^{32}P]ATP, and indicated amounts of a protein kinase, in the presence or absence of indicated concentrations of a polycation. After incubation for the indicated times, reactions were terminated by excess EDTA (14-29 mM), and mixtures combined with equal volumes of a sample buffer consisting of 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromophenol blue, and 285 mM β-mercaptoethanol. The mixture so obtained was boiled for 2 min and centrifuged for 2 min at room temperature in a microfuge at maximal speed, and then an aliquot of the supernatant was subjected to SDS-PAGE on a 12% acrylamide gel. The gel was dried and visualized by autoradiography. The radioactive band of the phosphorylated CaM was quantified by a BAS-2000 Bio-Imaging Analyzer (Fuji Film), and the incorporation of phosphate into CaM was calculated from the radioactivity. When biotinylated CaM was used as a substrate, the phosphorylation reactions were terminated by adding excess EDTA, followed by adding 1 M NaCl. As a control, biotinylated CaM was replaced with unlabelled CaM. Mixtures

were allowed to stand at least 15 min at room temperature, and then 25 µl of avidin-agarose (50% v/v) and 500 µl of B/W buffer consisting of 20 mM sodium phosphate buffer (pH 7.2), 0.5 M NaCl, 0.1% SDS, and 0.5% Nonidet P-40 were added. After gentle shaking at 4 ˚C for 1 hr, the resin was washed four times with B/W buffer. The radioactivity bound to the resin was determined by liquid scintillation counter.

Measurements of polarized fluorescence. Fluorescence polarization assay was carried out at 30 ˚C in a reaction mixture comprising 40 mM Hepes-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 0.01% Tween 20, 0.41 µM dansyl-CaM, and 50 µM ATP in the presence or absence of 0.5 mM $CaCl_2$. Prior to measurement, the mixture was preincubated for 2 min at 30 ˚C, and then the fluorescence polarization of dansyl-CaM was measured on a full range Beacon 2000 fluorescence polarization instrument (PanVera /Takara) with a 300 nm excitation filter and a 535 nm emission filter. Subsequently, CaM-kinase IV or a polycation was added to the mixture, as indicated and at the indicated time point, followed by further measurements. CaM-kinase IV(K71R)/CaM complex was prepared by mixing CaM-kinase IV(K71R) (959 µg/ml) with recombinant chicken CaM (19.7 µM) on ice in 33 mM Tris-HCl (pH 7.5) containing 8.2% ethylene glycol, 0.04% Tween 40, 0.8 mM DTT, 0.4 mM EDTA, 0.4 mM EGTA, and 1.64 mM $CaCl_2$ prior to the start of the measurements.

Phosphoamino acid analysis. CaM phosphorylated by activated CaM-kinase IV using [γ-^{32}P]ATP was subjected to SDS-PAGE on a 12% acrylamide gel as described above, and the gel was stained with Quick-CBB. The protein band corresponding to the phosphorylated CaM was excised, and the gel pieces hydrolyzed with 6 M HCl for 2 h at 110 ˚C. The hydrolysate was dried *in vacuo* and dissolved in H_2O. The solution was neutralized with 2.8% aqueous NH_3, and then dried again *in vacuo.* This operation was then repeated. The residue was dissolved in a minimum amount of a solution containing 0.94 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine as markers. The solution was centrifuged for 2 min at room temperature in a microfuge at maximal speed, and an aliquot of the supernatant was electrophoresed on a silica gel-precoated thin layer plate

in pH 3.5 buffer of pyridine/glacial acetic acid/H_2O (1/10/189) [42]. ^{32}P radioactivity and amino acids were located by autoradiography and by staining with ninhydrin, respectively.

BrCN cleavage and API digestion of CaM phosphorylated by CaM-kinase IV. CaM (20.4 µg) was phosphorylated by activated CaM-kinase IV (1 µg/ml) in the presence of 0.5 mM $CaCl_2$ and 10 µg/ml of poly(Lys)(128K) as described above. After incubation for 80 min at 30 ˚C, the reaction was terminated by 14 mM EDTA, and the mixture processed as above, followed by SDS-PAGE. The gel was stained with Quick-CBB, and the protein band corresponding to the phosphorylated CaM excised. In-gel BrCN cleavage was carried out according to the method of Jahnen-Dechent and Simpson [43]. The resulting dried gel pieces were rehydrated with 100 mM Tris-HCl (pH 8.0) containing 0.1% SDS (digestion buffer), and the pH of the suspension adjusted to about pH 8 with 1M Tris-HCl (pH 8.8). Subsequently, in-gel digestion with API was carried out essentially according to the method of Kawasaki et al. [44].

Reversed-phase HPLC fractionation of phosphopeptides and their sequence analyses. The digested peptide fragments were extracted from the gel pieces as described [44] with the following modifications. A second extraction was carried out after the gel pieces were allowed to stand for 18 h at 4 ˚C in the digestion buffer. Sonication (5 min) was also conducted in the second and the third extraction procedures. The extracts thus obtained were combined, and filtered through a 0.22 µm filter. The filtrate was then loaded on a TSKgel ODS-80TM column (0.46 X 25 cm, Tosoh), thermostated at 25 ˚C by a water-jacket, attached to an HPLC system (Tosoh) with a DEAE-Toyopearl precolumn (0.6 X 1.0 cm) [45]. The column was eluted, at a flow rate of 0.5 ml/min, with 0.1% trifluoroacetic acid for an initial 10 min, with a linear gradient of 0-50% acetonitrile in 0.1% trifluoroacetic acid for the next 80 min, and then with 50% acetonitrile in 0.1% trifluoroacetic acid for an additional 10 min. Peptide and phosphopeptide peaks were monitored spectrophotometrically at A_{215} using an on-line UV monitor UV-8000 (Tosoh), and radiometrically by Cerenkov counting using an on-line detector, Ramona 2000 (Raytest), respectively. The radioactive fractions corresponding to each peak were pooled and subjected to automated Edman sequence analysis with a G1005A peptide sequenator (Hewlett Packard).

Purification of phospho-CaM and non-phospho-CaM. CaM (224 μg) was phosphorylated by activated CaM-kinase IV (1 μg/ml) in the presence of 0.5 mM $CaCl_2$ and 10 μg/ml poly(Lys) as described above, except that cold ATP was used instead of [γ-^{32}P]ATP. After incubation for 80 min, the reaction mixture was adjusted to 0.8 M NaCl and 2 mM $CaCl_2$, and allowed to stand for at least 15 min at room temperature. The following purification procedures were then performed at 25 ˚C. Hydrophobic interaction chromatography on a phenyl-Sepharose column (1.5 X 3.7 cm) was carried out as described by Quadroni et al. [13, 15]. Fractions eluted with the EGTA buffer were directly applied to a DEAE-NPR column (0.46 X 10 cm, Tosoh) attached to an HPLC system, previously equilibrated with buffer A (comprising 20 mM Tris-HCl and 0.1 mM EGTA). The column was eluted, at a flow rate of 0.4 ml/min, with a linear gradient of NaCl in buffer A as follows: 0-5 min, 200 mM NaCl; 5-65 min, 200-400 mM NaCl; 65-70 min, 400 mM NaCl; 70-75 min, 400-1000 mM NaCl; 75-85 min, 1000 mM NaCl. Elution of proteins was monitored spectrophotometrically at A_{215}. Under these elution conditions, non-phospho-CaM and phospho-CaM were eluted at the retention times of 37-38 min and 40-41 min, respectively. Fractions eluted as the two sharp peaks were collected separately; adjusted to 1 M NaCl. After standing for at least 15 min at room temperature, they were applied to a POROS R2/M column (0.46 X 10 cm, PerSeptive) attached to an HPLC system, previously equilibrated with H_2O. The column was eluted, at a flow rate of 4 ml/min, with a linear gradient of acetonitrile in H_2O as follows: 0-1 min, 0% acetonitrile; 1-6 min, 0-50% acetonitrile; 6-11 min, 50% acetonitrile. Fractions eluted as a single peak of A_{215} were pooled and lyophilized. The desalted and lyophilized CaM preparations were dissolved in 20 mM Hepes-NaOH (pH 8.0) and frozen in aliquots at -80 ˚C until use. The concentrations of the two CaM preparations were determined by HPLC analysis on a DEAE-NPR column (0.46 X 3.5 cm, Tosoh) on the basis of the peak area using purified rat CaM as a standard.

Protein kinase assays. CaM-kinase II activity was determined using syntide-2 as a substrate in the presence of the indicated concentrations of CaM and $CaCl_2$ as described previously [30]. CaM-kinase I and CaM-kinase IV activities were determined using syntide-2 as a substrate as described previously [34] with slight modifications [46]. To

avoid the effects of any contamination of non-phospho-CaM derived from the reaction mixture for the kinase activation, non-activated CaM-kinases I and IV were used for determination of CaM activity. The activity of CaM-kinase kinase α was assayed using PKIV peptide as a substrate as described previously [29].

Other analytical procedures. SDS-PAGE was carried out according to the method of Laemmli [47]. Native PAGE was carried out essentially according to the method of Davis [48]. The concentrations of CaM-kinase II, CaM, and S100a were determined spectrophotometrically using absorption coefficients, A_{280}(1 mg/ml), of 1.30, 0.21 [41], and 0.525 [49], respectively. Other proteins were determined by the method of Lowry et al. [50] as modified by Peterson [51] with bovine serum albumin as a standard except for dansyl-CaM. Recombinant chicken CaM was used as a standard for dansyl-CaM. The molecular weights for CaM, S100a, and MAP2 were taken as 17,000, 21,000, and 300,000, respectively.

Results

Poly(Lys)-dependent phosphorylation of CaM by CaM-kinase IV

As shown in Fig. 1, CaM-kinase IV phosphorylated CaM in the presence of poly(Lys) (lane 7). S100a (1 μM), another Ca^{2+}-binding protein with EF-hand domain, was not phosphorylated by CaM-kinase IV under the same conditions (data not shown). In the absence of poly(Lys), CaM-kinase IV did not phosphorylate CaM (lane 8). We examined whether other protein kinases could phosphorylate CaM. CaM-kinase II and its constitutively active catalytic fragment could also phosphorylate CaM only in the presence of poly(Lys) (lanes 9, 11), but to a much lesser extent than CaM-kinase IV. Thus, we did not examine the phosphorylation by CaM-kinase II any further. Under the same conditions, PKA, CaM-kinase I, and CaM-kinase kinase α did not phosphorylate CaM (lanes 1, 5, 15). The stoichiometry of the phosphorylation of CaM by CaM-kinase IV was about 0.2 mol phosphate/mol CaM after 1 hr of the phosphorylation reaction when 1 μM CaM was

phosphorylated by 1 μg/ml of activated CaM-kinase IV under the standard phosphorylation conditions. In an attempt to obtain a higher degree of phosphorylated CaM, we carried out the phosphorylation reaction under the same conditions as above but with the following modifications. The phosphorylation mixture was incubated for 40 min, and then the same amount of activated CaM-kinase IV as that added at the start of the reaction was supplied to the reaction mixture, followed by a further 40 min of incubation. Under these conditions, about 0.5 – 0.8 mol/mol of phosphate incorporation was observed.

We next examined the effect of the concentration of poly(Lys) on the phosphorylation of CaM catalyzed by CaM-kinase IV. As shown in Fig. 2A and 2B, maximal phosphorylation occurred at about 10 μg/ml of poly(Lys) when the concentration of CaM was 1 μM. Essentially, under the phosphorylation conditions, no phosphorylation was observed in the presence of more than 50 μg/ml or less than 1 μg/ml of poly(Lys). Thus, the concentration of poly(Lys) was critical for the phosphorylation of CaM by CaM-kinase IV. Since phosphorylation of MAP2 by CaM-kinase IV was also greatly enhanced by poly(Lys) (Fig. 1, lanes 7, 8), we examined the effect of poly(Lys) on CaM-kinase IV activity using syntide-2 as a substrate. As shown in Fig. 2C, CaM-kinase IV activity was strongly inhibited by 10 μg/ml poly(Lys), which caused maximal phosphorylation of CaM by CaM-kinase IV. Therefore, the poly(Lys)-dependent phosphorylation of CaM is not due to the activation of the catalytic activity of CaM-kinase IV itself by poly(Lys), but to some substrate-dependent process.

Fig. 3 shows the effects of other polycations, including poly(Lys) having various molecular weights, on the phosphorylation of CaM. When phosphorylation was carried out in the presence of poly(Lys) with various molecular weights, poly(Lys) with a higher molecular weight resulted in a higher degree of phosphorylation (lanes 1-6). Poly(Lys)(10mer), poly(Lys)(20mer), and poly(Lys)(9.6K) did not result in phosphorylation (lanes 1-3). Poly(DL-Lys) was as effective as poly(Lys) (lanes 4, 7), and poly(Arg) could partially substitute for poly(Lys) (lane 8). However, no phosphorylation was observed when poly(Glu, Lys, Tyr), which is much less basic, was used instead of poly(Lys) (lane 9), suggesting that the presence of an activator molecule of high basicity was essential for

phosphorylation. Protamine and spermine did not act as a substitute for poly(Lys) under the conditions tested (lanes 10, 11).

Poly(Lys) mediates interaction of CaM-kinase IV/CaM complex with another CaM

In order to gain further insight into the role of polycations in the phosphorylation reaction, we examined interactions among CaM, poly(Lys), and CaM-kinase IV using a fluorescence polarization method [52, 53]. To avoid any progression of the phosphorylation reaction during the binding experiments, a mutant CaM-kinase IV, whose activity was lost on the replacement of Lys^{71} with Arg (CaM-kinase IV(K71R)), was used instead of wild type CaM-kinase IV. Fig. 4A shows changes in fluorescence polarization of dansyl-CaM induced by the sequential addition of CaM-kinase IV and poly(Lys) in the presence of Ca^{2+}. When CaM-kinase IV was directly added to dansyl-CaM at point I, an increase in the fluorescence polarization, probably due to binding of dansyl-CaM to CaM-kinase IV, was observed (square). Subsequent addition of poly(Lys) at point II resulted in a large increase in polarization, suggesting that poly(Lys) bound to the CaM-kinase IV/dansyl-CaM complex. When CaM-kinase IV/CaM complex, which had been prepared by prior incubation of CaM-kinase IV with unlabeled CaM in the presence of Ca^{2+}, was added to dansyl-CaM at point I, no significant increase in polarization was observed (circle). This indicated that additional CaM (= dansyl-CaM) could no longer bind to the CaM-kinase IV/CaM complex. Subsequent addition of poly(Lys) at point II again resulted in a large increase in polarization. In contrast, the addition of CaM-kinase IV/CaM complex at point II after the addition of poly(Lys) at point I resulted in a significant increase in fluorescence polarization, though the initial addition of poly(Lys) also caused a marked increase in polarization due to the binding of poly(Lys) to dansyl-CaM (triangle). These results indicate that the addition of CaM-kinase IV/CaM complex to dansyl-CaM resulted in a significant increase in fluorescence polarization only when poly(Lys) was added to the mixture. Since dansyl-CaM did not bind to the CaM-binding domain of CaM-kinase IV, which had been occupied by unlabeled CaM (circle), the poly(Lys)/dansyl-CaM complex was

thought to bind to a site distinct from the CaM-binding domain (triangle). Control incubation of dansyl-CaM with no other additions during the incubation period caused no changes in fluorescence polarization (data not shown). Together with the fact that CaM was phosphorylated by CaM-kinase IV only in the presence of poly(Lys) (Figs. 1 and 2), these results suggest that CaM as a substrate can interact with the substrate-binding site of CaM-kinase IV only when the CaM-kinase IV/CaM-poly(Lys)-CaM complex is formed.

We also carried out binding assays using fluorescence polarization in the absence of Ca^{2+} as shown in Fig. 4B. When CaM-kinase IV was initially added to dansyl-CaM at point I before addition of poly(Lys), no significant changes in fluorescence polarization of dansyl-CaM were observed, indicating that CaM-kinase IV did not bind dansyl-CaM in the absence of Ca^{2+} (square). However, previously, when poly(Lys) was added at point I, the subsequent addition of CaM-kinase IV at point II resulted in a significant increase in polarization (triangle). Thus, poly(Lys) could mediate the interaction between dansyl-CaM and CaM-kinase IV even in the absence of Ca^{2+} .

We next carried out binding assays using various polycations with different molecular weights as shown in Fig. 4C. Addition of poly(Lys)(20mer) and poly(Lys)(9.6K) to dansyl-CaM caused significant increases in fluorescence polarization (open bar), but subsequent addition of CaM-kinase IV/CaM complex resulted in little change in polarization (hatched bar). On the contrary, subsequent addition of CaM-kinase IV/CaM complex after the addition of poly(Lys)(23K), poly(Lys)(128K), and poly(Arg) caused significant increases in fluorescence polarization (hatched bar). These results suggest that poly(Lys)(23K), poly(Lys)(128K), and poly(Arg) can bind to dansyl-CaM to form a complex with CaM-kinase IV/CaM, whereas poly(Lys)(20mer) and poly(Lys)(9.6K), though capable of binding to dansyl-CaM, cannot form a complex with CaM-kinase IV/CaM. No significant increase in fluorescence polarization of dansyl-CaM was observed with poly(Glu, Lys, Tyr). These results are in close agreement with the observation that poly(Lys)(23K), poly(Lys)(128K), and poly(Arg) resulted in phosphorylation of CaM by CaM-kinase IV, whereas poly(Lys)(20mer), poly(Lys)(9.6K), and poly(Glu, Lys, Tyr) did not (Fig. 3). Therefore,

changes in fluorescence polarization observed here are closely related to poly(Lys)-dependent phosphorylation of CaM by CaM-kinase IV.

Identification of the phosphorylation site of CaM phosphorylated by CaM-kinase IV

We carried out phosphoamino acid analysis of CaM phosphorylated by CaM-kinase IV as described in "Materials and methods." The position of the major radioactive spot on thin-layer chromatogram coincided with that of phosphothreonine, but no radioactive spots coinciding with the positions of phosphoserine and phosphotyrosine were detected (data not shown). Thus, CaM was phosphorylated by CaM-kinase IV exclusively on Thr residues under the experimental conditions used here. In order to determine the exact site(s) of the phosphorylation, we initially cleaved the phosphorylated CaM with BrCN and subsequently with API, as described in "Materials and methods." The phosphorylated CaM was extremely refractory to enzymatic digestion without prior BrCN cleavage as described by Quadroni et al. [15]. The peptide fragments generated by in-gel BrCN cleavage and subsequent in-gel API digestion were separated by reversed-phase HPLC. Fig. 5A shows the elution profile for the peptide fragments of [^{32}P]labeled CaM phosphorylated by CaM-kinase IV. These radioactive peaks were fractionated and subjected to automated Edman sequencing. The sequences of $R_{13.6}S_{2.9}L_{8.9}G_{8.1}Q_{4.6}N_{5.1}P_{3.8}XE_{3.4}A_{3.7}E_{3.3}L_{2.6}Q_{1.2}D_{0.9}X$ (the subscript numbers indicate the picomole yields) for phosphopeptide *a* and $R_{14.0}S_{2.5}L_{9.2}G_{7.7}Q_{5.7}N_{4.5}P_{3.3}XE_{4.5}A_{4.0}E_{3.5}L_{3.2}Q_{1.7}D_{1.0}X$ for phosphopeptide *b* were obtained. Since BrCN cleavage is known to generate a mixture of peptides with either homoserine or homoserine lactone on their C-terminals, phosphopeptides *a* and *b* were both assigned to the fragment corresponding to R^{37}-M^{51} of the amino acid sequence of CaM (Fig. 5B). The sum of the radioactivity corresponding to phosphopeptides *a* and *b* was calculated to be at least 50% of the radioactivity recovered. The broad peaks in retention time ranging from 75 min to 85 min could be ascribed to incomplete digestion because their peak heights diminished as the digestion proceeded. The amounts of the peptides recovered from the other peaks were not sufficient for the sequence analysis. Considering

that CaM was phosphorylated exclusively on Thr residues, the major phosphorylation site appears to be Thr^{44}.

Effect of phosphorylation on the function of CaM

In an attempt to clarify the functional changes of CaM caused by the CaM-kinase IV-catalyzed phosphorylation, we isolated the non-phosphorylated form of CaM (non-phospho-CaM) and the phosphorylated form of CaM (phospho-CaM) from the phosphorylation reaction mixture as described in "Materials and methods." Non-phospho-CaM and phospho-CaM could be separated from each other by HPLC on a high performance anion exchange column, DEAE-NPR. The purified preparations of non-phospho-CaM and phospho-CaM were subjected to native and SDS-PAGE analysis as shown in Fig. 6A and 6B, respectively. Phospho-CaM showed reduced electrophoretic mobility both in native PAGE and in SDS-PAGE in the presence of excess EDTA. Using the non-phospho-CaM and phospho-CaM preparations thus obtained, we examined whether the activation activities of CaM toward CaM-kinases were altered by CaM-kinase IV-catalyzed phosphorylation. No significant differences were observed between phospho-CaM and non-phospho-CaM activation of CaM-kinase I, CaM-kinase IV, and CaM-kinase kinase α (data not shown). However, activation of CaM-kinase II by phospho-CaM at concentrations higher than 0.3 μM was significantly lower than that by non-phospho-CaM (Fig. 6C). The Ca^{2+}-dependence of the ability of phospho-CaM to activate CaM-kinase II was also examined (Fig. 6D). Although it is not clear whether the Ca^{2+}-dependence of CaM-kinase II activation was significantly affected by the phosphorylation of CaM, the reduced activation of CaM-kinase II by phospho-CaM was again observed. Thus, phosphorylation of CaM by CaM-kinase IV resulted in a reduction in the activation of CaM-kinase II.

Discussion

CaM has been reported to be phosphorylated by several protein kinases *in vitro* [2-10]. The present study has shown that CaM-kinase IV phosphorylates CaM, its own activator, in a poly(Lys)-dependent manner. Since CaM-kinase I and CaM-kinase kinase α did not phosphorylate CaM under the same conditions (Fig. 1), it is unlikely that CaM-dependent protein kinases generally possess poly(Lys)-dependent kinase activity toward CaM. It is reported that polycations are essential for *in vitro* phosphorylation of CaM by casein kinase II [5, 54], myosin light chain kinase [10], and insulin receptor kinase [3, 4]. Polycations such as poly(Lys) were also essential for the phosphorylation of CaM by CaM-kinase IV, and the degree of the phosphorylation was highly dependent on the concentration and the molecular weight of poly(Lys) (Figs. 2 and 3). When 1 μM CaM was used for the phosphorylation, the optimal concentration of poly(Lys)(128K) was about 10 μg/ml, which, given a molecular weight of poly(Lys) 128,000, corresponds to 0.078 in a poly(Lys)/CaM molar ratio. This value is very close to the value reported for phosphorylation of CaM by myosin light chain kinase [10], and 2.5 times lower than those reported for CaM phosphorylation by either insulin receptor kinase [55] or casein kinase II [54], though poly(Arg) is reported to be a better cofactor than poly(Lys) in the former case. It has been suggested that poly(Lys) binds to regulatory β-subunit of casein kinase II to relieve autoinhibition and thus to activate the kinase [56]. Poly(Lys) is reported to markedly stimulate casein kinase II activity toward not only CaM but also a variety of peptide substrates [57]. It has also been suggested that poly(Lys) activates insulin receptor kinase by interaction with the acidic domains of the insulin receptor, leading to stimulation of kinase activity toward both CaM and a *src*-related peptide [4, 58]. In contrast, CaM-kinase IV activity toward syntide-2 was strongly inhibited by poly(Lys) (Fig. 2C). Therefore, the phosphorylation of CaM by CaM-kinase IV was not due to a simple activation of the catalytic activity by poly(Lys). The mechanism of CaM phosphorylation by CaM-kinase IV appears different from those by casein kinase II and insulin receptor kinase.

Fluorescence polarization experiments indicated that poly(Lys) mediates interaction, which indeed cannot occur without poly(Lys), between CaM-kinase IV/CaM complex and another CaM as a substrate (Fig. 4A and 4B). This was also confirmed by the observation that when biotinylated CaM was added as a substrate to the phosphorylation reaction mixture containing CaM-kinase IV/CaM complex, which had been prepared by prior incubation of CaM-kinase IV with unlabeled CaM in the presence of Ca^{2+} to saturate the CaM binding site of CaM-kinase IV by CaM, a significant incorporation of phosphate into biotinylated CaM was observed during the initial 3 min (2.2 pmol when biotinylated CaM was used, and 0.18 pmol when unlabelled CaM was used as a control). Since exchange of free dansyl-CaM for unlabeled CaM that had previously bound to CaM-kinase IV was almost negligible for at least the initial 3 min (Fig. 4A, closed circle), this supported the contention that CaM-kinase IV/CaM complex phosphorylates another CaM molecule (= biotinylated CaM) as a substrate. The observation that poly(Lys) with low molecular weights could not cause CaM phosphorylation (Fig. 3) suggests that low-molecular-weight poly(Lys) cannot mediate the interaction between CaM-kinase IV/CaM complex and another CaM, which was in good agreement with the fluorescence polarization experiments (Fig. 4C). Poly(Lys) may bring CaM-kinase IV/CaM complex and the substrate CaM into close proximity by binding to both of them to enable the phosphorylation of CaM. Hardy et al. reported that poly(Lys) binds to CaM and alters its structure [59]. Thus, the poly(Lys)-induced structural alteration of CaM molecule may also contribute to the phosphorylation reaction. Alternatively, binding of poly(Lys) to the substrate CaM may cause a conformational change of the latter so that CaM-kinase IV/CaM complex can bind to and phosphorylate CaM. Some basic protein may substitute for poly(Lys) to phosphorylate CaM *in vivo*, as suggested for casein kinase II-catalyzed [13, 54, 56] or insulin receptor-catalyzed [4, 60] CaM phosphorylation. Quadroni et al. revealed that Thr^{79}, Ser^{81}, and Ser^{101}, the phosphorylation sites by casein kinase II *in vitro* [6], are actually phosphorylated *in vivo* [15]. This might imply the presence of some activator for casein kinase II *in vivo*, whose effect is mimicked *in vitro* by polycations, because phosphorylation of CaM by casein kinase II requires polycations [5, 54]. Some protein phosphatases are also known to be markedly activated by polycations such as

poly(Lys) [61, 62]. Physiological activators that substitute for poly(Lys) remain to be identified.

CaM-kinase IV phosphorylated CaM mainly on Thr^{44} (Fig. 5B). Although a number of phosphorylation sites of CaM have been reported [5, 6, 10, 11, 15, 19, 63], the phosphorylation of Thr^{44} has not. The amino acid sequence around Thr^{44} is not consistent with a consensus sequence for phosphorylation by CaM-kinase IV, which is reported to be "hydrophobic residue-X-Arg-X-X-Ser/Thr" [64], though there is a Leu at the residue 39 (the -5 position). In the presence of polycations, myosin light chain kinase phosphorylates CaM on Thr^{26} and Thr^{29}, which have not previously been shown to be consensus sequence sites for the kinase [10]. Furthermore, we found that CaM-kinase phosphatase, which is not phosphorylated by CaM-kinase II in the absence of poly(Lys), becomes a good substrate for CaM-kinase II in its presence [65]. These data indicate that polycations such as poly(Lys) can alter the substrate specificity of protein kinases. Although physiological activators that substitute for poly(Lys) are still unclear, CaM-kinase IV is reported to be abundant in nuclei [20-22], which contain large amounts of nuclear proteins with high basicity. Therefore, CaM-kinase IV might phosphorylate CaM using a highly basic protein in the nucleus as an activator. Whether or not Thr^{44} of CaM is phosphorylated *in vivo*, especially in tissue such as brain and thymus in which CaM-kinase IV occurs abundantly, is an interesting question.

Using an anion exchange HPLC, we isolated phospho-CaM and non-phospho-CaM from the phosphorylation reaction mixture. CaM phosphorylated by CaM-kinase IV showed a reduction in activation activity toward CaM-kinase II (Fig. 6C and 6D). It has been believed that CaM-kinase II activity is mainly regulated by Ca^{2+}/CaM, autophosphorylation, and dephosphorylation by protein phosphatases [20-22, 62]. Our present findings imply that CaM-kinase IV can modulate CaM-kinase II activity through phosphorylation of CaM (Fig. 6). Another CaM-dependent kinase, myosin light chain kinase, phosphorylates CaM on the Thr residues located in the first Ca^{2+} binding pocket of CaM [10]. However, no functional changes in CaM caused by this phosphorylation have been reported. Whether or not CaM-dependent enzymes other than CaM-kinases are

regulated by the CaM-kinase IV-catalyzed phosphorylation of CaM is a question yet to be answered. Further investigations are needed to clarify the physiological significance of the phosphorylation of CaM by CaM-kinase IV.

Acknowledgments

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Figure legends

Fig. 1. Phosphorylation of CaM by CaM-kinase IV in the presence of poly(Lys). CaM (1 μM) and MAP2 (0.01 μM) were phosphorylated by PKA (1 μg/ml, lanes 1-4), activated CaM-kinase I (1 μg/ml, lanes 5, 6), activated CaM-kinase IV (1 μg/ml, lanes 7, 8), CaM-kinase II (1 μg/ml, lanes 9, 10), 30-kDa constitutively active catalytic fragment of CaM-kinase II (1 μg/ml, lanes 11-14), and CaM-kinase kinase α (1 μg/ml, lanes 15, 16) in the presence or absence of 10 μg/ml poly(Lys)(128K) and 0.5 mM $CaCl_2$ as indicated. Since MAP2 is an extremely poor substrate for CaM-kinase kinase α, CaM-kinase IV(K71R) (6.7 μg/ml) was used instead of MAP2 for CaM-kinase kinase α. After 10 min of incubation, the reactions were terminated, and SDS-PAGE was carried out as described in "Materials and methods." The gel was visualized by autoradiography. The positions corresponding to MAP2, CaM, CaM-kinase II, CaM-kinase IV, and CaM-kinase IV (K71R) are indicated.

Fig. 2. Effect of varying concentrations of poly(Lys) on phosphorylation of CaM by CaM-kinase IV (A, B) and the effect of poly(Lys) on CaM-kinase IV activity (C). (A) CaM (1 μM) was phosphorylated by activated CaM-kinase IV (1 μg/ml) in the presence of the indicated concentrations of poly(Lys)(128K). After 10 min of incubation, the reactions were terminated, and the mixtures were subjected to SDS-PAGE, followed by autoradiography. (B) The radioactive bands of the phosphorylated CaM indicated by an arrow were quantified, and the incorporation of phosphate into CaM calculated from the radioactivity was plotted against the concentration of poly(Lys) used. (C) Activated CaM-kinase IV (0.2 μg/ml) was assayed in the presence of the indicated concentrations of poly(Lys)(128K) using syntide-2 as a substrate.

Fig. 3. Effect of polycations on phosphorylation of CaM by CaM-kinase IV. CaM (1 μM) was phosphorylated by activated CaM-kinase IV (1 μg/ml) in the presence of 10 μg/ml each of poly(Lys)(10mer) (lane 1), poly(Lys)(20mer) (lane 2), poly(Lys)(9.6K) (lane 3), poly(Lys)(23K) (lane 4), poly(Lys)(87K) (lane 5), poly(Lys)(128K) (lane 6), poly(DL-Lys)

(lane 7), poly(Arg) (lane 8), poly(Glu, Lys, Tyr) (lane 9), protamine sulfate (lane 10), and 0.67 mg/ml of spermine (lane 11). After incubation for 10 min, the reactions were terminated, and the mixtures were subjected to SDS-PAGE, followed by autoradiography. The positions corresponding to CaM-kinase IV, CaM, and protamine are indicated

Fig. 4. Interaction of CaM-kinase IV(K71R) with dansyl-CaM via poly(Lys). (A) Changes in fluorescence polarization of dansyl-CaM were monitored in the presence of 0.5 mM $CaCl_2$ as described in "Materials and methods." At point I (indicated by an arrow), CaM-kinase IV(K71R)/CaM (24 µg/ml as CaM-kinase IV, ●), poly(Lys)(128K) (10 µg/ml, ▲), and CaM-kinase IV(K71R) (24 µg/ml, G) were added to the mixture, and measurements continued. Next, poly(Lys)(128K) (10 µg/ml, ● G) and CaM-kinase IV(K71R)/CaM (24 µg/ml as CaM-kinase IV, ▲) were added at point II, followed by further measurements. (B) Changes in fluorescence polarization of dansyl-CaM were monitored in the presence of 1.1 mM EGTA without $CaCl_2$. At point I (indicated by an arrow), CaM-kinase IV(K71R) (24 µg/ml, G) and poly(Lys)(128K) (10 µg/ml, ▲) were added. Next, poly(Lys)(128K) (10 µg/ml, G) and CaM-kinase IV(K71R) (24 µg/ml, ▲) were added at point II. (C) Changes in fluorescence polarization of dansyl-CaM were measured in the presence of 0.5 mM $CaCl_2$, as in A, except that the indicated polycations were used instead of poly(Lys)(128K). Initial fluorescence polarization without addition (filled bar), fluorescence polarization after addition of the indicated polycation (10 µg/ml, open bar), and fluorescence polarization after further addition of CaM-kinase IV(K71R)/CaM (24 µg/ml as CaM-kinase IV, hatched bar) are shown.

special characters

J →

H G →

J G →

H →

G H →

G H →

Fig. 5. Identification of the phosphorylation site of CaM phosphorylated by CaM-kinase IV. (A) [^{32}P]Phosphopeptide fragments generated by BrCN cleavage followed by API digestion of CaM phosphorylated by CaM-kinase IV were separated by reversed-phase HPLC as described in "Materials and methods." A representative elution profile of the [^{32}P]radioactivity under the elution conditions as described is shown. The recovery of radioactivity upon HPLC was 60-80%. (B) A schematic representation of CaM and the

major phosphorylation site identified in this study. Loops I-IV show the Ca^{2+} binding domain with an EF-hand structure [1]. The amino acid sequence between Ca^{2+} loops I and II of mammalian CaM is shown below [1]. The boxed sequence indicates the phosphopeptide isolated and sequenced. The asterisk shows the major phosphorylation site identified.

Fig. 6. Activation of CaM-kinase II by non-phospho-CaM and phospho-CaM. The purified preparations of non-phospho-CaM (lane 1) and phospho-CaM (lane 2) were analyzed by native PAGE (A) and SDS-PAGE (B) on 12% acrylamide gels. The gels were visualized by protein staining with Quick-CBB. (C) CaM-kinase II assays were carried out in the presence of 0.15 mM $CaCl_2$ and the indicated concentrations of non-phospho-CaM (○) or phospho-CaM (●) using syntide-2 as a substrate. Kinase activity is expressed as a percentage of the activity attained with 1 μM non-phospho-CaM, which ranged from11,100 to 14,000 nmol/min/mg. (D) CaM-kinase II assays were carried out in the presence of non-phospho-CaM (0.4 μM, ○) or phospho-CaM (0.4 μM, ●) at the free Ca^{2+} concentrations indicated. The free Ca^{2+} concentrations in the assay mixtures were calculated by a freeware computer program, CALCON ver 3.3, which is based on the algorithm described by Goldstein [66]. pCa represents $-\log\{[Ca^{2+}]_{free}\ (M)\}$. The kinase activity is expressed as a percentage of the activity attained with non-phospho-CaM at 99 μM free Ca^{2+}, which ranged from 14,300 to 17,400 nmol/min/mg. All values are averages of at least three independent measurements ± SD. A comparison between CaM-kinase II activity in the presence of non-phospho-CaM and that in the presence of phospho-CaM under the same assay conditions was conducted using Student's *t*-test. Differences of $P < 0.05$ or $P < 0.02$ were considered significant. $^{*}P < 0.02$. $^{**}P < 0.05$.

Footnotes (for the title page)

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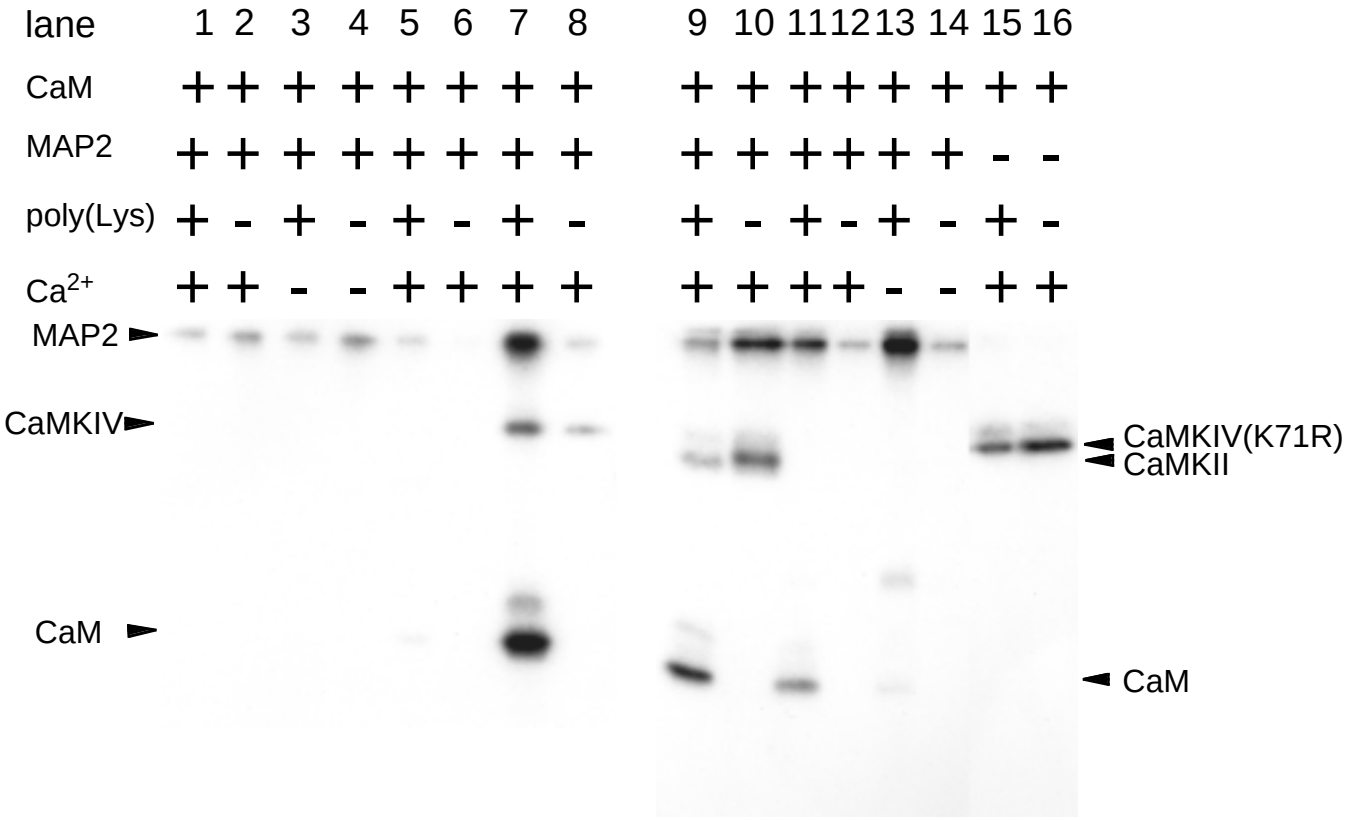
[3]Abbreviations used: API, Achromobacter protease I; CaM, calmodulin; CaM-kinase, Ca^{2+}/calmodulin-dependent protein kinase; MAP2, microtubule associated protein 2; PKA, catalytic subunit of cAMP-dependent protein kinase; poly(Arg), poly-L-arginine; poly(DL-Lys), poly-DL-lysine; poly(Glu, Lys, Tyr), poly-L-glutamic acid/lysine/tyrosine (6/3/1); poly(Lys), poly-L-lysine.

Fig. 1. **A. Ishida et al.**



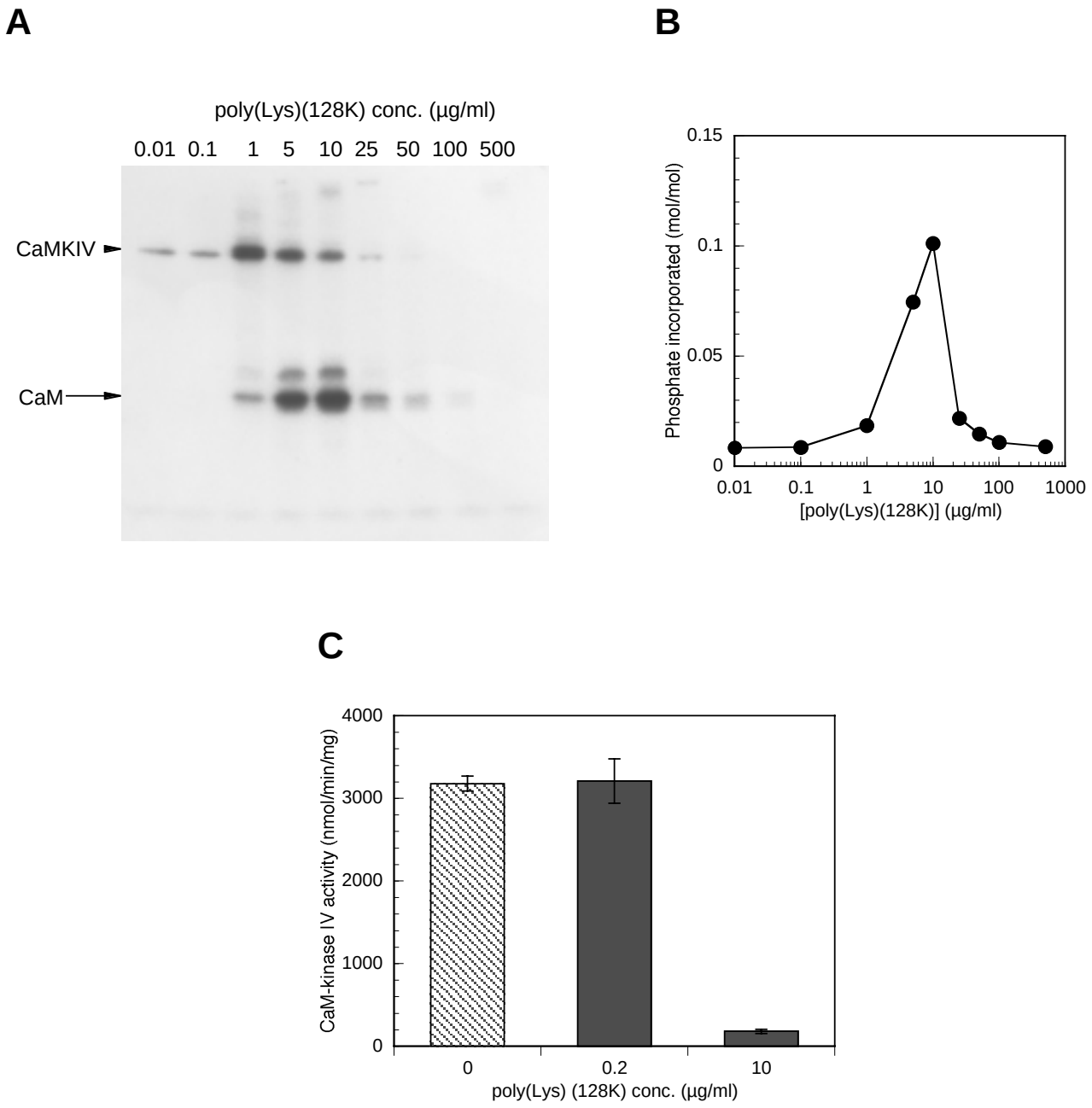
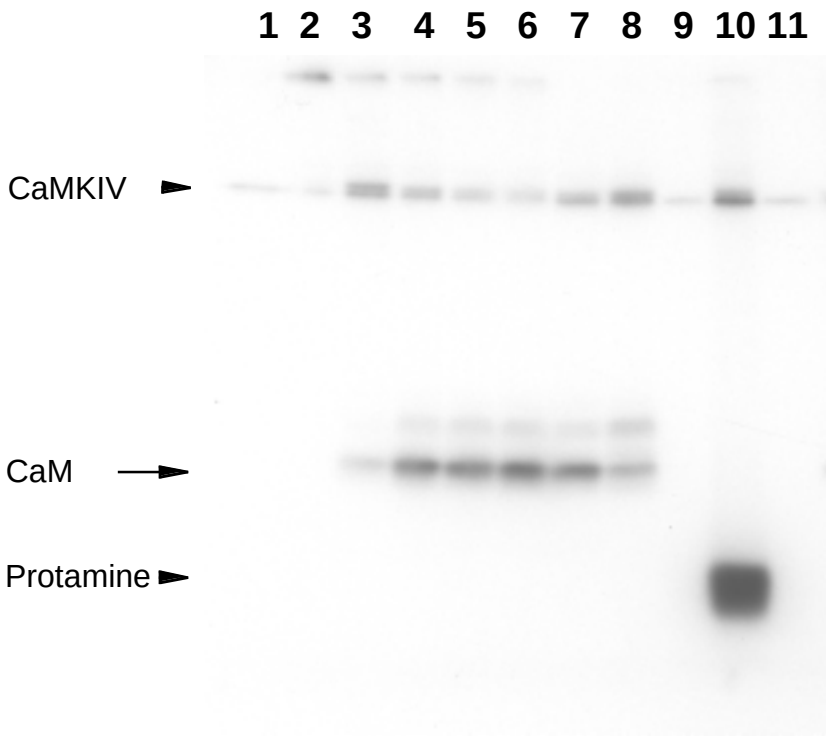
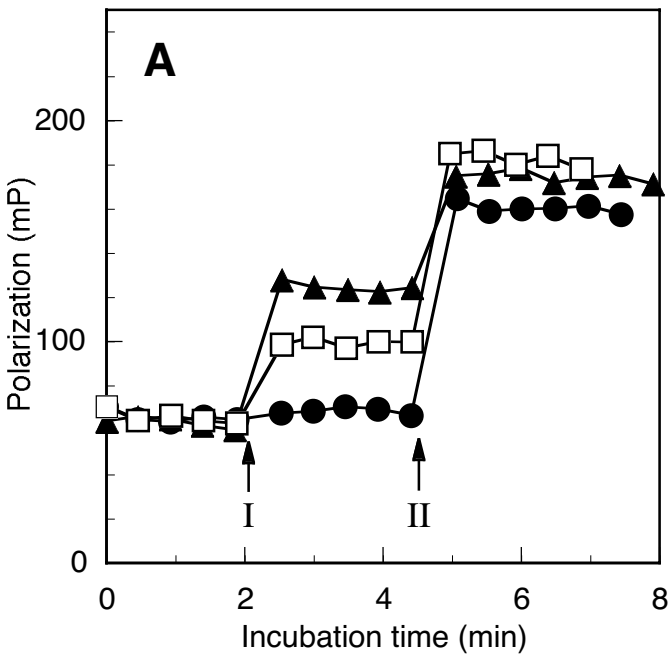
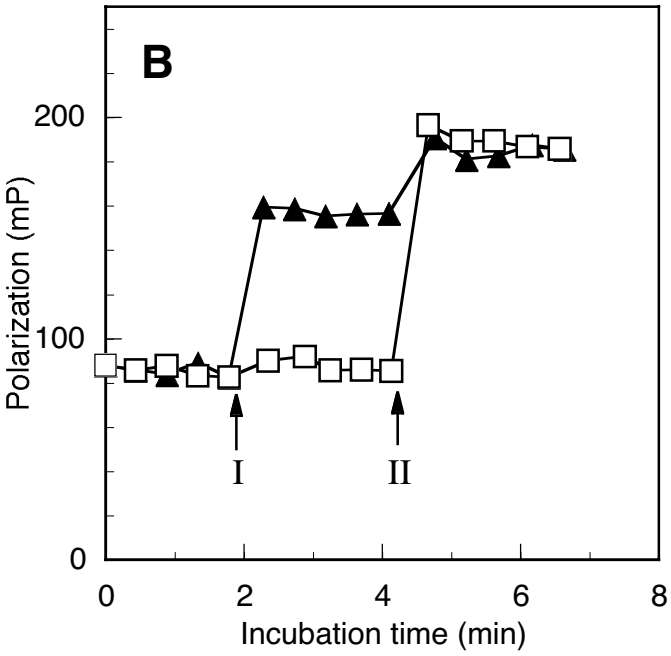

A
poly(Lys)(128K) conc. (μg/ml)
0.01 0.1 1 5 10 25 50 100 500
CaMKIV
CaM
B
Phosphate incorporated (mol/mol)
0.15
0.1
0.05
0
0.01 0.1 1 10 100 1000
[poly(Lys)(128K)] (μg/ml)
C
CaM-kinase IV activity (nmol/min/mg)
4000
3000
2000
1000
0
0 0.2 10
poly(Lys) (128K) conc. (μg/ml)

Fig. 3.

A. Ishida et al.

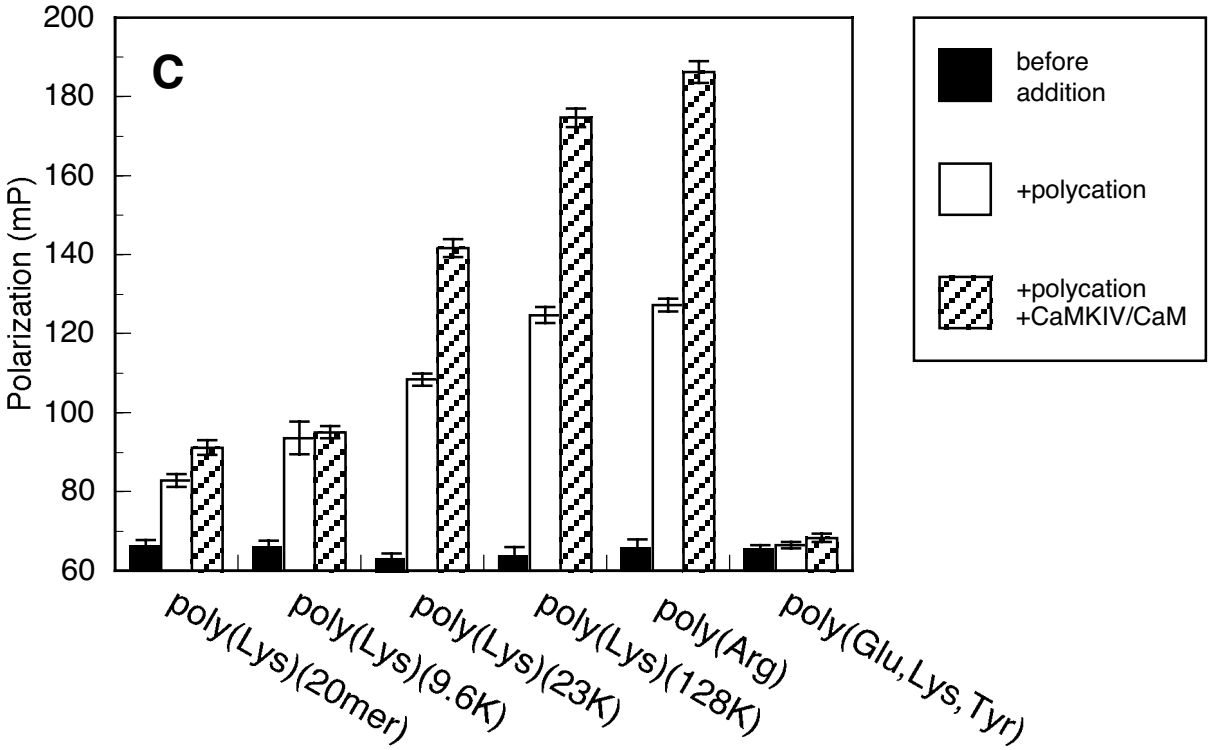


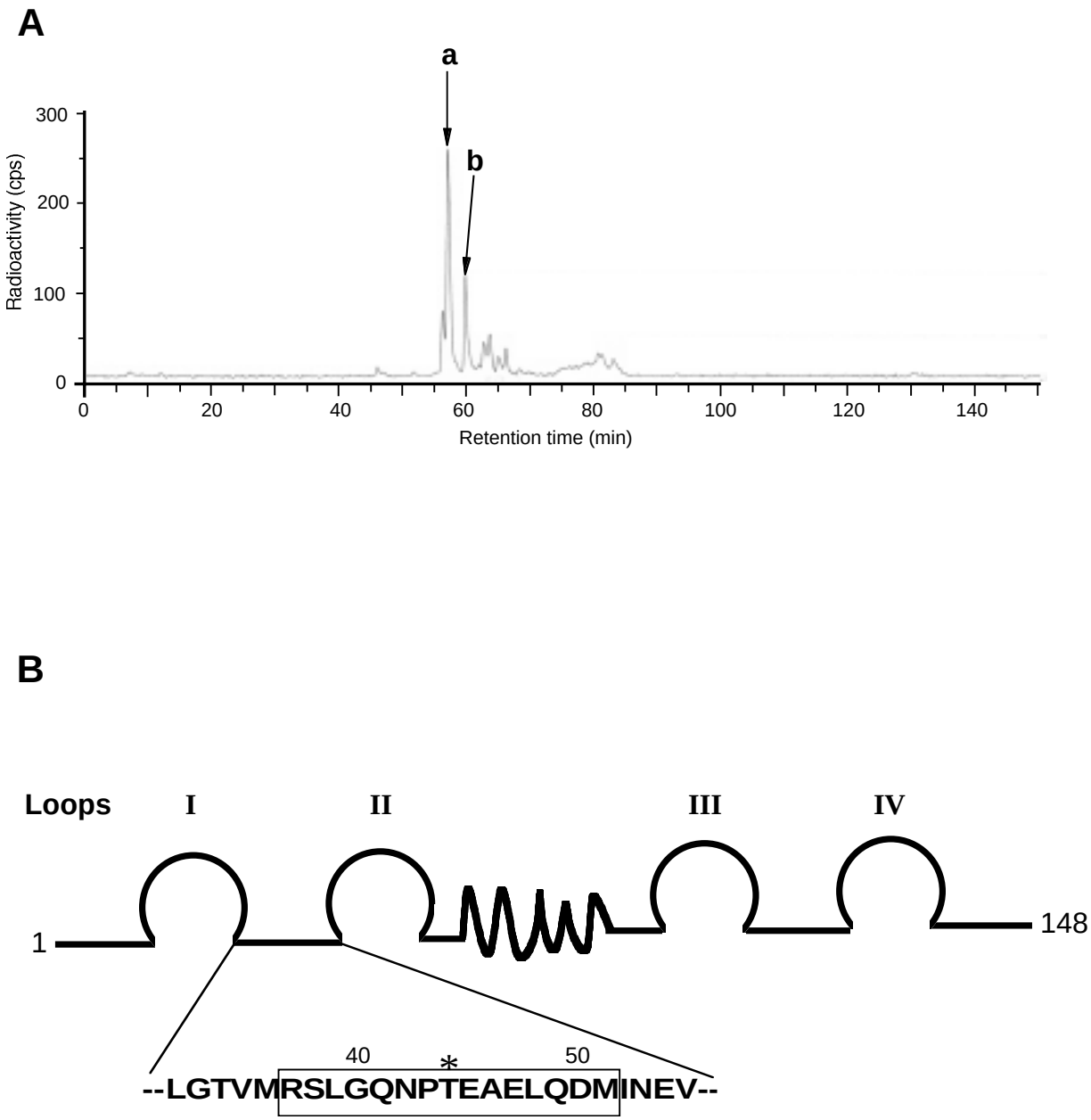


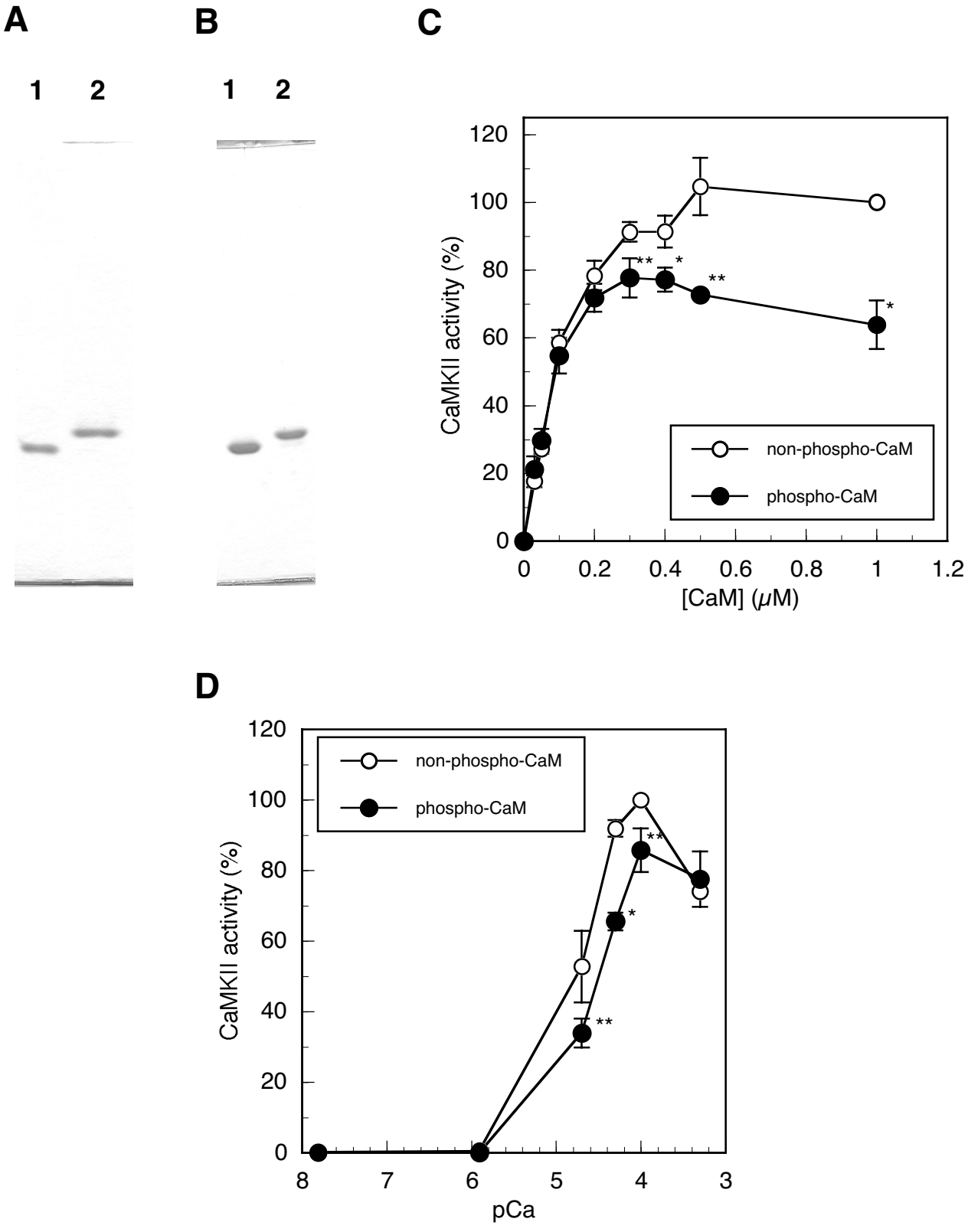


symbol	start	additions I	additions II
●	DNS-CaM/Ca^{2+}	CaMKIV/CaM	poly(Lys)
□	DNS-CaM/Ca^{2+}	CaMKIV	poly(Lys)
▲	DNS-CaM/Ca^{2+}	poly(Lys)	CaMKIV/CaM

symbol	start	additions I	additions II
□	DNS-CaM	CaMKIV	poly(Lys)
▲	DNS-CaM	poly(Lys)	CaMKIV




A
a
b
300
200
100
0
Radioactivity (cps)
0
20
40
60
80
100
120
140
Retention time (min)
B
Loops
I
II
III
IV
1
148
40
*
50
--LGTVMRSLGQNPTEAELQDMINEV--


A
B
C
1 2
1 2
CaMKII activity (%)
[CaM] (μM)
non-phospho-CaM
phospho-CaM
D
CaMKII activity (%)
pCa
non-phospho-CaM
phospho-CaM