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Stimulation of Ca^{2+}/calmodulin-dependent protein kinase phosphatase by polycations

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Abstract

Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase) dephosphorylates and regulates multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs). One of the prominent features of CaMKPase is stimulation of phosphatase activity by polycations such as poly-L-lysine (poly(Lys)). Using various polycations, basicity and molecular weight of the polymer proved to be important for the stimulation. Surface plasmon resonance (SPR) analysis showed that CaMKIV(T196D), which mimics CaMKPase substrate, and CaMKPase could form tight complexes with poly(Lys). Pull-down binding experiments suggested that the formation of a tightly associated ternary complex consisting of CaMKPase, poly(Lys), and phosphorylated CaMKIV is essential for the stimulation. Dilution experiments also supported this contention. Poly(Lys) failed to stimulate a CaMKPase mutant in which a Glu cluster corresponding to the residues 101-109 in the N-terminal domain was deleted, and the mutant could not interact with poly(Lys) in the presence of Mn^{2+}. Thus, the Glu cluster appeared to be the binding site for polycations, and to play a pivotal role in the polycation stimulation of the CaMKPase activity.

Key words: Ca^{2+}/calmodulin-dependent protein kinase; CaMKPase; dephosphorylation; phosphatase; polycation; stimulation.

Calcium ions are known to play important roles in the regulation of a variety of neuronal functions, and most of the diverse actions of Ca^{2+} may be mediated through protein phosphorylation by three multifunctional calmodulin-dependent protein kinases, calmodulin-dependent protein kinases (CaMK)2 I, II, and IV(see refs. 1-4 for reviews). CaMKII is known to be activated through autophosphorylation of Thr^{286}. In contrast, CaMKI and IV are activated through phosphorylation of Thr^{177} and Thr^{196}, respectively, by the upstream kinase, CaMK kinase. Therefore, protein phosphatases that dephosphorylate these residues are important for regulation of the multifunctional CaMKs. With a novel in-gel protein phosphatase assay [5], we detected three distinct protein phosphatases capable of dephosphorylating the phosphopeptide corresponding to the sequence around Thr^{286} of CaMKII in rat brain extract, and purified one of them [6]. The purified enzyme, designated as CaMK phosphatase (CaMKPase), is a calyculin A-insensitive and Mn^{2+}-dependent protein phosphatase which exhibits a much higher substrate specificity than do the well-known multifunctional protein phosphatases such as protein phosphatase (PP) 1, 2A, and 2C. The cDNA sequence of CaMKPase encodes a polypeptide consisting of 450 amino acid residues with a molecular weight of 49,165, which shows 19.6% sequence identity with rat PP2Cα [7]. CaMKPase ubiquitously distributes in various tissues, and localizes in the cytosol [7, 8]. CaMKPase can dephosphorylate and reversibly deactivate activated CaMKI, II, and IV *in vitro* [9], and can promote apoptosis when overexpressed in mammalian cells [10]. Recently, we found a novel nuclear protein phosphatase which shows similarities to CaMKPase, and designated it as CaMKP-N [11]. CaMKP-N shows similar biochemical properties to CaMKPase.

One of the prominent biochemical features of CaMKPase is marked stimulation of the protein phosphatase activity by polycations such as poly-L-lysine (poly(Lys)) and protamine [6]. It is well-known that PP2A is also markedly stimulated by polycations, but the mechanism and the physiological significance of the phenomenon is not yet fully understood [12-15]. Although the physiological significance of the stimulation of CaMKPase activity by polycations is also unclear, it may represent some regulatory mechanism of the enzyme in the cells as is suggested for PP2A [12, 14, 15]. In our previous paper, we showed that poly(Lys) stimulates the enzyme mainly through an increase in the Vmax values by use of various phosphopeptide substrates [16]. Since the magnitudes of the stimulation are extremely low compared with those using phosphoprotein substrates, another mechanism should also contribute to the marked stimulation when phosphoproteins are used as substrates. Thus, mechanisms for the stimulation of CaMKPase activity by polycations were investigated using phosphorylated CaMKIV or its analog as a model substrate.

Materials and methods

Materials. ATP, poly(Lys) (poly(Lys)(128K), poly(Lys)(87K), poly(Lys)(23K), and poly(Lys)(9.6K), of which average molecular weights are 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), poly-L-lysine/serine (3:1) (poly(Lys, Ser), average molecular weight 31,000), poly-L-lysine/tyrosine (4:1) (poly(Lys, Tyr), average molecular weight 24,000), and poly-D-glutamic acid/lysine (6:4) (poly(D-Glu,

D-Lys), average molecular weight 23,000) were purchased from Sigma. Unless otherwise specified, poly(Lys) represents poly(Lys)(87K). Polybrene was from Sigma-Aldrich. $(Lys)_5$ (KKKKK), $(Lys)_{10}$ (KKKKKKKKKK), and $(Lys)_{20}$ (KKKKKKKKKKKKKKKKKKKK) were synthesized by a Shimadzu PSSM8 automated peptide synthesizer and purified by reversed-phase HPLC. [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham International. Ni-NTA-agarose was from Qiagen. CaMKIV-specific antibody was obtained as described previously [17].

Protein preparations. Mixed histones (type II-A), myelin basic protein, and bovine serum albumin (BSA) were purchased from Sigma. Protamine sulfate was from nacalai tesque. Recombinant mouse p42 mitogen-activated protein kinase (Erk2)-GST (inactive) was purchased from Upstate Biotechnology, Inc. Recombinant rat CaMKIV(K71R), with Lys^{71} (ATP-binding site) replaced with Arg, expressed in Sf9 cells was purified as described [18]. Recombinant rat CaMKIV(T196D), with Thr^{196} replaced with Asp, expressed in Sf9 cells was prepared essentially as described above (Okuno, S., Kitani, T. and Fujisawa, H., unpublished work). Recombinant rat CaMK kinase-α [18] and recombinant rat CaMKPase [16], both of which were expressed in E. coli, were purified as described. Unless otherwise mentioned, this preparation of CaMKPase was used in this study. His_6-tagged wild type CaMKPase was prepared as follows. An *Nde*I restriction site was created in the start codon of the CaMKPase cDNA as described previously [7], and the cDNA fragment containing the entire coding sequence was subcloned into pUC119 to generate pUC-CaMKP. The 1.7 kb *Nde*I-*Bam*HI fragment of pUC-CaMKP was inserted into pET28a (Novagen) at *Nde*I and *Bam*HI sites to generate pET-His_6-CaMKP, which encodes full length CaMKPase with an N-

terminal His_6-tag. This construct was transformed into E. coli strain BL21(DE3). The transformed bacteria were grown to an A_{600} of 1.0 at 30 ˚C in M9ZB medium containing 30 μg/ml kanamycin, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. After shaking overnight at 16 ˚C, the bacteria were harvested by centrifugation. The cell pellet was suspended in the homogenization buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.2% Tween 40, 10 mM imidazole, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of microbial protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate a crude extract. RNase A and DNase I (20 μg/ml each) were added to the extract, and it was allowed to stand on ice for 30 min. After the streptomycin treatment was carried out as described [16], the recombinant protein was purified using Ni-NTA-agarose (Qiagen) chromatography according to the manufacturer's instruction. All the purification procedures were carried out at 4 ˚C unless otherwise mentioned. CaMKPase fractions eluted from the column were combined, and solid ammonium sulfate was added to the solution to give 55% saturation. The precipitate, collected by centrifugation at 395,000 X G for 30 min, was dissolved in dialysis buffer consisting of 40 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Tween 40, 5% ethylene glycol, 0.5 mM dithiothreitol, and 50 mM NaCl, followed by overnight dialysis against the same buffer. The purified enzyme was frozen in aliquots at –80 ˚C. His_6-tagged CaMKP (Δ101-109) mutant was prepared as follows. A cDNA for the mutant was prepared using the single-stranded DNA obtained from pUC119, into which the *Eco*RI-*Kpn*I fragment (0.84 kb) of pUC-CaMKP was inserted, as a template, and a synthetic

oligonucleotide, 5'-CTGAATTTAAGAGGTTGCCTGAACAAAGAGTACTCACGACCCTACTGGATGCC-3', as a mutagenic sense primer, essentially according to the method of Kunkel et al. [19]. The mutation was confirmed by DNA sequencing using dideoxynucleotide chain-termination method [20] with a DNA sequencer, LI-COR model 4000L (Aloka). The *Nde*I-*Kpn*I fragment (0.72 kb) of the pUC119 containing the mutated segment, and the *Kpn*I-*Bam*HI fragment (0.94 kb) of pUC-CaMKP were inserted into pET28a at *Nde*I and *Bam*HI sites to generate pET-His$_6$-CaMKP (Δ101-109), which encodes the full length CaMKP (Δ101-109) with an N-terminal His$_6$-tag. Transformation and cultivation of the bacteria, induction of the recombinant protein by IPTG, and purification of the protein were carried out as described above for the wild type His$_6$-tagged CaMKPase. His$_6$-tagged CaMKP (1-412) mutant was prepared as follows. A cDNA for the mutant was prepared using the single-stranded DNA obtained from pUC119, into which the *Kpn*I-*Bam*HI fragment (0.94 kb) of pUC-CaMKP was inserted, as a template, and a synthetic oligonucleotide, 5'-TTCCTTAGGGACCCCTAGGAGCTGCTGGAGGG-3', as a mutagenic sense primer, as described above. After the mutation was confirmed by direct sequencing, the *Kpn*I-*Bam*HI fragment (0.94 kb) of the pUC119 containing the mutated segment, and the *Nde*I-*Kpn*I fragment of pUC119-CaMKP (0.75 kb) was inserted into pET28a at *Nde*I and *Bam*HI sites to generate pET-His$_6$-CaMKP(1-412), which encodes the full length CaMKP (1-412) with an N-terminal His$_6$-tag. Transformation and cultivation of the bacteria, induction of the recombinant protein by IPTG, and purification of the protein were carried out as described above.

Protein phosphatase assay. Purified rat recombinant CaMKIV (K71R) was phosphorylated as described previously [9]. CaMKPase activity was determined with [^{32}P]CaMKIV (K71R) as a substrate essentially as described previously [21] except that KCl was omitted from the mixture and that [^{32}P]CaMKIV (K71R) was used instead of [^{32}P]CaMKII.

Surface plasmon resonance (SPR) analysis. SPR analysis of the interaction of CaMKPase and CaMKIV (T196D) with poly(Lys) was performed with a BIAcore X biosensor (BIAcore AB). Poly(Lys) was immobilized on a sensor chip CM5 (BIAcore AB) at 25 ˚C by a standard amine coupling method with HBS (BIAcore AB) consisting of 10 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v) as a running buffer according to the manufacturer's instruction. Binding analyses were performed at 25 ˚C with a flow rate of 20 µl/min using HBS containing 10 mM $MnCl_2$ as a running buffer. At time 0 sec, the indicated concentrations of the proteins in the same buffer (20 µl) were injected, and the association/dissociation kinetics was monitored. After each binding experiment, bound protein was completely removed by injecting 20 µl of 0.5% (w/v) SDS and 20 µl of 2.5 M NaCl. No detectable loss of binding activity was observed after the treatment. The nonspecific binding obtained under the same experimental conditions using a control sensor chip, which had been treated by the standard amine coupling method without poly(Lys), was subtracted from the sensorgram. Data were analyzed by BIA Evaluation 3.0 software (BIAcore AB), and the binding parameters were calculated by simultaneous global curve fitting analysis according to the manufacturer's instruction.

Pull-down binding assay of CaMKIV. His_6-tagged wild type CaMKPase (30 μg) was incubated with Ni-NTA agarose resin (Qiagen) (ca. 30% v/v) in 225 μl of 20 mM Tris-HCl (pH 7.5) containing 0.13% Tween 20, 10 mM imidazole, and 228 mM NaCl at 4 ˚C for 2 hr with gentle shaking. As a control without CaMKPase, BSA was used instead of CaMKPase. The resins were washed three times with ice-cold binding buffer consisting of 20 mM Tris-HCl (pH 7.5), 10 mM imidazole, 100 mM NaCl, and 0.01% Tween 40, and suspended into the same buffer as 50% (v/v) suspensions. To the suspensions (18 μl) were added 9 μg/ml poly(Lys) and 4.5 mM $MnCl_2$, and the mixtures were incubated for 2 min at 30 ˚C. After the resins were washed twice with ice-cold binding buffer containing 5 mM $MnCl_2$, they were resuspended into an equal volume of binding buffer containing 5 mM $MnCl_2$ and 0.75 mg/ml BSA. CaMKIV (T196D) (21 ng) was added to the suspensions, followed by incubation for 2 min at 30 ˚C. After the resins were washed twice with ice-cold binding buffer containing 5 mM $MnCl_2$, the bound proteins to the resin were solubilized by boiling for 2 min in the sample buffer for SDS-polyacrylamide gel electrophoresis [22]. They were subjected to Western blotting analysis using CaMKIV-specific antibody.

Other analytical procedures. Protein concentrations were determined by the method of Lowry et al. [23], as modified by Peterson [24], with BSA as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [22]. Western blotting analysis was performed as described by Harlow and Lane [25] using peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) as a secondary antibody (Cappel). The Western blot was developed by an enhanced chemiluminescence procedure

(Super Signal, Pierce). Chemiluminescence signals were analyzed by ChemiDoc documentation system (Bio-Rad).

Results

Stimulation of CaMKPase by polycations

When phosphoproteins and a phosphopeptide-magnetic particle conjugate are used as substrates, CaMKPase is markedly stimulated by polycations such as poly(Lys) [6, 9]. Fig. 1A shows the effect of varying the concentration of poly(Lys) on the activation of CaMKPase using [^{32}P]CaMKIV (K71R) as a substrate. Maximal activation occurred about 5 μg/ml of poly(Lys), and higher concentrations of poly(Lys) resulted in a decrease in the enzyme activity as previously reported for brain CaMKPase using a [^{32}P]phosphopeptide-magnetic particle conjugate as a substrate [6]. Next, we examined the effect of molecular weights of poly(Lys) on the stimulation using poly(Lys) with various molecular weights. As shown in Fig. 1B, the stimulation of CaMKPase increased with increasing molecular weight of poly(Lys). Various artificial and natural polycations also stimulated CaMKPase activity (Table 1). Poly (Lys, Ser), poly(Lys, Tyr), and poly(Arg) markedly stimulated CaMKPase, but an amino acid polymer containing a significant amount of Glu did not stimulate CaMKPase at all. Therefore, basicity of the polymer is essential for the stimulation. The observation that another artificial polymer, polybrene, also significantly stimulated CaMKPase supports this contention. The stimulation by poly(DL-Lys) was nearly equal to that by poly(Lys) with the same molecular weight, indicating that CaMKPase

does not distinguish between optical isomers of Lys on the stimulation. Basic proteins, protamine and histone, stimulated CaMKPase activity about 8-fold and 2-fold, respectively, but myelin basic protein had no significant effect on CaMKPase activity.

Fig. 2 shows effects of NaCl concentrations on CaMKPase activity. Poly(Lys)-stimulated CaMKPase activity markedly decreased with increasing concentrations of NaCl. In the presence of 515 mM NaCl, the activity was suppressed to about 5% of the maximum value (Fig. 2A). Thus, stimulation of CaMKPase by poly(Lys) was highly sensitive to the NaCl concentrations in the reaction mixture. Basal activity of CaMKPase also decreased with increasing concentrations of NaCl, but the extent of the inhibition was rather small (Fig. 2B).

SPR analysis of the interaction of CaMKPase and CaMKIV (T196D) with poly(Lys)

In order to clarify the mechanism of stimulation of CaMKPase by polycations, we examined an interaction of CaMKPase with poly(Lys) by means of SPR analysis. Poly(Lys) was immobilized on an SPR sensor chip, and CaMKPase was injected to the SPR biosensor as an analyte in the presence of 10 mM $MnCl_2$, which is absolutely required for CaMKPase activity. Fig. 3A shows sensorgrams obtained by such analyses. CaMKPase rapidly associated to the sensor chip and dissociated very slowly. Thus, CaMKPase formed a very stable complex with poly(Lys). BSA did not show significant binding even at 267 nM, suggesting that the binding of CaMKPase did not represent non-specific binding of proteins to poly(Lys) (Fig. 3A, dotted line). Curve fitting analysis of the sensorgrams yielded a Kd value of 39 nM for the binding of CaMKPase to the poly(Lys)-immobilized

sensor chip, suggesting that CaMKPase has very high affinity to poly(Lys). CaMKPase also tightly bound to the sensor chip to form a very stable complex with poly(Lys) in the absence of $MnCl_2$ as well as in its presence, though the SPR signals were somewhat larger than those in the presence of 10 mM $MnCl_2$ (data not shown).

Since CaMKPase efficiently dephosphorylates phosphorylated CaMKIV in a poly(Lys)-dependent manner [9], we also examined an interaction of a CaMKIV mutant, CaMKIV (T196D), which mimics activated CaMKIV by Thr^{196}→Asp mutation [18], with poly(Lys). In the presence of 10 mM $MnCl_2$, CaMKIV (T196D) also rapidly associated to the sensor chip on which poly(Lys) was immobilized, and formed a very stable complex with poly(Lys), as shown in Fig. 3B. A Kd value of 6.5 nM was obtained from a curve fitting analysis of the sensorgram. Erk2-GST was analyzed under the same conditions, but no significant binding was observed at 80 nM (data not shown). In the absence of $MnCl_2$, CaMKIV (T196D) also tightly bound to the sensor chip as well as in its presence (data not shown).

Binding of CaMKIV (T196D) to CaMKPase-Ni-NTA agarose via poly(Lys)

Fairly tight interaction between CaMKPase and poly(Lys), and that between CaMKIV (T196D) and poly(Lys) led us to the notion that CaMKPase might bind its substrate with the aid of poly(Lys) to form a tight complex. Therefore, we examined whether CaMKPase could bind CaMKIV (T196D) with or without poly(Lys). Ni-NTA-agarose resin itself did not significantly bind CaMKIV (T196D) irrespective of the presence of poly(Lys) (Fig. 4, lanes 1 and 2). The resin on which CaMKPase was immobilized through His_6-tag bound CaMKIV (T196D) only marginally in the absence of poly(Lys) (lane 3). In the presence of

poly(Lys), however, marked binding of CaMKIV (T196D) on the resin was observed (lane 4). Thus, it is suggested that CaMKPase effectively binds the substrate phosphoprotein via polycations such as poly(Lys). When $(Lys)_{10}$, which could not stimulate CaMKPase activity (Fig. 1B), was used instead of poly(Lys), binding of CaMKIV (T196D) to the resin was not significant (Fig. 4, lane 6). When 500 mM NaCl was included in the binding reaction mixture, under which conditions the stimulation of CaMKPase by poly(Lys) was severely inhibited (Fig. 2A), CaMKIV (T196D) bound to the resin only marginally even in the presence of poly(Lys) (Fig. 4, lane 8). These data suggest that the formation of a tightly associated ternary complex consisting of CaMKPase, poly(Lys), and phosphorylated CaMKIV is important for the stimulation of CaMKPase by poly(Lys).

Effect of dilution of CaMKPase-poly(Lys)-[^{32}P]CaMKIV (K71R) complex on CaMKPase activity

In order to know whether or not the tightly associated ternary complex could be formed during the phosphatase reaction, we examined the effect of dilution of the preincubation mixture containing CaMKPase, poly(Lys), and [^{32}P]CaMKIV (K71R) on its CaMKPase activity. If such a ternary complex is formed during the preincubation of CaMKPase with poly(Lys) and [^{32}P]CaMKIV (K71R), then the specific activity of CaMKPase should be invariable in spite of lowering the substrate concentration by dilution. Fig. 5A shows that the specific CaMKPase activity was approximately constant over a range of 4 nM to 20 nM of [^{32}P]CaMKIV (K71R) when the assay was carried out immediately after the dilution of the preincubation mixture. Under these assay conditions, the concentrations of [^{32}P]CaMKIV

(K71R) was sufficiently lower than the Km value for [^{32}P]CaMKIV (K71R) (85 nM, in the presence of poly(Lys)), indicating that CaMKPase was not saturated by the substrate. Van't Hoff plot of the data yielded a linear curve with a slope of 1.1 (Fig. 5B), suggesting that the phosphatase reaction proceeds by the "intramolecular " mechanism, as described by Todhunter and Purich [26]. Thus, it is suggested that CaMKPase does form a tightly associated ternary complex with poly(Lys) and [^{32}P]CaMKIV (K71R) during the phosphatase reaction.

Stimulation of mutant CaMKPases by poly(Lys)

In order to identify the region of CaMKPase responsible for the stimulation by polycations, we prepared two deletion mutants of CaMKPase, CaMKP (1-412) and CaMKP (Δ101-109), as shown in Fig. 6A. The expression plasmids for these mutants were constructed and expressed in E. coli as described under "Materials and methods." Since the mutant proteins were expressed sufficiently in the soluble fractions, we purified them by Ni-NTA-agarose affinity chromatography as described. Stimulation of the mutant CaMKPases by poly(Lys) is shown in Fig. 6B. The C-terminal truncated mutant, CaMKP (1-412) was markedly stimulated by 10 μg/ml poly(Lys) as well as the wild type CaMKPase, suggesting that the truncated C-terminal region, 413-450, is essential for neither the stimulation by poly(Lys) nor the catalytic function itself. In contrast, CaMKP (Δ101-109) mutant was not stimulated by poly(Lys) at all, suggesting that the amino acid residues 101-109, which is composed of a Glu cluster, is essential for the stimulation by poly(Lys).

Besides these, we constructed the expression plasmids for N-terminal truncation

mutants, CaMKP (51-450), CaMKP (96-450), and CaMKP (109-450), and for a C-terminal truncation mutant, CaMKP (1-395), to express in E. coli. However, they were exclusively expressed in the insoluble fractions, perhaps as inclusion bodies, and we failed to solubilize them under non-denaturing conditions (data not shown). Therefore, we could not characterize these mutants.

SPR analysis of the interaction of the mutant CaMKPases with poly(Lys)

In order to know whether the region 101-109 was also important for binding to poly(Lys), we carried out SPR analysis using CaMKP (Δ101-109) mutant. In the absence of $MnCl_2$ in the running buffer, CaMKP (Δ101-109) tightly bound to the sensor chip, on which poly(Lys) was immobilized, as well as the wild type CaMKPase (data not shown). In the presence of $MnCl_2$ in the running buffer, however, CaMKP (Δ101-109) mutant could not bind to the sensor chip even at 1495 nM, as shown in Fig. 3C. Thus, the Glu cluster 101-109 of CaMKPase is essential for binding to poly(Lys) in the presence of $MnCl_2$, which is absolutely required for CaMKPase activity. In contrast, the C-terminal truncation mutant CaMKP (1-412) could efficiently bind to the sensor chip in the absence (data not shown) and presence (Fig. 3D) of 10 mM $MnCl_2$. Therefore, it is suggested that the C-terminal residues 413-450 of CaMKPase is not necessary for binding to poly(Lys).

Discussion

Stimulation of protein phosphatase activity by polycations such as poly(Lys) is one of the prominent features of CaMKPase. The data obtained with various artificial polycations suggest that basicity and molecular weight of the activator molecule are important determinants for its potency of the stimulation (Fig. 1B and Table 1). From the binding analysis using an SPR biosensor, it is obvious that both CaMKPase and CaMKIV (T196D) tightly bind to poly(Lys) (Fig. 3A and B), suggesting that CaMKPase and phosphorylated CaMKIV mutually interact through tight binding to poly(Lys). This is also supported by the observation that CaMKIV (T196D) markedly bound to the Ni-NTA-agarose resin, to which CaMKPase was immobilized, only in the presence of poly(Lys) (Fig. 4). High concentrations of NaCl, which markedly reduced the stimulation of CaMKPase by poly(Lys), also interfered with poly(Lys)-dependent binding of CaMKIV (T196D) to CaMKPase-immobilized Ni-NTA-agarose resin. The binding was also suppressed by substituting poly(Lys) with $(Lys)_{10}$, which could not stimulate CaMKPase activity. Thus, the formation of a ternary complex composed of CaMKPase, phosphorylated CaMKIV and poly(Lys) seems to be important for the stimulation by poly(Lys). The dilution experiment of a preincubation mixture containing CaMKPase, poly(Lys), and [^{32}P]CaMKIV (K71R) also suggests the formation of such a tightly associated complex during the dephosphorylation reaction (Fig. 5). It is likely that poly(Lys) brings phosphorylated CaMKIV and CaMKPase into close proximity by binding to both of them to enable the dephosphorylation of the substrate. The observation that high concentrations of poly(Lys) resulted in a

decrease in the enzyme activity (Fig. 1A) can be ascribed to a decrease in the ternary complex favorable for the phosphatase reaction due to trapping of the substrate by excess poly(Lys). Inability of poly(Lys) with a low molecular weight to stimulate CaMKPase activity is interpreted as the result of an insufficient length of the polycationic chain to which CaMKPase and phosphorylated CaMKIV are bound to form the tight complex (Fig. 1B and Fig. 4, lanes 5 and 6). It is reported that a poly(Lys)- induced aggregation of casein is directly related to the mechanism of the stimulation of casein kinase II by poly(Lys) [27]. Since we also observed that [^{32}P]CaMKIV (K71R) was readily precipitated by poly(Lys) under the assay conditions (Kameshita, I., unpublished observation), CaMKPase, poly(Lys), and phosphorylated CaMKIV might form a large complex in which the phosphatase reaction proceeds favorably.

CaMKPase and CaMKP-N, nuclear CaMKPase, have unique N-terminal domains which are not shared by other PPM family phosphatases, though no significant homology between the two domains was found [11] (see also Fig. 6A). The functions of these domains are not clarified so far. The fact that poly(Lys) failed to stimulate CaMKP (Δ101-109) mutant strongly suggests that the Glu cluster 101-109 of CaMKPase, which is located in the N-terminal domain, is essential for the stimulation by poly(Lys) (Fig. 6B). Since the mutant could not bind to poly(Lys) in the presence of Mn^{2+}, as shown by the SPR analysis (Fig. 3C), the inability of poly(Lys) to stimulate CaMKP (Δ101-109) mutant seems to be ascribed to the inability of the mutant to bind poly(Lys) due to lack of the Glu cluster corresponding to the residues 101-109. Therefore, the Glu cluster is a binding site for polycations, and is responsible for polycation-stimulated activity of CaMKPase. This means

that one of the functions of the N-terminal domain of CaMKPase is regulation of the phosphatase activity. The reason why CaMKP (Δ101-109) mutant could bind to poly(Lys) in the absence of Mn^{2+} is now unclear. Since CaMKPase requires Mn^{2+} for activity, it seems likely that CaMKPase changes its conformation from an inactive form to an active form when Mn^{2+} is added. This conformational change may play an important role for the interaction of CaMKPase with poly(Lys). Since we could not obtain a soluble form of the N-terminal truncation mutants, CaMKP (51-450), CaMKP (96-450), and CaMKP (109-450), the N-terminal domain might also contribute to proper folding or stabilization of the higher-order structure of the enzyme. Tan et al. suggest that the N-terminal domain of hFEM-2, which is identical to human CaMKPase, has a negative regulatory function in inducing apoptosis [10]. On the other hand, the C-terminal region of CaMKPase, the residues 413-450, is not essential for the stimulation, because CaMKP (1-412) mutant was markedly stimulated by poly(Lys) as well as wild type CaMKPase (Fig. 6B).

Glu clusters are also found in various proteins [28], but the functions of the regions remain to be elucidated. CaMKP-N also possesses a Glu-rich sequence in its N-terminal region (90-102), though several neutral amino acid residues are included in the sequence [11]. Since CaMKP-N also shows polycation-dependent stimulation of the phosphatase activity [11], the Glu-rich sequence may be responsible for the polycation-dependence. Interestingly, rat CaMKIV also contains a Glu cluster in its C-terminal region (403-413) [29]. Since CaMKIV (T196D) tightly bound to poly(Lys) to form a complex with CaMKPase, the region might be also important for the binding to poly(Lys).

In our previous paper, we reported that poly(Lys) stimulates CaMKPase mainly through

an increase in the Vmax values using various phosphopeptide substrates [16]. In the present paper, we demonstrated that poly(Lys) tightly interacted with both CaMKPase and its substrate protein, leading to the stimulation of the phosphatase activity. Thus, poly(Lys) stimulates CaMKPase in two different mechanisms; one is the direct stimulation of the catalytic activity, and the other is the stimulation by improving availability of the substrate proteins through the formation of a ternary complex composed of the substrate, poly(Lys), and the enzyme. Low magnitude of the stimulation for the phosphopeptide substrates is likely to be due to lack of an interaction between the peptide and poly(Lys), because Km values for them are not significantly altered in the presence of poly(Lys) compared to those in its absence [16]. As to phosphoprotein substrates for CaMKPase, binding affinity of the protein to polycations such as poly(Lys) may be a critical determinant for the substrate activity in addition to the higher-order structure and the primary structure around the dephosphorylation site of the proteins.

Physiological activators that substitute for poly(Lys) remain to be identified. Since CaMKP(Δ101-109) mutant is poly(Lys)-insensitive as described above, it may be a useful tool for exploring a physiological role of the stimulation by polycations in intact cells. It is hypothesized that polycations such as poly(Lys) and protamine may be mimicking the actions of some as yet undetermined intracellular factors for PP2A [12, 14, 15]. Besides protein phosphatases, some protein kinases show polycation-stimulated protein kinase activity [21, 27, 30-35]. As to casein kinase II, it has been suggested that polycations mimic *in vitro* the action of some native components on the kinase *in vivo* [27, 36]. Moreno et al. presented an alternative hypothesis that polycations might build *in vitro* a particular structure, which is

able to simulate the structure in which the enzyme works *in vivo* [27]. We are presently attempting to identify binding proteins for CaMKPase to address the question what the physiological activator for CaMKPase is.

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Footnotes (for the title page)

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[2]Abbreviations used: BSA, bovine serum albumin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKPase, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; IPTG, isopropyl-β-D-thiogalactopyranoside; poly(Arg), poly-L-arginine; poly(D-Glu, D-Lys), poly-D-glutamic acid/lysine (6:4); poly(DL-Lys), poly-DL-lysine; poly(Glu, Lys, Tyr), poly-L-glutamic acid/lysine/tyrosine (6:3:1); poly(Lys), poly-L-lysine; poly(Lys, Ser), poly-L-lysine/serine (3:1); poly(Lys, Tyr), poly-L-lysine/tyrosine (4:1); PP, protein phosphatase; SPR, surface plasmon resonance.

Table 1

Effect of various polycations on CaMKPase activity

polycation	molecular weight	concentration (μg/ml)	stimulation (fold)
poly(Lys)	23,000*	5	36.1
poly(DL-Lys)	23,000*	5	44.5
poly(Lys, Ser)	31,000*	5	59.0
poly(Lys, Tyr)	24,000*	5	22.6
poly(Glu, Lys, Tyr)	23,000*	5	0.94
poly(D-Glu, D-Lys)	23,000*	5	1.1
poly(Arg)	40,000*	2	57.1
protamine	4,200-4,400	20	7.2
histone	21,500	20	2.5
myelin basic protein	18,300	20	1.0
polybrene	**	20	10.1

Notes: The activity of CaMKPase was measured in the presence of the indicated concentrations of the indicated polycations in place of poly(Lys) using 20 nM [^{32}P]CaMKIV(K71R) as a substrate. The results are expressed as the ratio of the activity in the presence of the indicated polycations to that in its absence. The data shown are the mean of two independent experiments. *Average molecular weight. ** Molecular weight is not determined.

Figure legends

Fig. 1. (A) Effect of varying the concentration of poly(Lys) on CaMKPase activity. The activity of CaMKPase was measured using 20 nM [^{32}P]CaMKIV(K71R) as a substrate in the presence of the indicated concentrations of poly(Lys)(23K) (■), poly(Lys)(87K) (●), and poly(Lys)(128K) (○). The results are expressed as the ratio of activity in the presence of poly(Lys) to that in its absence. (B) Effect of varying the molecular weight of poly(Lys) on CaMKPase activity. The activity of CaMKPase was measured using 20 nM [^{32}P]CaMKIV(K71R) as a substrate in the presence of 5 μg/ml of poly(Lys) with the indicated molecular weight. Poly(Lys)s used for this experiment were as follows: poly(Lys)(128K), poly(Lys)(87K), poly(Lys)(23K), poly(Lys)(9.6K), $(Lys)_{20}$, $(Lys)_{10}$, and $(Lys)_5$. The molecular weights of poly(Lys) except for $(Lys)_{20}$, $(Lys)_{10}$, and $(Lys)_5$ are average molecular weights. The results are expressed as the ratio of activity in the presence of poly(Lys) to that in its absence. Data are mean ± SD values from three independent determinations.

Fig. 2. (A) Effect of NaCl concentrations on CaMKPase activity in the presence of poly(Lys). The activity of CaMKPase was measured in the presence of 5 μg/ml of poly(Lys) and the indicated concentrations of NaCl using 20 nM [^{32}P]CaMKIV(K71R) as a substrate. (B) Effect of NaCl concentrations on CaMKPase activity in the absence of poly(Lys). The activity of CaMKPase was measured in the absence of poly(Lys) and in the presence of the indicated concentrations of NaCl using 20 nM [^{32}P]CaMKIV(K71R) as a

substrate. Data are mean ± SD values from three independent determinations.

Fig. 3. Analysis of interactions with poly(Lys) by SPR. (A) Interactions of His_6-tagged wild type CaMKPase with poly(Lys) in the presence of $MnCl_2$ were analyzed by SPR as described under "Materials and methods." The concentrations of the injected CaMKPase were as follows: a, 534 nM; b, 267 nM; c, 134 nM; d, 80 nM. Dotted line shows the sensorgram obtained for 267 nM BSA. (B) Interactions of CaMKIV(T196D) with poly(Lys) in the presence of $MnCl_2$ were analyzed by SPR. The concentrations of the injected CaMKIV(T196D) were as follows: a, 267 nM; b, 134 nM; c, 80 nM; d, 50 nM; e, 30 nM. (C) Interactions of His_6-tagged CaMKP (Δ101-109) mutant with poly(Lys) in the presence of $MnCl_2$ were analyzed by SPR. The concentrations of the injected mutant CaMKPase were as follows: a, 1495 nM; b, 800 nM; c, 580 nM. (D) Interactions of His_6-tagged CaMKP (1-412) mutant with poly(Lys) in the presence of $MnCl_2$ were analyzed by SPR. The concentrations of the injected mutant CaMKPase were as follows: a, 587 nM; b, 249 nM; c, 147 nM; d, 88 nM. The data presented are representative of several identical experiments with similar results.

Fig. 4. Binding of CaMKIV(T196D) to CaMKPase via poly(Lys). Binding of CaMKIV(T196D) to Ni-NTA-agarose resin, which was treated with the indicated additions and omissions, was analyzed by pull-down binding assay as described under "Materials and methods." "+K10" means that $(Lys)_{10}$ was used in place of poly(Lys). "NaCl" means that binding buffer containing 500mM NaCl was used throughout the procedure.

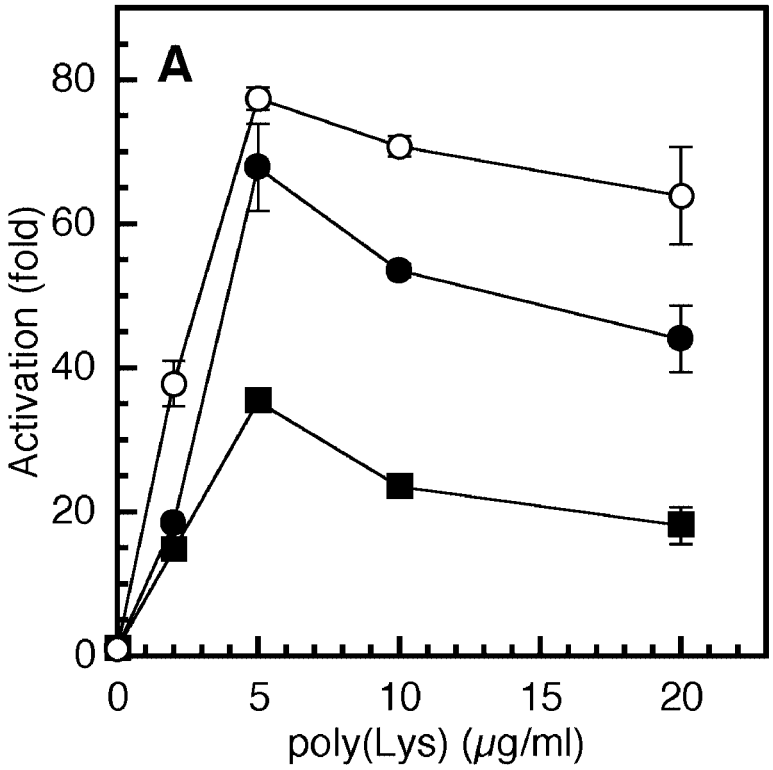
CaMKIV(T196D) bound to the resin was detected by Western blotting analysis using CaMKIV-specific antibody. Upper panel shows the Western blot developed by an enhanced chemiluminescence procedure, and the lower panel shows quantification of the corresponding chemiluminescence signal of the blot. The data presented are representative of three independent experiments with similar results.

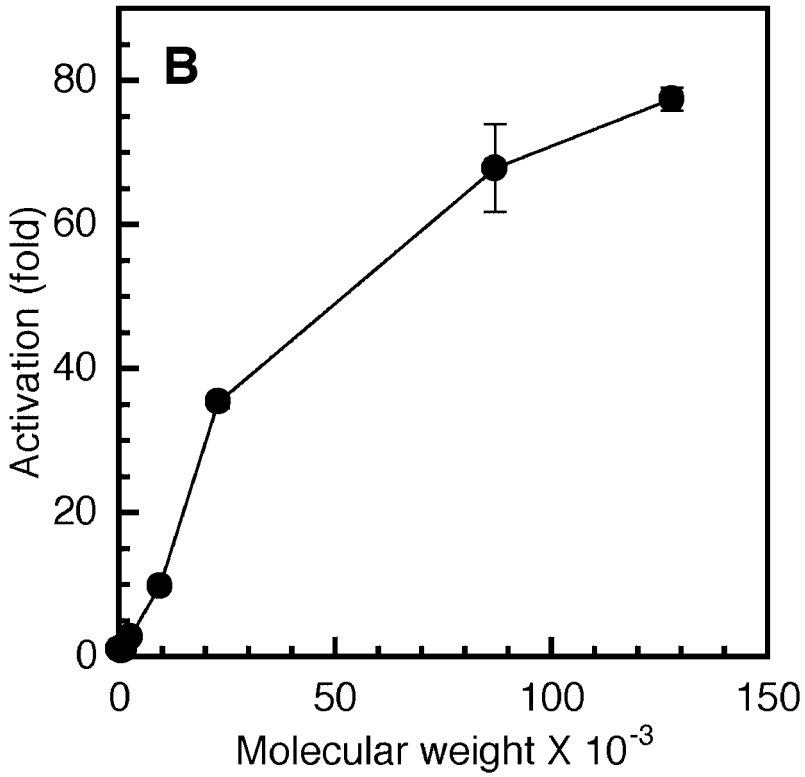
Fig. 5. Effect of dilution of CaMKPase-poly(Lys)-[^{32}P]CaMKIV(K71R) on CaMKPase activity. (A) CaMKPase (10 ng/ml) was incubated at 30 ˚C for 2 min with [^{32}P]CaMKIV(K71R) (20 nM) and poly(Lys) (10 μg/ml) in 50 mM Tris-HCl (pH7.5) containing 0.1 mM EGTA and 0.01% Tween 20, followed by dilution to the indicated concentrations of [^{32}P]CaMKIV(K71R) with the same buffer containing 2 mM $MnCl_2$. Immediately after the dilution, the phosphatase reaction was started, and CaMKPase activity was determined as described under “Materials and methods.” (B) The data in A were replotted as the logarithm of the initial velocity (pmol/min/ml) versus the logarithm of the enzyme concentration (ng/ml). Data are mean ± SD values from three independent determinations.

Fig. 6. Stimulation of mutant CaMKPases by poly(Lys). (A) Schematic diagram of deletion mutants of CaMKPase. Domain structures of rat PP2Cα and CaMKPase are shown. Phosphatase domains of PP2Cα and CaMKPase, which show significant sequence homology (28% identity), were aligned (hatched box) with the PP2C motifs located within these regions being indicated. A Glu cluster located within the N-terminal domain of CaMKPase (101-

109, closed box) and a large portion of its C-terminal domain (412-450, closed box) were deleted to generate CaMKP(Δ101-109) mutant and CaMKP(1-412) mutant, respectively. (B) Effect of poly(Lys) on CaMKPase activity of the mutants. His_6-tagged wild type CaMKPase, CaMKP(1-412) mutant, and CaMKP(Δ101-109) mutant were prepared as described under "Materials and methods." The CaMKPase activities were determined in the presence (closed bars) and absence (open bars) of 10 μg/ml poly(Lys) using 7.5 nM [^{32}P]CaMKIV(K71R) as a substrate. Data are mean ± SD values from three independent determinations.

A. Ishida et al. Fig. 1





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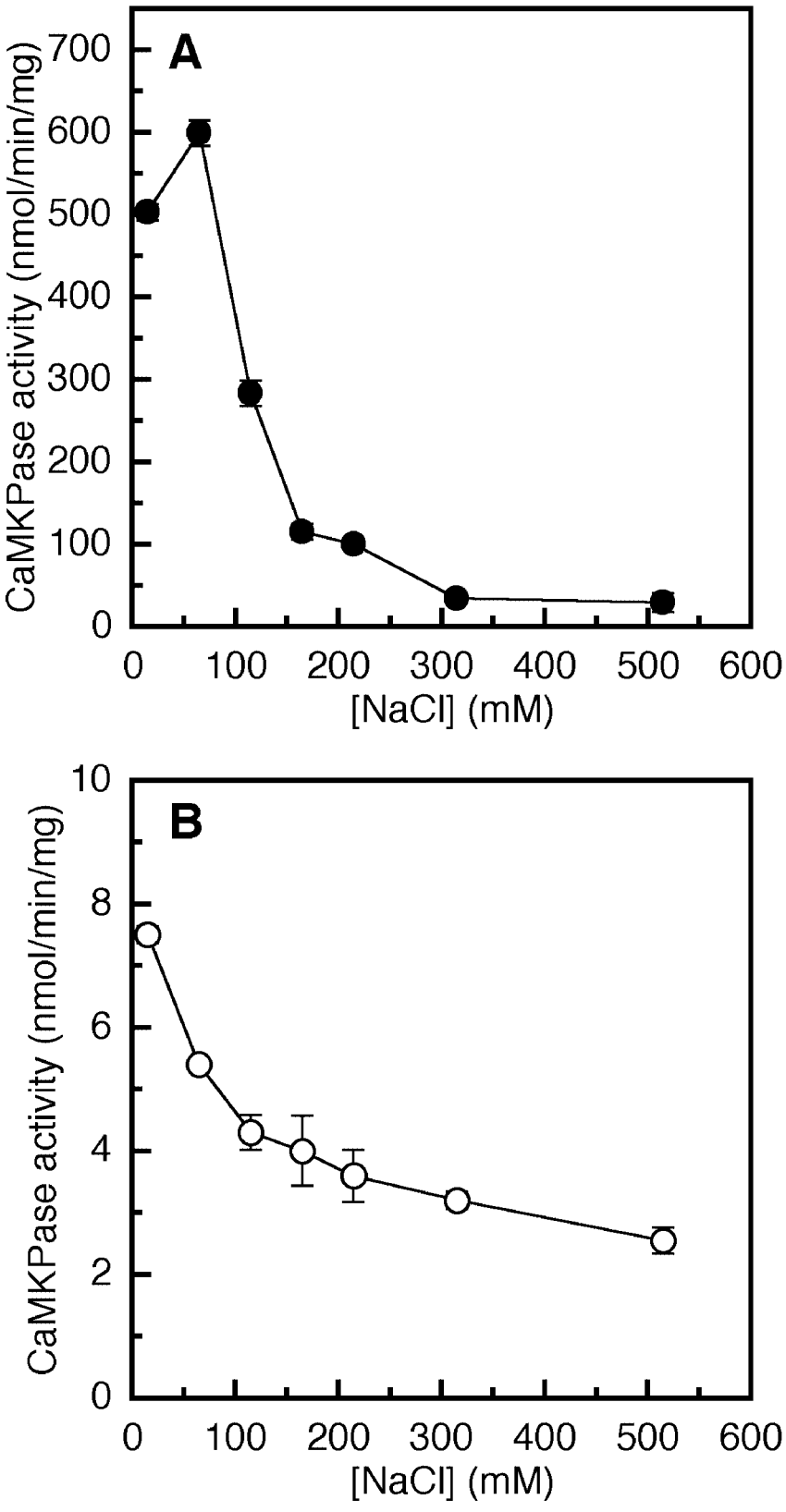

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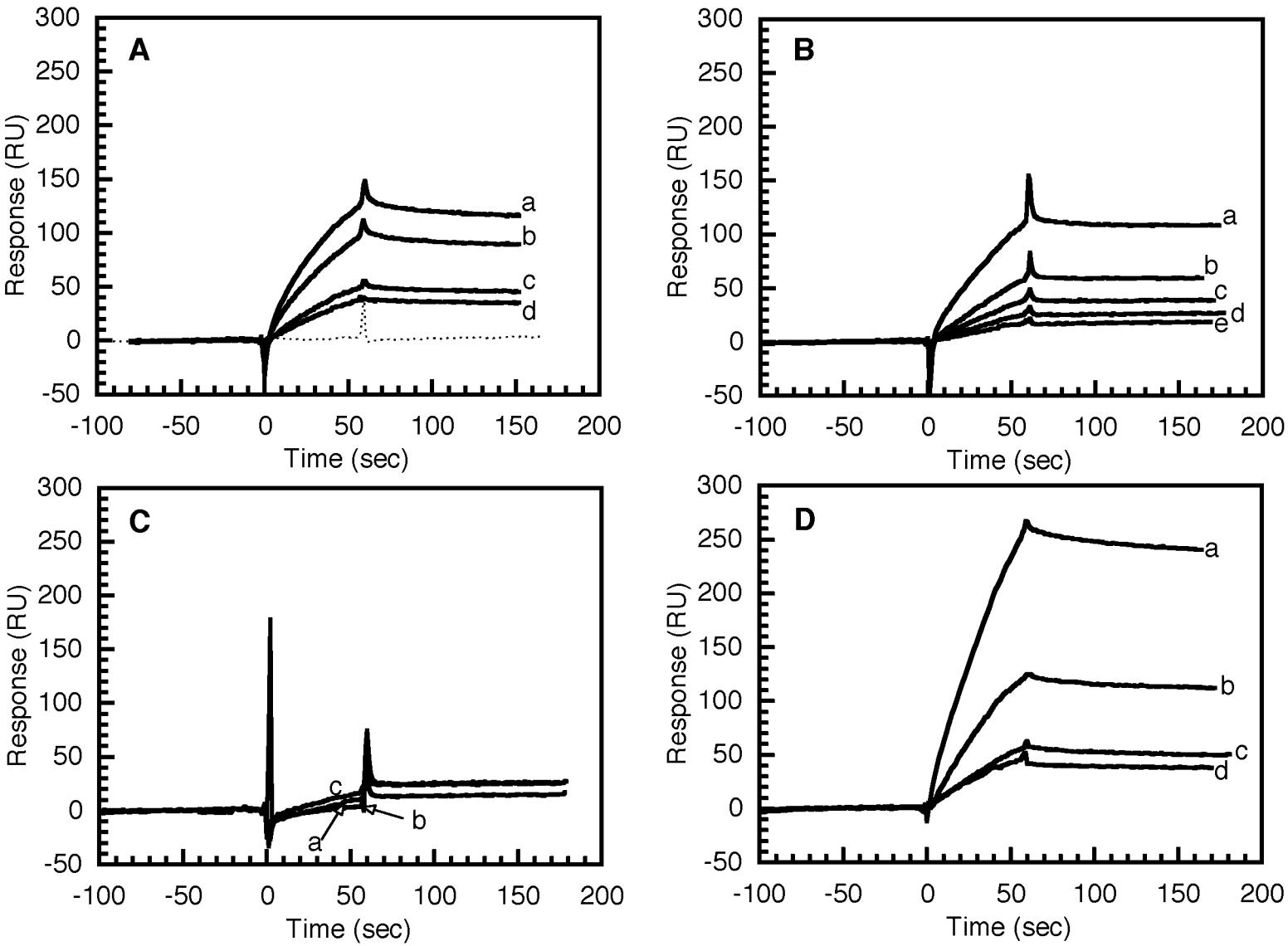

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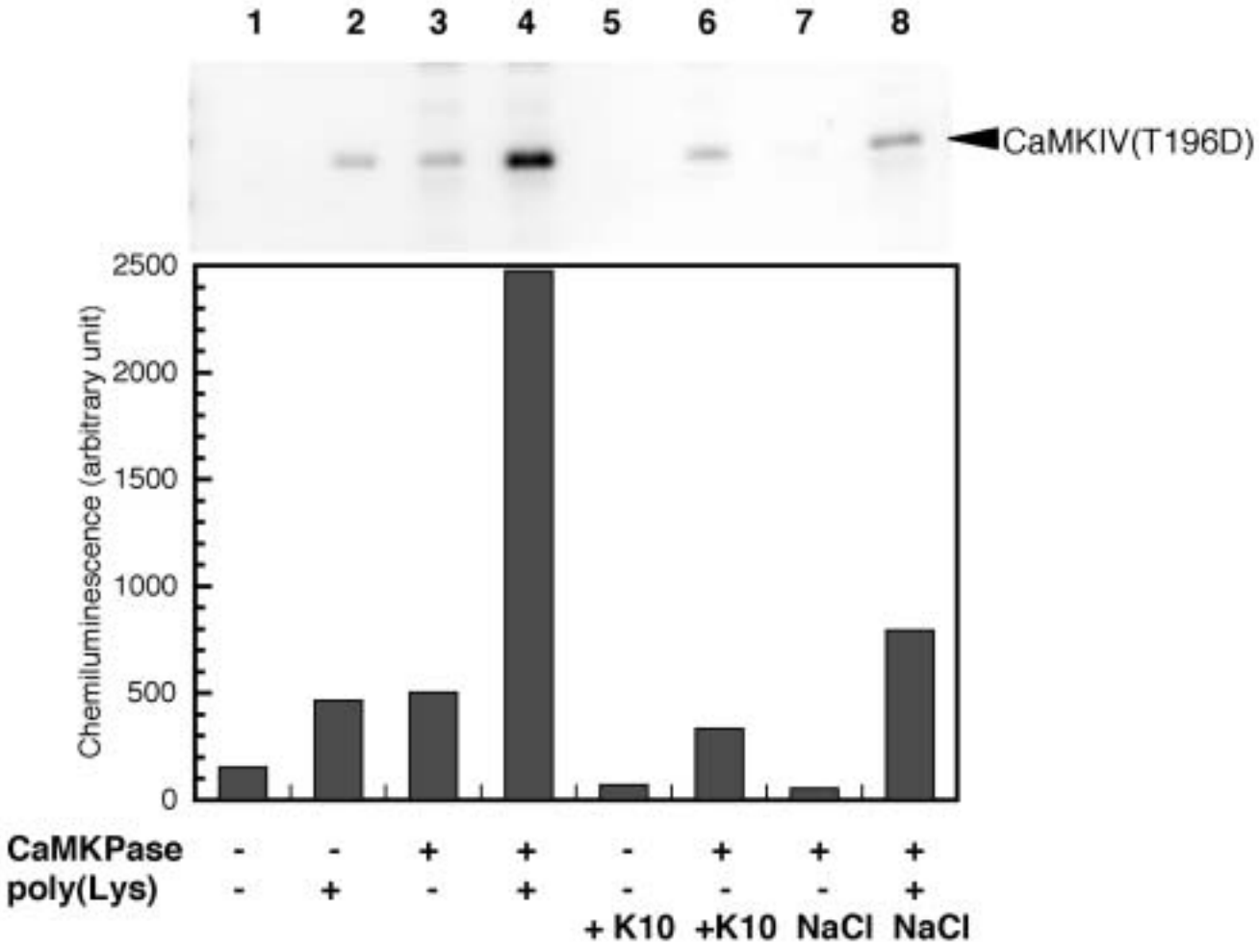

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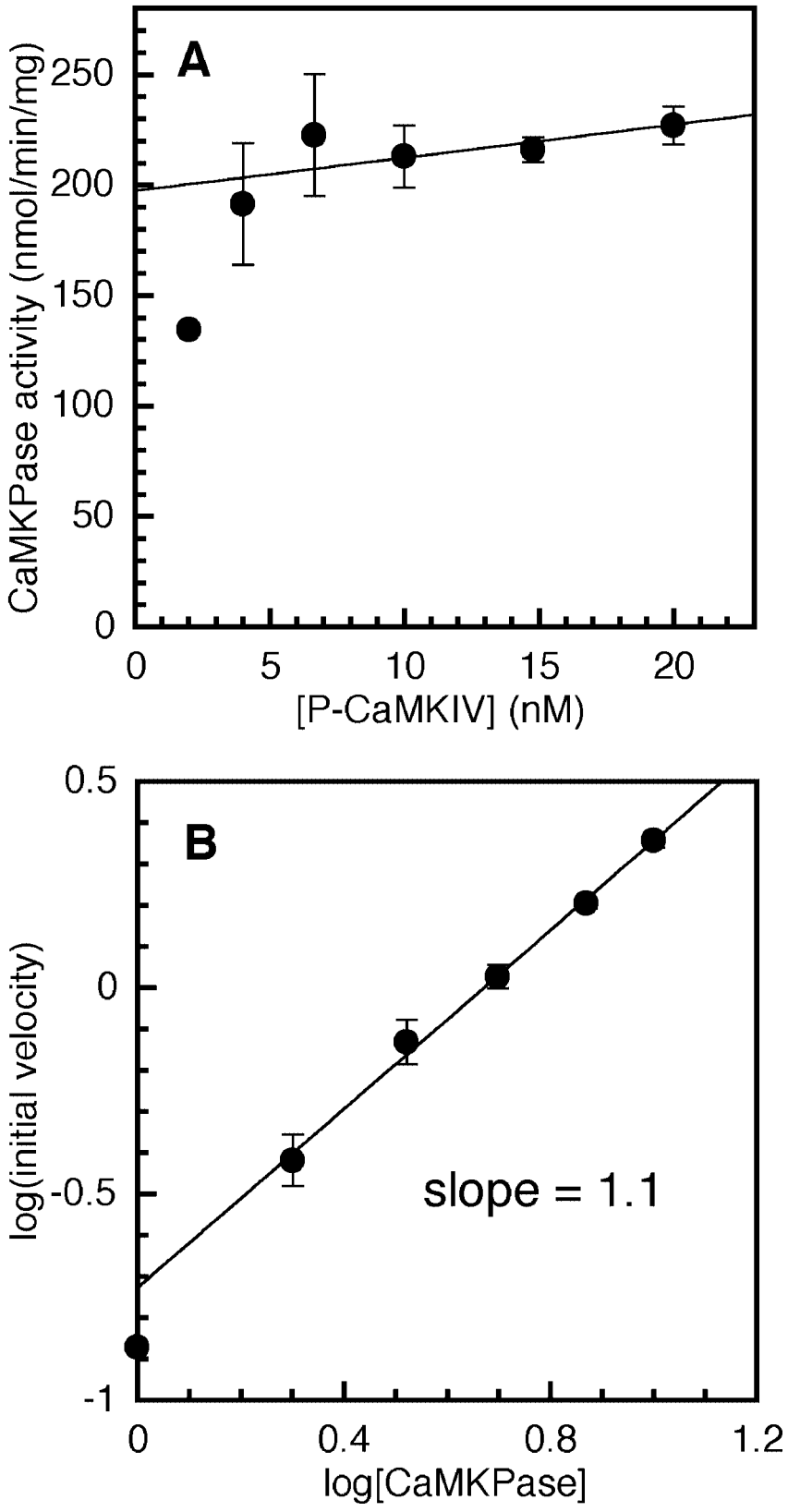
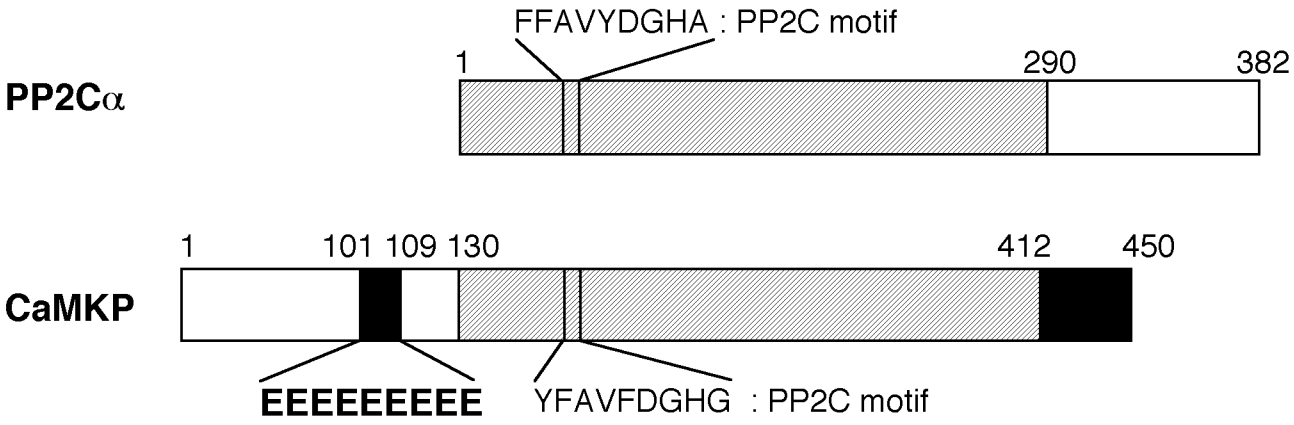


A. Ishida et al. Fig. 6

A



B