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Substrate Specificity of Ca^{2+}/Calmodulin-Dependent Protein Kinase Phosphatase: Kinetic Studies Using Synthetic Phosphopeptides as Model Substrates[1]

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Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase) dephosphorylates and regulates multifunctional Ca^{2+}/calmodulin-dependent protein kinases. In order to elucidate the mechanism of substrate recognition by CaMKPase, we chemically synthesized a variety of phosphopeptide analogs and carried out kinetic analysis using them as CaMKPase substrates. This is the first report using systematically synthesized phosphopeptides as substrates for kinetic studies on substrate specificities of protein Ser/Thr phosphatases. CaMKPase was shown to be a protein Ser/Thr phosphatase having a strong preference for a phospho-Thr residue. A Pro residue adjacent to the dephosphorylation site on the C-terminal side and acidic clusters around the dephosphorylation site had detrimental effects on dephosphorylation by CaMKPase. Deletion analysis of a model substrate peptide revealed that the minimal length of the substrate peptide was only 2 to 3 amino acid residues including the dephosphorylation site. The residues on the C-terminal side of the dephosphorylation site were not essential for dephosphorylation, whereas the residue adjacent to the dephosphorylation site on the N-terminal side was essential. Ala-scanning analysis suggested that CaMKPase did not recognize a specific motif around the dephosphorylation site. Myosin light chain phosphorylated by protein kinase C and Erk2 phosphorylated by MEK1 were poor substrates for CaMKPase, while a synthetic phosphopeptide corresponding to the sequence around the phosphorylation site of the former was not dephosphorylated by CaMKPase but that of the latter was fairly good substrate. These data suggest that substrate specificity of CaMKPase is determined by higher-order structure of the substrate protein rather than by the primary structure around its dephosphorylation site. Use of phosphopeptide substrates also revealed that poly-L-lysine, an activator for CaMKPase, activated the enzyme mainly through increase in the V_{max} values.

Key words: CaM-kinase, kinetic analysis, protein phosphatase, substrate specificity, synthetic phosphopeptide.

Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II is a Ca^{2+}-responsive multifunctional protein kinase occurring abundantly in the brain (*1*) and is thought to be involved in a variety of neuronal functions mediated by Ca^{2+} (*2*, *3*). Since CaMKII is markedly activated by autophosphorylation at Thr^{286}, protein phosphatases that dephosphorylate this residue are important for regulation of CaMKII activity. With a novel in-gel protein phosphatase assay (*4*), we detected three distinct protein phosphatases capable of dephosphorylating the phosphopeptide corresponding to the sequence around Thr^{286} of CaMKII in the rat brain extract, and purified one of the three phosphatases (*5*). The purified phosphatase, designated as Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase), is a calyculin A–insensitive, Mn^{2+}-dependent, and poly-L-lysine [poly(Lys)]–stimulated protein phosphatase that dephosphorylates and regulates not only autophosphorylated CaMKII but also CaMKI and CaMKIV, which are phosphorylated and activated by the upstream kinase, CaMK kinase (*5*, *6*). 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% homology with rat protein phosphatase (PP) 2Cα (*7*). Since CaMKPase does not significantly dephosphorylate phosphorylase kinase, mixed his-

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Abbreviations: CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKPase, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; MAP kinase, mitogen-activated protein kinase; MLC, myosin light chain; PKC, protein kinase C; poly(Lys), poly-L-lysine; PP, protein phosphatase; PTPase, protein Tyr phosphatase.

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tones, myelin basic protein, and α-casein (which are phosphorylated by cAMP-dependent protein kinase), phosphorylase *a* (phosphorylated by phosphorylase kinase), and autophosphorylated CaMK kinase α (*5*, *6*), the substrate specificity of the phosphatase is much higher than those of the four well-known major classes of Ser/Thr protein phosphatases, PP1, PP2A, PP2B, and PP2C (*8*, *9*). Therefore, it is interesting to clarify the mechanism of substrate recognition by CaMKPase.

The use of phosphopeptide substrates for elucidation of substrate specificity of protein phosphatases is a useful approach, especially for protein Tyr phosphatases (PTPases) (*10*). Unlike phosphotyrosyl peptides used as PTPase substrates, however, it was difficult until recently to chemically synthesize phosphopeptides containing phospho-Ser/Thr used as substrates for protein Ser/Thr phosphatases. Recent development of a simple protocol for the synthesis of peptides containing phospho-Ser/Thr (*11*, *12*) enabled us to prepare a variety of phosphopeptide substrates for protein Ser/Thr phosphatases in sufficient quantities and purities for kinetic analysis. In this study, we carried out kinetic analysis of CaMKPase using a variety of phosphopeptide substrates with systematically designed sequences to clarify the determinant for the substrate specificity of CaMKPase in the amino acid sequence surrounding the dephosphorylation site of the substrate.

MATERIALS AND METHODS

Materials—Malachite green hydrochloride, ATP, 1,2-dioleoyl-rac-glycerol, and poly(Lys) (average molecular weight 87,000) were purchased from Sigma. [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham International. *N*-α-Fmoc-*O*-benzyl-L-phosphothreonine, *N*-α-Fmoc-*O*-benzyl-L-phosphoserine, and *N*-α-Fmoc-*O*-benzyl-L-phosphotyrosine were purchased from Novabiochem. Other Fmoc-L-amino acids used for the peptide synthesis were obtained from Shimadzu.

Protein Preparations—Recombinant mouse p42 mitogen–activated protein kinase (MAP kinase, Erk2)-GST (inactive) and recombinant human MEK1 (active) were purchased from Upstate Biotechnology. Myosin light chain (MLC) was prepared from chicken gizzard myosin by the method of Perrie and Perry (*13*). Chicken gizzard myosin was prepared by the method of Ebashi (*14*). Protein kinase C (PKC) was purified from rat cerebral cortex essentially according to Woodgett and Hunter (*15*). Recombinant rat CaMK kinase α expressed in *Escherichia coli* (*16*) was purified as described previously (*17*). Recombinant rat CaMKPase was purified from the crude extract of *E. coli* transformed with expression vector pET11a carrying cDNA encoding rat brain CaMKPase (*7*) by the following procedures. Rat CaMKPase cDNA was expressed in *E. coli*, and the crude extract was prepared as described previously (*7*). To the crude extract of the bacteria was added 1/25 volume of 5% streptomycin sulfate solution. After standing for 15 min on ice, the mixture was centrifuged at 20,000 ×*g* for 10 min. The supernatant was subjected to the following purification procedures at 4°C. The supernatant was applied to a DEAE cellulose column (Whatman DE52), which had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 10 mM EDTA, and 10 mM EGTA. A DEAE cellulose column chromatography and a subsequent TSKgel phenyl-5PW HPLC (Tosoh) were carried out as described previously (*5*). CaMKPase fractions eluted from the HPLC column were pooled and dialyzed overnight against 40 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Tween 40, 5% ethylene glycol, 0.5 mM DTT, and 50 mM NaCl with two changes of buffer. The purified enzyme was frozen in aliquots at −80°C. The enzyme was about 90% pure as judged by SDS-PAGE analysis. Recombinant rat CaMKIV-(K71R) mutant expressed in *E. coli* was prepared as follows. A cDNA for CaMKIV(K71R) was prepared essentially according to the method of Kunkel *et al.* (*18*) as described previously (*17*). A cDNA fragment containing the entire coding sequence of the mutant CaMKIV(K71R) was introduced into an expression vector pET11a. The construct was transformed into *E. coli* strain BL21(DE3). Cultivation of the bacteria, induction of the recombinant protein by isopropyl-β-D-thiogalactoside, and preparation of the crude extract were carried out as described previously (*17*). Partial purification of the CaMKIV(K71R) protein was carried out at 4°C as follows. Streptomycin treatment was carried out as described above. Solid ammonium sulfate was added to the supernatant to give 50% saturation, and the mixture was allowed to stand for 30 min while being stirred. The precipitate, collected by centrifugation at 20,000 ×*g* for 15 min, was dissolved in buffer A [consisting of 40 mM Tris-HCl (pH 7.5), 10% ethylene glycol, 0.05% Tween 40, 0.2 mM $CaCl_2$, 1 mM DTT] containing 50 mM NaCl and 10 μg/ml each of pepstatin, leupeptin, antipain, and chymostatin. The supernatant obtained by centrifugation at 20,000 ×*g* for 10 min was mixed with about 1.7× volume of 50% (v/v) suspension of calmodulin-Sepharose resin, which had been prepared as described (*19*) and equilibrated with the same buffer. The suspension was gently shaken overnight, then poured into a glass column and washed with the same buffer. The column was extensively washed with buffer A containing 2 M NaCl, then with buffer A without ethylene glycol containing 50 mM NaCl. The mutant protein was eluted with 40 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 0.05% Tween 40, 1 mM DTT, and 1 mM EGTA. The eluted fractions were combined and concentrated by ammonium sulfate precipitation (50% saturation), followed by dialysis against 40 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 0.05% Tween 40, and 1 mM DTT. The dialysate was made 10% (v/v) in ethylene glycol and stored at −80°C. Recombinant rat CaMKIV(K71R/T196S) mutant expressed in *E. coli*, in which Lys^{71} and Thr^{196} were replaced with Arg and Ser, respectively, was prepared as follows. A cDNA for CaMKIV(T196S), which was introduced into an expression vector, pET11a, was prepared as previously described (*17*). This construct was digested with *Eag*I to generate a 5,145 bp *Eag*I–*Eag*I fragment. A cDNA for CaMKIV(K71R), which was introduced into pET11a as described above, was digested with *Eag*I to generate a 2,305 bp *Eag*I–*Eag*I fragment. The two fragments were ligated, and the constructs were transformed into *E. coli* strain BL21(DE3). The nucleotide sequences of several clones were determined by the dideoxynucleotide chain termination method (*20*) using a DNA sequencer, LI-COR model 4000L to confirm direction of the ligation. A clone possessing the proper sequence was selected. The bacteria were grown, and the mutant protein was induced by isopropyl-β-D-thiogalactoside as described previously (*17*). Preparation of the crude extract and par-

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tial purification of CaMKIV(K71R/T196S) were carried out by the procedures described above for CaMKIV(K71R) expressed in *E. coli.*

Preparation of Protein Phosphatase Substrates—Phosphopeptides were synthesized by use of a Shimadzu PSSM-8 automated peptide synthesizer according to the method of Wakamiya *et al.* (*11*, *12*). All these peptides were purified by reversed-phase HPLC, and their molecular masses were confirmed by mass spectrometry on a time-of-flight mass spectrometer (Shimadzu/Kratos Kompact MALDI II) with matrix-assisted laser desorption ionization. The concentrations of the phosphopeptides containing phospho-Ser or phospho-Thr were determined by measuring P_i release by alkali hydrolysis, which was carried out at 50°C in 3 M NaOH for 24 h (*21*). After neutralization of the mixture with HCl, the P_i released from the peptides was determined using the malachite green assay as described below. The concentrations of phosphopeptides containing phospho-Tyr were determined by measuring P_i release by acid hydrolysis (*22*) using the malachite green assay. These values were in good agreement with those obtained by amino acid analysis. Recombinant rat CaMKIV(K71R) (127 μg/ml) and CaMKIV(K71R/T196S) (187 μg/ml) were phosphorylated by CaMK kinase α (0.24 μg/ml) at 30°C for 20 min in a reaction mixture comprising 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 5 μM calmodulin, 0.4 mM $CaCl_2$, 2 mM DTT, and 50 μM [γ-^{32}P]ATP. MLC (200 μg/ml) was phosphorylated by PKC (1.03 μg/ml) at 30°C for 20 min in a reaction mixture consisting of 30 mM Tris-HCl (pH 7.5), 30 mM KCl, 1 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP, 2.02 μg/ml 1,2-dioleoyl-rac-glycerol, 150 μg/ml phosphatidylserine, and 0.5 mM $CaCl_2$ (*23*). MAP kinase-GST (inactive, 20 μg/ml) was phosphorylated by active MEK1 (78 ng/ml) at 30°C for 20 min in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT, and 50 μM [γ-^{32}P]ATP. After the reactions had been terminated with excess EDTA (12.3 mM) and bovine serum albumin (1 mg/ml), the phosphoproteins were desalted on a Sephadex G-50 spin column as described previously (*5*) except that the equilibration buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 0.1 mM EGTA, and 0.01% Tween 20 was used. The concentrations of the phosphoprotein substrates presented in the text represent the concentrations of ^{32}P bound to the substrate proteins.

Protein Phosphatase Assay—Dephosphorylation of a phosphopeptide substrate was carried out at 30°C for 6 min in a reaction mixture (50 μl) containing an appropriate amount of CaMKPase, 50 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 10 μg/ml poly(Lys), and various concentrations of a phosphopeptide substrate. The reaction was started by adding the enzyme, and terminated by adding 12.5 μl of malachite green solution prepared by the method of Baykov *et al.* (*24*) . The mixture was allowed to stand at 25°C for 10 min, then 7 μl of 34% (w/v) sodium citrate·$2H_2O$ was added (*25*), followed by vortexing. The mixture was allowed to stand at 25°C for a further 30 min, then the absorbance at 620 nm was measured. Values for P_i release were calculated by comparison with the standard curve obtained by use of phosphoric acid that was treated as above. CaMKPase assays were carried out with six different concentrations of the phosphopeptide substrate so that the K_m value might be within this concentration range. Michaelis-Menten kinetic parameters were determined from a direct fit of the *v versus* [S] data to the Michaelis-Menten equation (Eq. 1) using a nonlinear regression program (DeltaGraph PRO3, DeltaPoint).

$$v = \frac{V_{max}\,[S]}{K_m + [S]} \qquad \text{(Eq. 1)}$$

The data presented are the averages of two to three independent determinations with similar results. Dephosphorylation of a phosphoprotein substrate was carried out at 30°C for 1 min in a reaction mixture containing an appropriate amount of CaMKPase, 50 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 10 μg/ml poly(Lys), and 7.35 nM phosphoprotein substrate. The reaction was started by adding CaMKPase, and terminated by adding 22.7 mM EDTA, followed by mixing with an equal volume 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 was boiled for 2 min, centrifuged for 2 min at room temperature in a microcentrifuge at maximum speed, and an aliquot of the supernatant was subjected to SDS-PAGE on a 10% acrylamide gel. The gel was dried and visualized by autoradiography.

Other Analytical Procedures—SDS-PAGE was carried out according to Laemmli (*26*). Protein concentrations were determined by the method of Lowry *et al.* (*27*), as modified by Peterson (*28*), with bovine serum albumin as a standard. Specific activity of CaMKPase was calculated on the assumption that the CaMKPase preparation used in this study was of 90% purity as judged by SDS-PAGE analysis.

RESULTS

Deletion Analysis of a Model Substrate Peptide Corresponding to the Sequence around the Autophosphorylation Site of CaMKII—We carried out kinetic analysis of CaMKPase using chemically synthesized phosphopeptides as substrates and a sensitive assay of inorganic phosphate by a malachite green procedure (*24*, *25*). CaMKPase effectively dephosphorylates ^{32}P-labeled CaMKII(281-289) peptide conjugated to paramagnetic particles and autophosphorylated CaMKII in the presence of poly(Lys) (*5*). Therefore, we chose phospho-CaMKII(281-289) peptide (termed as pp2) as a model substrate for CaMKPase and examined the effect of amino acid sequence around the dephosphorylation site of the substrate on the CaMKPase reaction. Figure 1 shows dephosphorylation of pp2 by CaMKPase. From a direct fit of the reaction rate *versus* the pp2 concentration as described under "MATERIALS AND METHODS," the K_m and V_{max} values were calculated to be 16 ± 0.4 μM and 2.22 ± 0.13 μmol/min/mg, respectively (n = 3). Kinetic parameters for other phosphopeptides were similarly determined. At first, we examined effect of the deletion of the residues on the N- and C-terminal sides of the dephosphorylation site on the kinetic parameters. To simplify the discussion, the phospho-Thr residue to be dephosphorylated is numbered zero in the amino acid sequence, and the adjacent amino acid residues on the N- and C-terminal sides are numbered −1 and +1, respectively. Kinetic parameters for the pp2 analogs which were truncated at the residues on the C-terminal side (pp32, pp43, and pp36) or the N-terminal side (pp42, pp41, pp40, pp39, and pp37) of the dephosphoryla-

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tion site of pp2 are listed in Table I. When the residues on the C-terminal side were completely removed, the analog (pp36) was still active as a CaMKPase substrate but showed a decrease of about 4-fold in the V_{max}/K_m value, which is an index of substrate specificity, compared to pp2. Thus, the residues on the C-terminal side of the dephosphorylation site were favorable, but not essential for substrate activity. In contrast to the residues on the C-terminal side, sequential deletion of the residues on the N-terminal side of the dephosphorylation site did not result in the decrease in the V_{max}/K_m value, but instead some increase in it was observed (pp39). However, complete deletion of the residues on the N-terminal side caused complete loss of substrate activity (pp37). Therefore, the residues ranging from the −5 position to the −2 position were rather detrimental, but the residue adjacent to the N-terminal side of the dephosphorylation site (the −1 position) was essential for substrate activity.

To determine the minimal length of the phosphopeptide substrate, the residues on the C-terminal side of the dephosphorylation site of pp39 were further deleted (pp46–48). As shown in Table I, complete deletion of the C-terminal residues (pp48) resulted in a large increase in the K_m value without significantly changing the V_{max} value as compared with pp39. This result is in good agreement with that obtained with pp2 and pp36. Phospho-Thr itself did not function as a CaMKPase substrate. Judging from kinetic parameters, the minimal length of a phosphopeptide substrate for CaMKPase was only 2 to 3 amino acid residues containing the phospho-Thr to be dephosphorylated (pp47 or pp48); only one amino acid residue on the N-terminal side of the phospho-Thr was essential and the amino acid residue(s) on the C-terminal side were desirable. Thus, the Glu residue at the −1 position was necessary and sufficient for catalysis, but additional amino acid residue(s) adjacent to the C-terminal side of the dephosphorylation site were required for binding to the enzyme with high affinity.

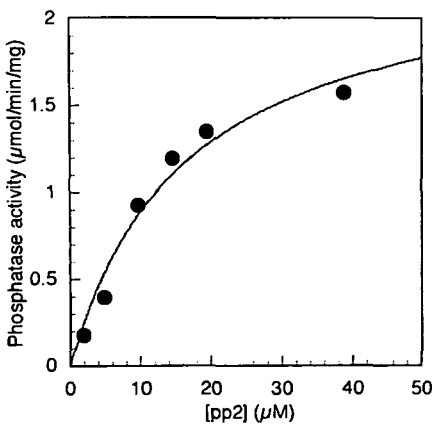

Fig. 1. **Dephosphorylation of a model substrate peptide pp2, which corresponds to the sequence around the autophosphorylation site of CaMKII, by CaMKPase.** The indicated concentrations of pp2 (MHRQET(p)VDC) were dephosphorylated by CaMKPase at 30°C for 6 min under the standard assay conditions as described under "MATERIALS AND METHODS." The reaction was terminated, and the CaMKPase activity was determined from the released P_i as described. The indicated curve was obtained by a direct fit of the data to Michaelis-Menten equation using a nonlinear regression program. The data shown are those in a representative experiment of three independent determinations.

Ala-Scanning Analysis of a Model Substrate Peptide pp2—Another approach to estimate the contribution of each residue to substrate activity is Ala-scanning analysis. The kinetic parameters for the pp2 analogs in which individual amino acid residues were systematically replaced by Ala are summarized in Table II. Although minor variations were observed, the kinetic parameters for these analogs were not significantly different from those for the parent peptide pp2. This suggested that no specific amino acid residues around the dephosphorylation site of the substrate significantly contribute to substrate activity.

Substitution Analysis on the Residue at the −1 Position—Since kinetic analysis with pp39 and pp37 indicated that the Glu residue at the −1 position was essential for substrate activity (Table I), we carried out kinetic analysis using analogs in which the Glu residue was replaced with Ala, Arg, or Asp. As shown in Table III, the Glu residue could be substituted by Ala or Asp without significant loss of substrate activity (pp52 and pp55). In contrast, replacement with Arg caused a significant increase in the K_m value (4.5-fold) without affecting the V_{max} value (pp53). Thus, introduction of a positive charge at the −1 position had detrimental effect on the binding affinity. Judging from the V_{max}/K_m values of the analogs, an acidic residue at the −1 posi-

TABLE I. **Deletion analysis of a model substrate peptide pp2.** Standard CaMKPase assays were carried out using phosphopeptide substrates listed, and the kinetic parameters were calculated as described under "MATERIALS AND METHODS." For extremely poor substrates, phosphatase activities assayed at the indicated concentrations are shown in parentheses. T(p) represents phospho-Thr. ND, not determined.

Peptide	Sequence	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/μM)
pp2	MHRQET(p)VDC	16	2.22	0.14
pp32	MHRQET(p)VD	34	1.97	0.058
pp43	MHRQET(p)V	22	0.90	0.041
pp36	MHRQET(p)-NH_2	50	1.54	0.032
pp42	HRQET(p)VDC	10	1.93	0.19
pp41	RQET(p)VDC	17	2.36	0.14
pp40	QET(p)VDC	18	3.26	0.19
pp39	ET(p)VDC	13	3.94	0.35
pp37	T(p)VDC	ND	(0.03)*	—
pp46	ET(p)VD	37	4.82	0.13
pp47	ET(p)V	37	2.65	0.072
pp48	ET(p)-NH_2	184	3.96	0.022
—	T(p)	ND	(0.04)**	—

*At 515 μM, **at 400 μM.

TABLE II. **Ala-scanning analysis of a model substrate peptide pp2.** Standard CaMKPase assays were carried out using phosphopeptide substrates listed, and the kinetic parameters were calculated as described under "MATERIALS AND METHODS."

Peptide	Sequence	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/μM)
pp2	MHRQET(p)VDC	16	2.22	0.14
pp24	AHRQET(p)VDC	17	2.11	0.13
pp25	MARQET(p)VDC	25	2.68	0.11
pp26	MHAQET(p)VDC	10	1.91	0.19
pp27	MHRAET(p)VDC	15	2.68	0.18
pp28	MHRQAT(p)VDC	17	2.55	0.15
pp29	MHRQET(p)ADC	21	2.49	0.12
pp30	MHRQET(p)VAC	17	3.02	0.18
pp31	MHRQET(p)VDA	21	2.09	0.10

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tion seemed to be favorable for substrate activity (pp39 and pp55). Therefore, a cluster of Glu residues was introduced into the N-terminal side of the dephosphorylation site (pp56). However, such an acidic cluster resulted in a marked decrease in the V_{max} value, without significantly changing the K_m value. An analog in which the −1 Glu residue was replaced with an acetyl group (pp54) was significantly active as a CaMKPase substrate, with the K_m and V_{max} values being about one-third of those of pp39. Thus, the acetyl group could substitute for Glu at the −1 position without significantly reducing the V_{max}/K_m value.

Preference of the Residue at the Dephosphorylation Site— Next, we examined whether or not dephosphorylation by CaMKPase was affected by the replacement of the dephosphorylatable phospho-Thr with phospho-Ser (pp4) or phospho-Tyr (pp6). As shown in Table IV, pp4 showed a markedly reduced V_{max} value compared with the parent pp2, whereas its K_m value was essentially the same as that of pp2, suggesting that the phosphoseryl peptide results in inefficient catalysis without significantly affecting the binding affinity. Another phosphopeptide substrate pp45, which was obtained by replacement of the phospho-Ser residue in the phosphorylated syntide-2 (pp44) with phospho-Thr residue, could be dephosphorylated by CaMKPase, though it was about 4-fold worse than pp2 as judged by its V_{max}/K_m value. However, the phosphorylated syntide-2 (pp44), of which phosphorylation site was Ser residue, could not be significantly dephosphorylated by CaMKPase even at a concentration about 8-fold higher than the K_m value for pp45. Thus, CaMKPase strongly preferred phospho-Thr residues to phospho-Ser residues as the residue to be dephosphorylated. This is also the case for phosphoprotein substrates. As shown previously, CaMKIV phosphorylated by CaMK kinase α at the Thr residue within the activation loop is a good substrate for CaMKPase in the presence of poly(Lys), as are phosphorylated CaMKI and CaMKII (*6*). The mutant CaMKIV in which the Thr residue was replaced with Ser could be phosphorylated by CaMK kinase α, and the phosphorylated mutant kinase [CaMKIV(K71R/T196S)] was used as CaMKPase substrate in the presence of poly(Lys). As shown in Fig. 2, the mutant CaMKIV (lanes 1 and 2) was more resistant to dephosphorylation by CaMKPase compared with the wild-type CaMKIV (lanes 3 and 4), in good agreement with the results obtained with phosphopeptide substrates.

The phosphotyrosyl peptide pp6 was not dephosphorylated by CaMKPase at all, suggesting that CaMKPase did

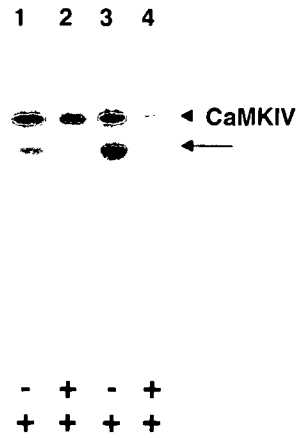

Fig. 2. **Dephosphorylation of CaMKIV(K71R) and CaMKIV(K71R/T196S) by CaMKPase.** CaMKIV(K71R/T196S) (7.35 nM) (lanes 1 and 2) and CaMKIV(K71R) (7.35 nM) (lanes 3 and 4), which had been partially purified from the transformed *E. coli* cells and phosphorylated by CaMK kinase α as described under "MATERIALS AND METHODS," were incubated at 30°C with (lanes 2 and 4) or without (lanes 1 and 3) CaMKPase (0.14 μg/ml) as described. After incubation for 1 min, the reaction was terminated, and aliquots were analyzed by SDS-PAGE followed by autoradiography. The arrowhead indicates the position corresponding to the CaMKIV mutants. Western blotting analysis using CaMKIV-specific antibody (*19*) revealed that the bands shown by the arrow were proteolytic fragments generated from the CaMKIV protein during the purification (data not shown).

TABLE III. **Substitution analysis on the residue at the −1 position.** Standard CaMKPase assays were carried out using phosphopeptide substrates listed, and the kinetic parameters were calculated as described under "MATERIALS AND METHODS." For an extremely poor substrate, phosphatase activity assayed at the indicated concentration is shown in a parenthesis. Ac represents acetyl group. ND, not determined.

Peptide	Sequence	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/μM)
pp37	T(p)VDC	ND	(0.03)*	—
pp39	ET(p)VDC	13	3.94	0.35
pp52	AT(p)VDC	30	7.07	0.25
pp53	RT(p)VDC	58	4.12	0.071
pp55	DT(p)VDC	17	6.54	0.45
pp54	AcT(p)VDC	4.4	1.31	0.30
pp56	EEEEET(p)VDC	7.7	0.40	0.073

*At 515 μM.

TABLE IV. **Preference of the residue at the dephosphorylation site.** Standard CaMKPase assays were carried out using phosphopeptide substrates listed, and the kinetic parameters were calculated as described under "MATERIALS AND METHODS." For extremely poor substrates, phosphatase activities assayed at the indicated concentrations are shown in parentheses. S(p) and Y(p) represent phospho-Ser and phospho-Tyr, respectively. ND, not determined.

Peptide	Sequence	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/μM)
pp2	MHRQET(p)VDC	16	2.22	0.14
pp4	MHRQES(p)VDC	19	0.30	0.016
pp6	MHRQEY(p)VDC	ND	(0.00)**	—
pp45	PLARTLT(p)VAGLPGKK	52	1.93	0.037
pp44	PLARTLS(p)VAGLPGKK	ND	(0.07)*	—

*At 400 μM, **at 200 μM.

TABLE V. **Negative determinants for the dephosphorylation in the amino acid sequence around the dephosphorylation site.** Standard CaMKPase assays were carried out using phosphopeptide substrates listed, and the kinetic parameters were calculated as described under "MATERIALS AND METHODS." For an extremely poor substrate, phosphatase activity assayed at the indicated concentration is shown in a parenthesis. ND, not determined.

Peptide	Sequence	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/μM)
pp38	RRAT(p)VA	24	2.06	0.089
pp49	RRPT(p)VA	26	4.04	0.16
pp50	RRAT(p)PA	ND	(0.02)*	—
pp51	RRREEET(p)EEEAA	24	0.45	0.021

*At 200 μM.

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not possess PTPase activity (Table IV).

Negative Determinants for the Dephosphorylation in the Amino Acid Sequence around the Dephosphorylation Site—It is reported that four major classes of protein Ser/Thr phosphatases (PP1, PP2A, PP2B, and PP2C) differentially dephosphorylated a variety of ^{32}P-labeled phosphopeptide substrates (*10*, *29–32*). Among these, RRAT(p)VA (pp38) is useful for sensitive assay of PP2A and PP2C activities (*10*). Some of its analogs were also used for characterization of the major protein Ser/Thr phosphatases (*10*, *29–32*). Therefore, we chemically synthesized these phosphopeptide substrates (pp38, pp49, pp50, and pp51), and examined whether or not they were dephosphorylated by CaMKPase. As shown in Table V, pp38 was dephosphorylated by CaMKPase, and the V_{max}/K_m value was comparable to that of pp2. pp50, an analog of pp38 in which the Val residue at the +1 position is replaced with Pro, is reported not to be dephosphorylated by PP2A and PP2C (*10*, *31*). CaMKPase also could not dephosphorylate pp50. Thus, Pro residue at the +1 position had a detrimental effect on dephosphorylation by CaMKPase. In contrast, it is reported that Pro residue at the −1 position does not affect PP2A- and PP2C-catalyzed dephosphorylation (*31*). When the pp38 analog pp49, in which the Ala residue at the −1 position of pp38 was replaced with Pro, was used as a CaMKPase substrate, an increase of about 2-fold in the V_{max} value was observed, while the K_m value was unchanged as compared with that of pp38. It is reported that pp51 is an excellent substrate for PP2A but is not dephosphorylated at all by PP2C (*31*). As shown in the table, CaMKPase poorly dephosphorylated pp51, with the V_{max} value being about 5 times lower than that of pp38. Since the K_m values for pp51 and pp38 were almost the same, introduction of acidic clusters around the dephosphorylation site caused inefficient catalysis without loss of binding affinity to the enzyme. A similar effect of acidic clusters was also observed in the set of pp39 and pp56 (Table III).

Effect of poly(Lys) on CaMKPase Activity—Previously, we reported that CaMKPase is markedly activated by polycations including poly(Lys) (*5*). Since we used ^{32}P-labeled phosphoproteins or phosphopeptide-paramagnetic particle conjugates as substrates in the previous studies, we could not determine the detailed kinetic parameters upon activation by poly(Lys) due to limitation of the quantity of the phosphoprotein substrates or due to insolubility of the conjugate. Therefore, using synthetic phosphopeptide substrates, we examined how the kinetic parameters changed upon activation by poly(Lys) in the present study. Table VI shows the rates of activation by poly(Lys) determined using several phosphopeptides as substrates. As shown in the table, poly(Lys) activated CaMKPase mainly by increase in the V_{max} value rather than by decrease in the K_m value.

Dephosphorylation of Phosphorylated CaMKIV, MAP kinase, MLC, and the Corresponding Phosphopeptides by CaMKPase—Since phospho-Thr at the dephosphorylation site is a critical determinant for dephosphorylation by CaMKPase as mentioned above, we examined whether or not other phosphoproteins containing phospho-Thr residues, which were not related to CaMKs, were also dephosphorylated by CaMKPase. MAP kinase (Erk2) is phosphorylated by activated MAP kinase kinase (MEK) at Thr^{183} and Tyr^{185} within its activation loop, leading to activation of the kinase (*33*). The activated MAP kinase is dephosphorylated by PP2A and a specific protein phosphatase, MAP kinase phosphatase, and thereby deactivated (*34*, *35*). On the other hand, MLC is phosphorylated by protein kinase C mainly at Thr^{9} (*23*, *36*) and dephosphorylated by unidentified aortic protein phosphatases (*23*, *37*). As shown in Fig. 3, the phosphorylated MAP kinase and MLC were not significantly dephosphorylated by CaMKPase under conditions where CaMKIV(K71R) phosphorylated by CaMK kinase α was almost completely dephosphorylated. The kinetic parameters of CaMKPase for phosphopeptides corresponding to the amino acid sequences around the phosphorylation sites of MAP kinase, MLC and CaMKs I, II, and IV were determined and are summarized in Table VII. A phosphopeptide corresponding to the phosphorylation site of CaMKIV (pp12) was efficiently dephosphorylated by CaMKPase, with the V_{max}/K_m value being comparable to that of pp2. A phosphopeptide corresponding to the phosphorylation site of CaMKI (pp14) was a much better substrate for CaMKPase than pp2. A phosphopeptide corresponding to the phosphorylation site of MLC (pp58) was not dephosphorylated by CaMKPase at

TABLE VI. **Activation of CaMKPase activity by poly(Lys).** Standard CaMKPase assays were carried out using phosphopeptide substrates listed in the presence or absence of 10 μg/ml poly(Lys), and the kinetic parameters were calculated as described under "MATERIALS AND METHODS." The ratios of the parameters in the presence of poly(Lys) to those in its absence are shown. Values are the averages of three independent determinations ± SD.

Peptide	Sequence	$K_m(+)/K_m(-)$	$V_m(+)/V_m(-)$
pp2	MHRQET(p)VDC	0.89 ± 0.02	1.73 ± 0.10
pp40	QET(p)VDC	1.2 ± 0.22	2.44 ± 0.29
pp39	ET(p)VDC	0.59 ± 0.29	2.35 ± 0.21
pp52	AT(p)VDC	1.1 ± 0.39	1.64 ± 0.15
pp53	RT(p)VDC	1.0 ± 0.12	1.42 ± 0.09
pp55	DT(p)VDC	1.1 ± 0.60	2.14 ± 0.28

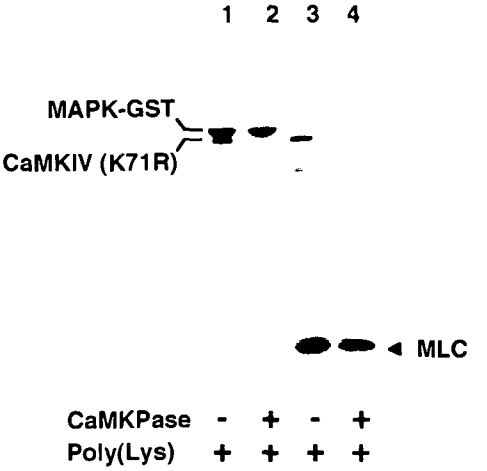

Fig. 3. **Dephosphorylation of CaMKIV(K71R), MAP kinase, and MLC by CaMKPase.** MAP kinase-GST (7.35 nM, lanes 1 and 2), which had been phosphorylated by activated MEK1, and MLC (7.35 nM, lanes 3 and 4), which had been phosphorylated by PKC, were incubated at 30°C with CaMKIV(K71R), which had been phosphorylated by CaMK kinase α, in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of CaMKPase (0.14 μg/ml), as described under "MATERIALS AND METHODS." After incubation for 1 min, the reaction was terminated, and aliquots were analyzed by SDS-PAGE followed by autoradiography.

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TABLE VII. **Kinetic parameters for phosphopeptide substrates corresponding to the sequences around the phosphorylation sites of some phosphoproteins.** Standard CaMKPase assays were carried out using phosphopeptide substrates listed, and the kinetic parameters were calculated as described under "MATERIALS AND METHODS." For an extremely poor substrate, phosphatase activity assayed at the indicated concentration is shown in a parenthesis. ND, not determined.

Peptide	Origin	Sequence	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/μM)
pp14	CaMKI(165-185)	LSKMEDPGSVLST(p)ACGTPGYV	5.7	4.17	0.77
pp2	CaMKII(281-289)	MHRQET(p)VDC	16	2.22	0.14
pp12	CaMKIV(184-204)	LSKIVEHQVLMKT(p)VCGTPGYC	24	3.86	0.16
pp58	MLC(5-16)	AKAKT(p)TKKRPQR	ND	(0.01)*	—
pp59	Erk2(179-189)	TGFLT(p)EY(p)VATR	4.2	1.86	0.45

*At 500 μM.

all, suggesting the presence within this peptide of a particular amino acid or amino acid sequence that suppresses the dephosphorylation. In contrast, a phosphopeptide corresponding to the phosphorylation site of MAP kinase (pp59) was a good substrate, better than pp2 and pp12, suggesting that the failure of CaMKPase to dephosphorylate the phosphorylated MAP kinase is not due to the primary structure around the phosphorylation site of MAP kinase but due to the higher-order structure surrounding the phosphorylation site.

DISCUSSION

The use of a synthetic phosphopeptide corresponding to the amino acid sequence around the dephosphorylation site of the phosphoprotein substrate is a powerful means of analyzing phosphatase activity (*10*). Chemically synthesized phosphopeptides containing phospho-Tyr are widely used as substrates for PTPases, and substrate specificities of a variety of PTPases have been studied using such synthetic phosphopeptides (*10*, *22*, *38–41*). On the other hand, phosphopeptide substrates for Ser/Thr phosphatases are usually prepared by phosphorylation of synthetic peptides with [γ-^{32}P]ATP by suitable protein kinases because of the difficulty of chemical synthesis of phosphopeptides containing phospho-Ser/Thr. There have been several studies on substrate specificities of the four major classes of protein Ser/Thr phosphatases using enzymatically phosphorylated synthetic peptides (*10*, *29–32*). However, since the substrates are prepared by enzymatic phosphorylation of synthetic peptides by protein kinases, it is difficult to obtain arbitrary substrates in sufficient quantities and purities for kinetic analysis. Furthermore, the short half-life of [^{32}P]phosphate is very inconvenient in kinetic analysis using a large number of phosphopeptide analogs as substrates. Recently, Wakamiya *et al.* established a convenient method for the synthesis of phosphoseryl or phosphothreonyl peptides by a simple Fmoc strategy (*11*, *12*). Using this protocol, we can arbitrarily prepare a variety of phosphoseryl or phosphothreonyl peptides for exhaustive analyses of substrate specificities of protein Ser/Thr phosphatases.

Recently, we purified a novel protein phosphatase designated as CaMKPase from rat brain (*5*). CaMKPase shows fairly high degree of substrate specificity toward multifunctional CaMKs (*5*, *6*). To study the mechanism of substrate recognition of CaMKPase, we synthesized a variety of phosphopeptide analogs of a model substrate pp2, which corresponds to the amino acid sequence within the autoinhibitory domain of CaMKII (the residues 281–289). To our knowledge, this is the first report on the use of systematically synthesized phosphopeptides as substrates for kinetic studies on substrate specificities of protein Ser/Thr phosphatases.

The fact that the dephosphorylating activity of CaMKPase was not completely lost even through the deletion of all amino acid residues on the C-terminal side of the dephosphorylation site of pp2 (pp36 in Table I) indicates that the residue(s) on the C-terminal side is not essential for substrate activity. In contrast, the deletion of glutamate numbered −1 on the N-terminal side (pp37 in Table I) resulted in complete loss of substrate activity, indicating that the residue adjacent to the phosphorylation site on the N-terminal side is essential for substrate activity. However, Ala-scanning analysis (pp28 in Table II) and substitution analysis (Table III) revealed that the −1 amino acid residue is not important but that an amido bond on the α-amino group of the phospho-Thr is essential for substrate activity. Interaction between the phosphoryl group and the amide proton of the residue through hydrogen bonding as revealed by NMR studies (*42*) may participate in the catalytic mechanism. The importance of the −1 residue is also reported with a PTPase substrate, but an acidic side chain at this position is required for binding of the substrate to PTPases with high affinity (*10*, *41*). Deletion analysis of pp2 (Table I) indicated that the minimal length of a phosphopeptide substrate for CaMKPase was only 2 to 3 amino acid residues.

From the substitution study of the dephosphorylation sites of phosphopeptide substrates (Table IV), CaMKPase appears to be a protein Ser/Thr phosphatase having a strong preference for phospho-Thr. PP1, PP2A, and PP2C, but not PP2B, are reported to strongly prefer phospho-Thr to phospho-Ser for dephosphorylation (*10*).

A Pro residue at the +1 position makes a strong negative contribution to dephosphorylation by CaMKPase (pp50 in Table V). Such detrimental effect of Pro at the +1 position is also reported for PP2A and PP2C (*10*, *31*). Acidic clusters of Glu around the Thr residue (pp51 in Table V) had a negative effect on dephosphorylation. Such a negative effect is also observed for PP2C, but not for PP2A (*10*, *31*). Since PP2C and CaMKPase belong to the PPM gene family of protein phosphatases as judged by sequence analysis (*7*, *9*, *43*), this effect may be a common feature of PPM phosphatases. It should be noted that the motif Ser/Thr-Pro is specifically recognized by Pro-directed protein kinases and that acidic clusters of Glu around the Thr residue are favored by casein kinase II (*10*, *44*). Therefore, phosphoproteins phosphorylated by these protein kinases are predicted to be resistant to dephosphorylation by CaMKPase. A peptide corresponding to the sequence surrounding Thr^9 of

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MLC, pp58, could not be dephosphorylated by CaMKPase at all (Table VII). This may be in part due to the detrimental effect of Lys at the −1 position of pp58, since a basic residue at the −1 position is unfavorable for substrate binding as suggested by pp53 (Table III).

One of the characteristics of CaMKPase is a marked activation by polycations such as poly(Lys) (*5*), but the mechanism of the activation is unclear. When several phosphopeptide substrates were used as CaMKPase substrates, poly(Lys) activated CaMKPase mainly through increase in the V_{max} values (Table VI), suggesting that poly(Lys) directly interacts with CaMKPase to activate the enzyme. On the other hand, the activation magnitudes are extremely low compared with those obtained using phosphopeptide-magnetic particle conjugate (*ca.* 10-fold) or autophosphorylated CaMKII (*ca.* 90-fold) as substrates (*5*), suggesting the direct interaction of polycations with these substrates. It is suggested that protamine stimulates PP2A through direct interaction with phosphoprotein substrates (*45*, *46*). Thus, the mechanism for the activation of CaMKPase by polycations still remains to be clarified.

Since CaMKPase is highly specific for multifunctional CaMKs, it is important to know whether or not such high degree of substrate specificity is due to recognition of a specific sequence motif around the dephosphorylation site of the substrate by CaMKPase. Ala-scanning analysis suggests that no specific amino acid residues around the dephosphorylation site play an essential role in substrate activity (Table II). Therefore, unlike consensus sequences for protein kinases, CaMKPase does not recognize a specific and critical motif around the dephosphorylation site of the substrate. It is likely that substrate specificity of CaMKPase is determined by higher-order structure of the substrate protein rather than by the primary structure around its dephosphorylation site. The observation that phosphorylated MAP kinase is a very poor substrate for CaMKPase irrespective of the fairly high substrate activity of the phosphopeptide corresponding to the sequence around the phosphorylation site of MAP kinase strongly suggests that higher-order structure of the substrate protein is important for substrate recognition by CaMKPase. However, since substrate activity of a phosphopeptide is largely influenced by the amino acid residue to be dephosphorylated, a Pro residue at the +1 position, and acidic clusters of Glu residues around the dephosphorylation site, these residues in the primary structure can also affect substrate activity of the phosphoprotein substrate, as illustrated by the phosphorylated mutant CaMKIV (Fig. 2). It is suggested that the higher-order structure of the N-terminal region of MLC including the phosphorylation site is flexible in myosin subfragment-1 (*47*). Therefore, the failure of CaMKPase to dephosphorylate the phosphorylated MLC may be ascribed to the extremely low substrate activity of the phosphopeptide corresponding to MLC(5-16) (pp58 in Table VII). In addition, polycations may also participate in the recognition of the protein substrate by CaMKPase through the direct interaction with the substrate as discussed above. Thus, the feature in the higher-order structure of CaMKs recognized by CaMKPase and the interaction of polycations with CaMKs should be clarified for understanding of the molecular mechanism by which CaMKPase specifically recognizes its substrates.

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