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Studies on the Site of Phosphorylation of Ca^{2+}/Calmodulin-Dependent Protein Kinase (CaM-Kinase) IV by CaM-Kinase Kinase[1]

Takako Kitani,[2] Sachiko Okuno, and Hitoshi Fujisawa

Department of Biochemistry, Asahikawa Medical College, Asahikawa, Hokkaido 078

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The phosphorylation site(s) involved in the activation of CaM-kinase IV by CaM-kinase kinase α was studied using a mutant CaM-kinase IV (K71R) in which Lys^{71} (ATP-binding site) was replaced with Arg, because the autophosphorylation of CaM-kinase IV occurring at multiple sites made it difficult to study phosphorylation of the enzyme by CaM-kinase kinase. Sequence analysis of the phosphopeptide from the trypsin digest of CaM-kinase IV (K71R) phosphorylated by CaM-kinase kinase α suggested that the phosphorylation of CaM-kinase IV by CaM-kinase kinase only occurred at Thr^{196}. The recombinant mutant CaM-kinase IV in which Thr^{196} or Thr^{200} was replaced with nonphosphorylatable alanine showed little activity in the presence and absence of the kinase kinase. The mutant enzyme in which Thr^{196} was replaced with negatively charged aspartic acid showed almost 25 times as high activity as the wild-type enzyme in the absence of the kinase kinase, and no more activation was observed in its presence. In contrast, the enzyme in which Thr^{200} was replaced with aspartic acid showed little enzyme activity. Thus, it may be concluded that the phosphorylation of Thr^{196} in CaM-kinase IV by CaM-kinase kinase is necessary for the subsequent autophosphorylation and activation of CaM-kinase IV.

Key words: Ca^{2+}/calmodulin-dependent protein kinase, CaM-kinase IV, CaM-kinase kinase, protein phosphorylation, phosphorylation site.

Ca^{2+}/calmodulin-dependent protein kinases (CaM-kinases) I, II, and IV are thought to play important roles as Ca^{2+}-responsive multifunctional protein kinases (see Refs. *1-4* for reviews) controlling a variety of functions in response to an increase in intracellular Ca^{2+} in the cell, and regulation of their activities is therefore very important. Among the three CaM-kinases, CaM-kinases IV (*5-7*) and I (*8, 9*) are markedly activated through phosphorylation by other Ca^{2+}/calmodulin-dependent protein kinases, such as CaM-kinase kinases α (*10, 11*) and β (*11-13*). Amino acid sequence analysis (*14*) and mutational analysis (*15*) of the phosphorylation site of CaM-kinase I revealed that the phosphorylation of Thr^{177} of CaM-kinase I by CaM-kinase kinase is involved in the marked activation of CaM-kinase I. It was reported that replacement of the equivalent amino acid residue in CaM-kinase IV, Thr^{196}, with nonphosphorylatable alanine abolishes both the phosphorylation and activation of CaM-kinase IV (*16*), suggesting that the phosphorylation of Thr^{196} of CaM-kinase IV by CaM-kinase kinase is involved in the activation. In the present study, detailed analysis of the phosphorylation of CaM-kinase IV by CaM-kinase kinase was performed using a mutant CaM-kinase IV, the activity of which was lost on replacement of Lys^{71} (ATP-binding site) with arginine, because autophosphorylation of CaM-kinase IV occurring at many sites following activation by CaM-kinase kinase (*17, 18*) makes it difficult to observe the phosphorylation of CaM-kinase IV by CaM-kinase kinase. The results indicated that CaM-kinase kinase only phosphorylated Thr^{196} of CaM-kinase IV, which is involved in the activation.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham International. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). DEAE-cellulose (DE52), phosphocellulose paper (P81), and 3MM paper were from Whatman. Fluorotrans, a polyvinylidene difluoride (PVDF) membrane, was from Pall BioSupport. Sephacryl S-300 HR, blue-Sepharose CL-6B, and Protein A Sepharose 4 Fast Flow were from Pharmacia-LKB Biotechnology. Peroxidase-conjugated goat anti-rabbit immunoglobulin was from Organon Teknika. TPCK-trypsin was from Cooper Biomedical. Cellulose-precoated thin layer plates (TLC plates) were from Merck. Peptide-γ (KSDGGVKKRKSSSS) (*19*) was synthesized with a Shimadzu PSSM-8 automated peptide synthesizer. Wistar rats were purchased from the Shizuoka Laboratory Animal Center. All other reagents were of the highest grade commercially available.

Protein Preparations—Calmodulin was purified from

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[2] Affiliated with the Laboratory for Radioactive Isotope Research, Asahikawa Medical College.

Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; Mops, 3-(*N*-morpholino)propanesulfonic acid; TPCK-trypsin, tosylphenylalanyl chloromethyl ketone-treated trypsin.

Escherichia coli cells transformed with expression vector pET11d carrying a cDNA encoding chicken brain calmodulin (*20*), essentially as described by Gopalakrishna and Anderson (*21*). The cDNA encoding chicken brain calmodulin was kindly donated by A.R. Means (*22*).

Recombinant rat brain CaM-kinase kinase α expressed in *E. coli* cells (*11*) was purified as follows. Approximately 7.6 g of the bacterium was suspended in 76 ml of 20 mM Tris-HCl (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin), and then disrupted by sonic oscillation. To the supernatant (75.5 ml) obtained on centrifugation was added 9.1 ml of 2.5% streptomycin sulfate. After standing for 15 min on ice, the supernatant obtained on centrifugation was applied to a column of DE52 (20 ml) equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, and 10% glycerol. The column was washed with the equilibration buffer (about 280 ml), and then with the same buffer containing 0.07 M NaCl until the A_{280} of the eluate became negligible (about 250 ml), and then the enzyme was eluted with 140 ml of the equilibration buffer containing 0.17 M NaCl. To the eluate was added solid ammonium sulfate to a final saturation level of 60%. The resulting precipitate, collected on centrifugation, was dissolved in 6 ml of 10 mM Hepes-NaOH (pH 7.2 at 4°C) containing 0.1 mM dithiothreitol, 1 mM $CaCl_2$, 10% glycerol, 0.05% Tween 80, and 10 μg/ml each of microbial protease inhibitors, and then applied to a column of calmodulin-Sepharose (5 ml), prepared as described previously (*6*), equilibrated with the same buffer. The column was washed extensively with 10 mM Hepes-NaOH (pH 7.2) containing 0.1 mM dithiothreitol, 0.2 mM $CaCl_2$, 10% glycerol, 0.05% Tween 80, and 2 M NaCl, and then with the same buffer without NaCl, and then the enzyme was eluted with 10 mM Hepes-NaOH (pH 7.2) containing 0.1 mM dithiothreitol, 1 mM EGTA, 10% glycerol, 0.05% Tween 80, and 0.2 M NaCl. Approximately 4.5 mg of the purified CaM-kinase kinase α, which gave a single protein band on SDS-PAGE, as shown in Fig. 1A (lane 5), was obtained.

A cDNA for CaM-kinase IV (K71R) in which Lys^{71} was replaced with Arg was prepared by site-directed mutagenesis, using the single-stranded DNA obtained from pUC118, into which the *Xma*I-*Bam*HI fragment (495 bp) of CaM-kinase IV cDNA was inserted, as a template, and a synthetic oligonucleotide, 5′-TATGCTCTCAGAGTGTTAA-AG-3′ (the substitution site of the oligonucleotide is underlined), as a sense primer, essentially according to the method of Kunkel *et al.* (*23*). A cDNA fragment containing the entire coding sequence of the mutant CaM-kinase IV (K71R) was introduced into a vaculovirus, AcNPV, using the Bac-To-Bac Baculovirus Expression System (Life Technologies). Sf9 cells infected with the recombinant baculovirus were grown and recombinant CaM-kinase IV (K71R) was purified as described previously for the wild-type enzyme (*20*). Approximately 643 μg of the purified CaM-kinase IV (K71R), which gave a single band coinciding with the wild-type enzyme, as shown in Fig. 1A (lane 3), was obtained from 565 mg of the Sf9 cells.

cDNAs for CaM-kinase IV (T196A), CaM-kinase IV (T196S), CaM-kinase IV (T196D), CaM-kinase IV (T200A), CaM-kinase IV (T200S), and CaM-kinase IV (T200D), whose Ala^{196}, Ser^{196}, Asp^{196}, Ala^{200}, Ser^{200}, and Asp^{200}, respectively, were substituted for Thr, were prepared using synthetic oligonucleotides, 5′-GCTCATGAAG-GCGGTGTGTGGAA-3′, 5′-GCTCATGAAGTCGGTGTG-TGGAA-3′, GTGCTCATGAAGGACGTGTGTGGAACC-3′,5′-CGGTGTGTGGAGCCCCGGGGTAC-3′,5′-CGGTG-TGTGGATCCCCGGGGTAC-3′, and 5′-CGGTGTGTGGA-GACCCGGGGTACTGT-3′, as sense primers. The mutations were confirmed by the dideoxynucleotide chain-termination method (*24*) using a DNA sequencer, LI-COR model 4000L. Each cDNA containing the entire coding sequence of mutant CaM-kinase IV (T196A), (T196S), (T196D), (T200A), (T200S), or (T200D) was introduced into an expression vector, pET11a, and then the constructs were transformed into *E. coli* strain BL21(DE3) (*25*). The bacteria were grown to an A_{600} of 0.6 to 1.0 at 30°C in 10 ml of M9ZB medium containing 200 μg/ml ampicillin, and then isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After 4 h, the bacteria were harvested by centrifugation, suspended in 0.4 ml of 20 mM Tris-HCl (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of the microbial protease inhibitors, and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate a crude extract. The electrophoretic profiles of the crude extracts of the bacteria are shown in Fig. 1B.

An antibody against CaM-kinase kinase α was prepared by immunization of Japanese white rabbits with a peptide corresponding to the carboxyl-terminal 20 amino acids of CaM-kinase kinase α, and purified by affinity chromatography on peptide-coupled Cellulofine, as described previously (*11*).

Phosphorylation of CaM-Kinase IV (K71R) by CaM-Kinase Kinase α—Phosphorylation of CaM-kinase IV (K71R) by CaM-kinase kinase α was carried out at 30°C in the standard phosphorylation mixture comprising 50 mM Mops-NaOH (pH 7.0 at 30°C), 5 mM $Mg(CH_3COO)_2$, 0.1 mM [γ-^{32}P]ATP, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 1 μM calmodulin, 2 mM dithiothreitol, and the indicated amounts of CaM-kinase IV (K71R) and CaM-kinase kinase α. After incubation for the indicated times, the incorporation of [^{32}P]phosphate into CaM-kinase IV (K71R) was determined by the 3 MM paper method of Corbin and Reimann (*26*), except that the filter papers were washed with ice-cold 10% trichloroacetic acid containing 2 mM ATP.

Assaying of CaM-Kinase IV—The CaM-kinase IV activity of the crude extracts of the transformed bacteria obtained as described above was determined by measuring the phosphorylation of peptide-γ at 30°C, essentially as described previously (*19*). The standard assay mixture contained, in a final volume of 50 μl, 50 mM Mops-NaOH (pH 7.0 at 30°C), 5 mM $Mg(CH_3COO)_2$, 0.1 mM [γ-^{32}P]-ATP (approximately 230 cpm/pmol), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 1 μM calmodulin, 2 mM dithiothreitol, 40 μM peptide-γ, and 4 μl of a bacterial crude extract. After incubation for 5 min at 30°C in the presence and absence of 10 ng of CaM-kinase kinase α, 30-μl aliquots were withdrawn and the incorporation of [^{32}P]phosphate into peptide-γ was determined by the phosphocellulose paper method (*27*).

Analysis of Phosphorylation Sites of CaM-Kinase IV—

Approximately 100 μg (20 μg/ml) of CaM-kinase IV (K71R) was incubated with 10 μg (2 μg/ml) of CaM-kinase kinase α, in a final volume of 5 ml, in the standard phosphorylation mixture containing 0.1 mM [γ-^{32}P]ATP (250 cpm/pmol) at 30°C for 1 min. The reaction was stopped by the addition of ATP and ice-cold trichloroacetic acid at final concentrations of 10 mM and 10%, respectively. The precipitate obtained on centrifugation was washed by sonication for 2 min in 1 ml of ice-cold acetone for four times and then for 1 min in 0.3 ml for three times, and then air-dried at room temperature. The resulting protein was dissolved in 50 μl of 50 mM Tris-HCl (pH 8.0 at 30°C) containing 8 M urea, and the mixture was incubated for 30 min at 30°C. Then 3 μl of 0.5 M dithiothreitol (final concentration, 28 mM) was added under a stream of argon, and the mixture was incubated at 37°C for 2 h under an argon atmosphere. To the mixture was added 6 μl of 0.4 M iodoacetamide (41 mM), and then the mixture was incubated at 24°C for 30 min in the dark. The mixture was diluted with 120 μl of 50 mM Tris-HCl (pH 8.0 at 37°C) and, after the addition of 20 μl of 0.1 M $CaCl_2$ and 3.9 μl of 2 mg/ml TPCK-trypsin in 1 mM HCl, the mixture was incubated at 37°C for 25 h. The mixture was filtered through a 0.22-μm filter, and then loaded onto a C_{18} reverse-phase HPLC column (0.46×25 cm, TSK gel ODS-80Ts; Tosoh) equilibrated with buffer A (2% acetonitrile in 20 mM triethylamine phosphate, pH 3). The column was eluted successively, at the flow rate of 0.5 ml/min, with buffer A for the initial 30 min, then linear gradients of 0-50% (v/v) buffer B (80% acetonitrile in 20 mM triethylamine phosphate, pH 3) for 100 min and then 50-100% buffer B for 5 min, and finally buffer B for 5 min. The peptide and phosphopeptide peaks were monitored spectrophotometrically at 215 nm using an on-line UV monitor, Tosoh UV-8000, and radiometrically by Cerenkov counting using an on-line detector, Raytest Ramona 90, respectively. The fractions corresponding to each radioactive peak were pooled and subjected to the second HPLC on the same column equilibrated with buffer C (2% acetonitrile in 20 mM triethylamine acetate, pH 6.5). The column was eluted with buffer C for the initial 10 min, then linear gradients of 0-50% buffer D (80% acetonitrile in 20 mM triethylamine acetate, pH 6.5) for 100 min and 50-100% buffer D for 10 min, and finally 100% buffer D for 5 min. The amino acid sequences of the radioactive phosphopeptides thus obtained were determined with a Hewlett Packard G1005A peptide sequenator.

Other Analytical Procedures—SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (*28*). Immunoblotting analysis was performed essentially as described by Winston *et al.* (*29*). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*30*), and a molecular weight of 16,700 (*31, 32*). Other proteins were determined by the method of Lowry *et al.* (*33*), as modified by Peterson (*34*), with bovine serum albumin as a standard. The amount of CaM-kinase IV was corrected for overestimation by a factor of 1.15 by means of Lowry's method (*35*). The molar concentration of CaM-kinase IV (K71R) purified from Sf9 cells was calculated using a molecular weight value of 53,160, which was estimated from the molecular weight of 53,133 calculated from the deduced amino acid sequence of the wild-type enzyme (*36, 37*). The molar concentration of CaM-kinase kinase α was estimated, taking the molecular weight to be 55,900 (*11*). Phosphoamino acid analysis was performed by the method of Cooper *et al.* (*38*).

RESULTS

Phosphorylation of CaM-Kinase IV (K71R) by CaM-Kinase Kinase α—CaM-kinase IV undergoes autophosphorylation at many sites (*17, 18*) upon phosphorylation by CaM-kinase kinase, and accordingly it is difficult to study the phosphorylation sites of CaM-kinase IV for CaM-kinase kinase. Since Lys^{71} in the putative ATP-binding site of CaM-kinase IV (*10, 11*) is thought to be essential for the binding of ATP, the difficulty can be overcome by the use of an inactive enzyme in which Lys^{71} is replaced by another amino acid residue. The recombinant enzyme, CaM-kinase IV (K71R), in which Lys^{71} was replaced by Arg, an amino acid residue similar to Lys in hydrophobicity and charge, was expressed in Sf9 cells and purified to apparent homogeneity, and was used to study the phosphorylation sites of CaM-kinase IV for CaM-kinase kinase in this study. There was no significant difference in mobility on SDS-PAGE of the wild-type and mutant enzymes, as shown in Fig. 1A. The CaM-kinase kinase α used in the present study was a recombinant enzyme purified from transformed *E. coli* cells. The purified enzyme gave one major band and one faint one on SDS-PAGE (Fig. 1A), both of which reacted with an antibody against CaM-kinase kinase α, as determined by immunoblot analysis (data not shown). The specific activity of the purified recombinant CaM-kinase kinase α was significantly higher than that of the enzyme purified from rat brain, presumably reflecting its higher purity.

CaM-kinase IV and CaM-kinase IV (K71R) were incubated with CaM-kinase kinase α under the phosphorylation conditions and then subjected to SDS-PAGE followed by autoradiography, as shown in Fig. 2. Both CaM-kinase IV and CaM-kinase IV (K71R) were phosphorylated in the presence of Ca^{2+}/calmodulin, and the extent of the phosphorylation of wild-type CaM-kinase IV was much greater than that of CaM-kinase IV (K71R). CaM-kinase IV gave a doublet on SDS-PAGE after incubation for phosphorylation, one corresponding in mobility to the original enzyme and the other being slower than that of the original enzyme (Fig. 2A), and the slower protein stained band coincided with the major radioactive band (Fig. 2B). Thus, the mobility of phosphorylated CaM-kinase IV on SDS-PAGE was lower than that of the original unphosphorylated enzyme, but such a shift in mobility upon phosphorylation was not observed with CaM-kinase IV (K71R). These results indicate that the phosphorylation by CaM-kinase kinase does not cause a shift in mobility but that the subsequent autophosphorylation causes such a change in mobility on SDS-PAGE of CaM-kinase IV. The reason why a significant amount (about half) of CaM-kinase IV remained unphosphorylated after incubation with CaM-kinase kinase α is not yet understood, but it means that some of the CaM-kinase IV in our preparation existed in an inactive form. Figure 3 shows the time course of the phosphorylation of CaM-kinase IV (K71R) by CaM-kinase kinase α. When 20 μg/ml CaM-kinase IV (K71R) (3.8 pmol) was incubated with 2 μg/ml CaM-kinase kinase α (0.35 pmol) under Ca^{2+}/calmodulin-dependent phosphor-

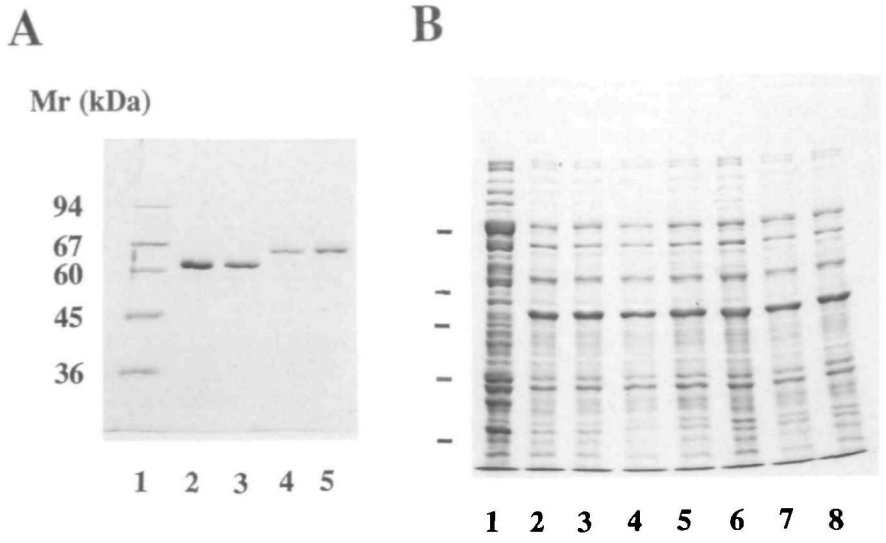

Fig. 1. **SDS-PAGE of CaM-kinase IV.** (A) Approximately 0.5 μg of CaM-kinase IV (lane 2) and CaM-kinase IV (K71R) (lane 3) purified from the transformed Sf9 cells, and CaM-kinase kinase α purified from rat brain (lane 4) and purified from the transformed *E. coli* cells (lane 5), and molecular weight standards (lane 1) were subjected to SDS-PAGE on a 7.5% acrylamide gel, and then stained with Coomassie Brilliant Blue R-250. (B) Approximately 4 μl of crude extracts of untransformed bacteria (lane 1), and the bacteria transformed with pET11a carrying CaM-kinase IV (wild type) (lane 2), (T196A) (lane 3), CaM-kinase IV (T196S) (lane 4), CaM-kinase IV (T196D) (lane 5), CaM-kinase IV (T200A) (lane 6), CaM-kinase IV (T200S) (lane 7), and CaM-kinase IV (T200D) (lane 8) were subjected to SDS-PAGE on a 7.5% acrylamide gel, and then stained with Coomassie Brilliant Blue R-250. The molecular weight standards used were phosphorylase *b* (M_r 94,000), bovine serum albumin (M_r 67,000), catalase (M_r 60,000), ovalbumin (M_r 45,000), and lactate dehydrogenase (M_r 36,000).

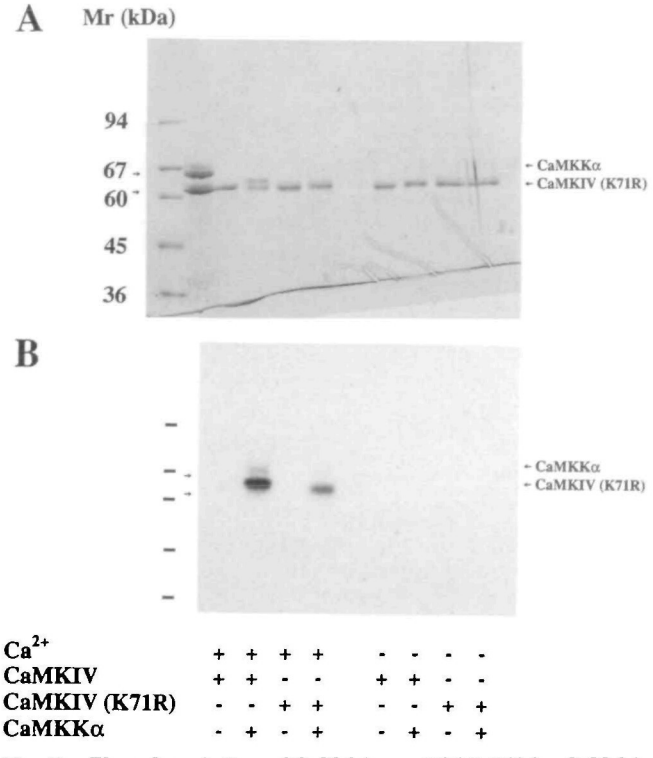

Fig. 2. **Phosphorylation of CaM-kinase IV (K71R) by CaM-kinase kinase α.** The reaction was carried out in a final volume of 10 μl of the standard phosphorylation mixture comprising 20 μg/ml CaM-kinase IV or CaM-kinase IV (K71R), 2 μg/ml CaM-kinase kinase α, and 0.1 mM [^{32}P]ATP (500 cpm/pmol), with the indicated omissions, in the presence and absence of Ca^{2+}. After incubation for 5 min at 30°C, the reactions were terminated by boiling for 3 min in Laemmli's sample buffer, and then the mixtures were subjected to SDS-PAGE on 7.5% gels. The molecular weight standards, and a mixture of CaM-kinase IV (K71R) and CaM-kinase kinase α were run in the first and second lanes from the left, respectively. (A) Proteins were visualized by staining with Coomassie Brilliant Blue. (B) Incorporation of [^{32}P]phosphate into proteins was examined by autoradiography.

ylation conditions at 30°C, initial rapid incorporation occurred, followed by the gradual incorporation of phosphate. Approximately 2.4 pmol of phosphate had been incorporated 1 min after the addition of CaM-kinase IV (K71R) regardless of whether CaM-kinase kinase α had previously been incubated under the autophosphorylation conditions or not, and an additional 2 pmol of phosphate was gradually incorporated after 1 h. The fact that incubation of CaM-kinase kinase α without CaM-kinase IV (K71R) for 30 min under the phosphorylation conditions caused the incorporation of approximately 0.4 pmol of phosphate (approximately 1 mol of phosphate was incorporated into 1 mol of the enzyme), but did not affect the rate of phosphorylation of CaM-kinase IV (K71R), suggests that the autophosphorylation of CaM-kinase kinase α caused no activation of the enzyme. Thus, approximately 0.6 mol of phosphate was incorporated into 1 mol of CaM-kinase IV (K71R) through the action of CaM-kinase kinase α after incubation for 1 min under the experimental conditions. In order to determine the phosphorylation site(s) of CaM-kinase IV for CaM-kinase kinase, 100 μg (1.88 nmol) of CaM-kinase IV (K71R) was phosphorylated by CaM-kinase kinase α for 1 min at 30°C under the conditions used in the experiment shown in Fig. 3, the phosphorylated protein was digested with TPCK-trypsin, and then the digested phosphopeptides were purified by reverse-phase HPLC in a triethylamine phosphate (pH 3)/acetonitrile system, as shown in Fig. 4. The HPLC gave two major (A and C) and two minor (B and D) peaks of radioactive phosphopeptides. The phosphorylated amino acids in the four peaks were all identified as threonine residues according to the method of Cooper *et al.* (*38*) (data not shown). When each of the four radioactive phosphopeptides was purified by the second HPLC in a triethylamine acetate (pH 6.5)/acetonitrile system and its amino acid sequence was analyzed with an automated peptide sequenator, the sequences of $XV_{25}XG_{16}T_{10}P_{10}G_6$ (the subscript numbers indicate the picomole yields) for phosphopeptide A, $L_{1.2}Q_{0.6}E_{0.6}XE_{0.5}V_{0.3}$ for B, $XV_{59}XG_{27}T_{16}P_{22}G_{25}Y_{36}XA_{38}P_{20}E_{24}I_{13}L_{16}R_9$ for C, and $D_9L_9K_6P_4E_5N_2Y_2$ for D were obtained. The sequences of phos-

phopeptides A and C are both assigned to the proteolytic peptide, TVCGTPGYCAPEILR, corresponding to T^{196}-R^{210} of the predicted amino acid sequence of CaM-kinase IV (*36, 37*). The reasons why the peptide appeared as two different species, phosphopeptides A and C, upon HPLC and the sequence determination of peptide A stopped at Gly are not yet known. The first amino acid residue of both major phosphopeptides A and C could not be identified but the fifth amino acid was identified as a threonyl residue, indicating that the first threonyl residue, corresponding to Thr^{196} of the enzyme, was phosphorylated and the other threonyl residue, corresponding to Thr^{200} of the enzyme, was not phosphorylated. On the other hand, the sequences of minor phosphopeptides B and D were not found in the predicted amino acid sequence of CaM-kinase IV (*36, 37*) or that of CaM-kinase kinase α (*10, 11*), indicating that the two minor phosphopeptides were derived from contaminants in the enzyme preparations used in this experiment. Thus, almost all the phosphate incorporated into CaM-kinase IV (K71R) through the action of CaM-kinase kinase α under the experimental conditions was incorporated into a threonine residue in the T^{196}-R^{210} region, possibly Thr^{196}, of the enzyme. The combined amount of the major phosphopeptides, A (25 pmol) and C (59 pmol), was 84 pmol. Since the total amount of phosphate incorporated into the proteins was 405 pmol, the recovery of the phosphopeptides with the experimental procedures including proteolytic digestion and HPLCs was estimated to be approximately 40% from the initial yield of 51% determined with the sequenator using human serum albumin, as described in the application note from Hewlett Packard.

The Enzyme Activity of Mutant CaM-Kinase IV—The phosphopeptide from the trypsin digest of CaM-kinase IV (K71R) phosphorylated by CaM-kinase kinase α contained two threonine residues, corresponding to Thr^{196} and Thr^{200} of the enzyme, and the results of amino acid sequence analysis with an automated peptide sequenator suggested that Thr^{196} was the more likely candidate for the phosphorylation site, as described above. In order to further confirm the phosphorylation site of CaM-kinase IV, the activities of recombinant mutant enzymes in which Thr^{196} or Thr^{200} was replaced by other amino acid residues were examined in the presence and absence of CaM-kinase kinase α, as shown in Table I. The mutant enzymes, T196A and T200A, in which Thr^{196} and Thr^{200}, respectively, were replaced by nonphosphorylatable alanine, showed little, if any, enzyme activity in both the presence and absence of CaM-kinase kinase α,

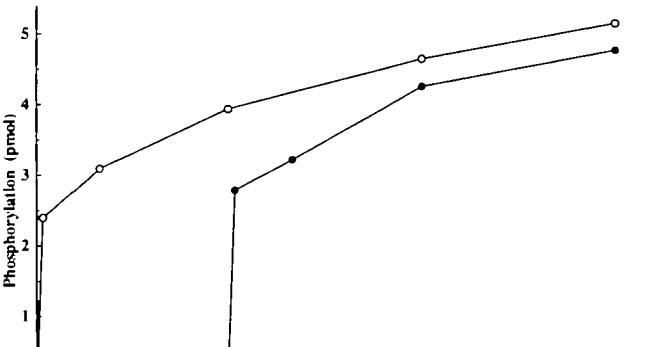

Fig. 3. **Time course of phosphorylation of CaM-kinase IV (K71R) by CaM-kinase kinase α.** (○) The reaction mixture comprised 20 μg/ml CaM-kinase IV (K71R), 2 μg/ml CaM-kinase kinase α, and 0.1 mM [^{32}P]ATP (2,335 cpm/pmol), in a final volume of approximately 0.1 ml of the standard phosphorylation mixture, and the reaction was initiated by the addition of CaM-kinase IV (K71R) and CaM-kinase kinase α after preincubation for 2 min at 30°C. Aliquots (one-tenth of the reaction mixture) containing 3.8 pmol of CaM-kinase IV (K71R) and 0.35 pmol of CaM-kinase kinase α were withdrawn at the indicated times for measurement of the incorporation of phosphate into proteins. (●) The reaction was started by the addition of CaM-kinase kinase α, and CaM-kinase IV (K71R) was added after 30 min.

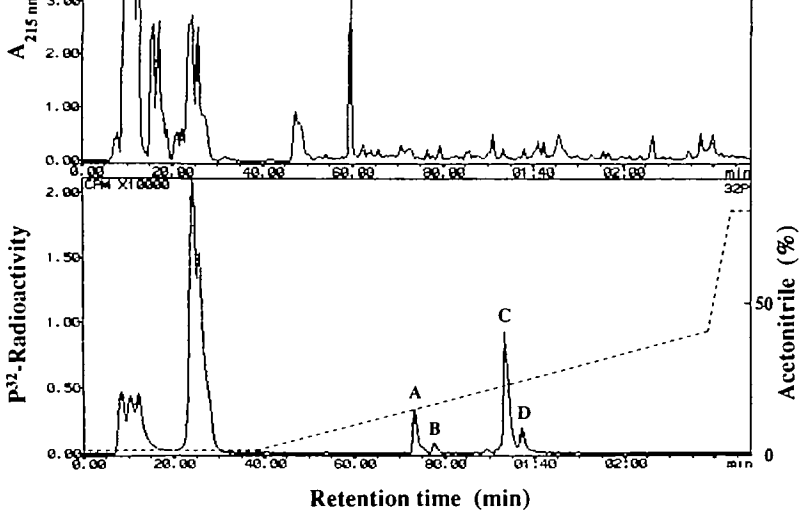

Fig. 4. **HPLC elution profiles of phosphopeptides from a trypsin digest of CaM-kinase IV (K71R) phosphorylated by CaM-kinase kinase α.** Approximately 100 μg (1.88 nmol) of CaM-kinase IV (K71R) was phosphorylated with 10 μg of CaM-kinase kinase α for 1 min at 30°C, as described under "EXPERIMENTAL PROCEDURES." The amount of phosphate incorporated into proteins was approximately 405 pmol, as measured by the 3MM paper method (*27*). The trypsin digest of the radioactive proteins was subjected to HPLC in the triethylamine phosphate/acetonitrile system, as described under "EXPERIMENTAL PROCEDURES." The fractions corresponding to each of radioactive peaks A, B, C, and D were pooled and subjected to the second HPLC in the triethylamine acetate/acetonitrile system, and then the amino acid sequences of the purified radioactive phosphopeptides were determined. The sequences obtained for the peptides from peaks A, B, C, and D were $XV_{25}XG_{16}T_{10}P_{10}G_5$ (the subscript numbers indicate the picomole yields), $L_{1.2}Q_{0.6}E_{0.6}XE_{0.5}V_{0.3}$, $XV_{59}XG_{27}T_{16}P_{22}G_{25}Y_{36}XA_{38}$-$P_{20}$-$E_{24}I_{13}L_{16}R_9$, and $D_9L_9K_6P_4E_5N_2Y_2$, respectively.

TABLE I. **The activity of mutant CaM-kinase IV.** The CaM-kinase IV activity of crude extracts of the transformed bacteria was measured with peptide-γ as a substrate in the presence and absence of CaM-kinase kinase α, as described under "EXPERIMENTAL PROCEDURES." The contents of the enzyme in the respective crude extracts were almost the same on the basis of the results of SDS-PAGE, as shown in Fig. 1B.

CaM-kinase IV	CaM-kinase kinase α: (−)	(+)
	(pmol/5 min/μl)	
Wild type	0.19	39.64
T196A	0.09	0.41
T196S	0.74	11.05
T196D	4.70	3.34
T200A	0.04	0.25
T200S	0.09	19.75
T200D	0.11	0.28

in agreement with the finding of Selbert *et al.* (*16*) that replacement of Thr-196 with alanine abolishes both the phosphorylation and activation of CaM-kinase IV by CaM-kinase kinase. The mutant enzyme, T196D, in which Thr^{196} was replaced by negatively charged aspartic acid, showed almost 25 times as high activity as the wild-type enzyme in the absence of CaM-kinase kinase α, and no more activation was observed in the presence of CaM-kinase kinase α, it presumably mimicking the enzyme phosphorylated at Thr^{196} by CaM-kinase kinase. In contrast to T196D, the T200D showed little enzyme activity. These results, taken together, suggest that Thr^{196} of CaM-kinase IV is the phosphorylation site involved in the marked activation by CaM-kinase kinase α. The results also indicate that Thr^{196} and Thr^{200} can be replaced by a serine residue for the activity.

DISCUSSION

Both CaM-kinases IV and I are markedly activated through phosphorylation by CaM-kinase kinases such as CaM-kinase kinase α (*10*), CaM-kinase Ia kinase (*16*), and CaM-kinase kinase β (*13*). The phosphorylation site of CaM-kinase I has been studied by amino acid sequence analysis (*14*) and mutational analysis (*15*). The stoichiometry of phosphorylation of CaM-kinase I in the presence of CaM-kinase kinase is 1 mol of phosphate/mol of CaM-kinase I, and a threonine residue corresponding to Thr^{177} of CaM-kinase I has been suggested as the phosphorylation site by sequence analysis (*14*). This conclusion has been confirmed by the results of mutational analysis, that replacement of Thr^{177} with Ala or Asp prevents both the phosphorylation and activation by CaM-kinase kinase, and the latter replacement leads to partial activation in the absence of CaM-kinase kinase (*15*). In contrast to CaM-kinase I, CaM-kinase IV is phosphorylated at many sites (*17, 18*) in the presence of CaM-kinase kinase, and this has hampered studies on the phosphorylation site(s) of CaM-kinase IV for CaM-kinase kinase. Selbert *et al.* (*16*) recently reported that replacement of Thr^{196} of CaM-kinase IV, which is located at an equivalent position to Thr^{177} of CaM-kinase I, with alanine abolishes both the phosphorylation and activation of CaM-kinase IV by CaM-kinase kinase, concluding that the phosphorylation of Thr^{196} is absolutely required for the activation of CaM-kinase IV by CaM-kinase kinase. Chatila *et al.* (*39*) also reported that the replacement of Thr^{200} of human CaM-kinase IV, which corresponds to Thr^{196} of the rat enzyme, with alanine completely prevents the activation of the enzyme by CaM-kinase I kinase, suggesting the phosphorylation of human CaM-kinase IV at Thr^{200} (equivalent to Thr^{196} of the rat enzyme) by the CaM-kinase kinase. However, since T196A and T200A both showed little activity in the presence and absence of CaM-kinase kinase, as shown in Table I, similar results would also be expected on replacement of Thr^{200} with alanine. To overcome the difficulty of multiple phosphorylation of CaM-kinase IV, mutant CaM-kinase IV (K71R), whose autophosphorylation was prevented by replacement of Lys^{71} (ATP-binding site) with arginine, was used for identification of the phosphorylation site in the present study. Sequence analysis suggested that the phosphorylation of CaM-kinase IV (K71R) by CaM-kinase kinase α only occurred at Thr^{196}, indicating that CaM-kinase IV is markedly activated upon phosphorylation at Thr^{196} by CaM-kinase kinase and then undergoes autophosphorylation at many sites. This conclusion was well confirmed by the results of mutational analysis. Although T196A and T200A both showed little activity in the presence and absence of CaM-kinase kinase, T196D showed almost 25 times as high activity as the wild-type enzyme in the absence of CaM-kinase kinase, and it was no more activated by the kinase kinase. In contrast, T200D showed little enzyme activity.

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