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# Identification of major $Ca^{2+}$/calmodulin-dependent protein kinase phosphatase-binding proteins in brain. Biochemical analysis of the interaction.

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Short title: CaMKP-binding proteins in brain

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**Abstract**

$Ca^{2+}$/calmodulin-dependent protein kinase phosphatase (CaMKP) is a unique protein phosphatase that specifically dephosphorylates and regulates multifunctional $Ca^{2+}$/calmodulin-dependent protein kinases (CaMKs). To clarify the physiological significance of CaMKP, we identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose bisphosphate aldolase as major binding partners of CaMKP in a soluble fraction of rat brain using the two-dimensional far-Western blotting technique, in conjunction with peptide mass fingerprinting analysis. We analyzed the affinities of these interactions. Wild type CaMKP-glutathione S-transferase (GST) associated with GAPDH in a GST pull-down assay. Deletion analysis suggested that the N-terminal side of the catalytic domain of CaMKP was responsible for the binding to GAPDH. Further, anti-CaMKP antibody coimmunoprecipitated GAPDH in a rat brain extract. GAPDH was phosphorylated by CaMKI or CaMKIV *in vitro*; however, when CaMKP coexisted, the phosphorylation was markedly attenuated. Under these conditions, CaMKP significantly dephosphorylated CaMKI and CaMKIV, which had been phosphorylated by CaMK kinase, whereas it did not dephosphorylate the previously phosphorylated GAPDH. The results suggest that CaMKP regulates the phosphorylation level of GAPDH in the CaMKP-GAPDH complex by dephosphorylating and deactivating CaMKs that are responsible for the phosphorylation of GAPDH.

*Key words*: CaM kinase phosphatase; binding proteins; GAPDH; aldolase; far-Western blotting; peptide mass fingerprinting; interaction analysis; phosphorylation; dephosphorylation

It is widely accepted that multifunctional $Ca^{2+}$/calmodulin-dependent protein kinases (CaMKs)[1] mediate various $Ca^{2+}$-signaling pathways in cells [1-4]. CaMKI, CaMKII, and CaMKIV are known as multifunctional CaMKs; CaMKII is known to be activated through autophosphorylation of Thr-286, whereas CaMKI and CaMKIV 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 CaMKs and thus for $Ca^{2+}$-signaling systems [5]. Using unique techniques to identify protein phosphatases involved in dephosphorylation of CaMKs [6, 7], we purified and isolated a $Mn^{2+}$-dependent, okadaic acid/calyculin A-insensitive protein phosphatase with a molecular weight of 54,000, and designated it as CaMK phosphatase (CaMKP) [8, 9]. CaMKP belongs to the PPM family of Ser/Thr protein phosphatases, with homology to PP2Cα being 28% in the catalytic domain of the phosphatase; and it can dephosphorylate and regulate multifunctional CaMKs *in vitro* [5, 9, 10]. Recently, a human homolog of CaMKP was shown to be involved in the regulation of CaMKII activity [11]. Another phosphatase having certain homology with CaMKP was found in the human database and named CaMKP-N as it had similar biochemical properties to CaMKP and its localization was in the nucleus [12]. CaMKP and CaMKP-N are thought to be involved in the negative regulation of multifunctional CaMKs [5]. Recently, a possible involvement of CaMKP in apoptotic signaling has been suggested [13]. In addition, it has been suggested that CaMKP and CaMKP-N participate in the negative regulation of signaling pathways mediated by p21-activated protein kinase to regulate cellular morphology [14].

To clarify the physiological significance of CaMKP, we identify binding proteins of CaMKP in rat brain extract. Using two-dimensional far-Western blotting analysis [15] in combination with peptide mass fingerprinting analysis [16], glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose bisphosphate aldolase (aldolase), are identified as major CaMKP-binding proteins in a soluble fraction of rat brain. We carry out biochemical analysis of the interaction between CaMKP and the binding proteins, and discuss the physiological significance of the interaction.

**Materials and methods**

*Materials.* [$\gamma$-$^{32}$P]ATP (5,000 Ci/mmol) was from Amersham Biosciences. India Ink (Fount India) was from Pelikan. ATP, rabbit muscle aldolase, rabbit muscle GAPDH, bovine serum albumin (BSA), and $\alpha$-casein were from Sigma. $His_6$-tagged wild type CaMKP was prepared as described previously [17]. Recombinant rat CaMKI [18] and recombinant rat CaMKIV [19] were expressed in Sf9 cells and purified as described. Recombinant rat CaMK kinase $\alpha$ expressed in *E. coli* [20] was purified as described previously [21]. *Achromobacter* protease I (Lysyl Endopeptidase®) was obtained from Wako Pure Chemical Industries. Anti-GAPDH monoclonal antibody and anti-aldolase polyclonal antibody were purchased from Chemicon. Anti-CaMKP polyclonal antibody was prepared essentially according to the procedure of Kitani et al [10]. The antiserum was used as an antibody without further purification.

*Two-dimensional far-Western blotting analysis using digoxigenin-labeled CaMKP.* A 100,000 x g supernatant fraction of the crude extract from rat brain stem was prepared essentially as described [8]. The fraction was diluted with 8 M urea containing 0.5% Triton X-100, 0.6% dithiothreitol, and 0.5% Ampholine (pH 3.5-9.5). Immobiline™ DryStrip (pH 3-10, 7cm) (Amersham Biosciences) was rehydrated for 12 hr at 20 °C with the sample, and then isoelectric focusing as the first dimension was carried out at 20 °C using the IPGphor system (Amersham Biosciences) essentially according to the manufacturer's instructions. After the strip was equilibrated as described, SDS-PAGE (10%) [22] was carried out as the second dimension. The separated proteins were electrotransferred onto PVDF membrane (Immobilon P, 0.45 μm, Millipore) [23], blocked for 2 hr at room temperature in 10 mM sodium phosphate buffer containing 0.85% NaCl (PBS) and 0.2% Tween 20, and subsequently used for far-Western blotting analysis. $His_6$-tagged CaMKP was labeled with digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (Boehringer-Mannheim) to prepare digoxigenin-labeled CaMKP (DIG-CaMKP) essentially according to the method as described for the preparation of digoxigenin-labeled PP1c [24].

DIG-CaMKP was overlaid onto the membrane by incubating the blot at 4 °C overnight in a DIG-CaMKP solution (107 nM) diluted with PBS containing 0.02% Tween 20 (PBST) and 5% non-fat milk. The membrane was extensively washed three times for 10 min with PBST, followed by incubation in PBS containing 0.2% Tween 20 for 30 min at room temperature. Subsequently, the membrane was incubated for 1 hr at 25 °C with anti-Digoxigenin-POD, Fab fragments (Boehringer-Mannheim) diluted 1: 900 in PBS containing 0.2% Tween 20. After the membrane was extensively washed three times for 10 min with PBST, DIG-CaMKP-binding proteins were detected by chemiluminescence using Super Signal BLAZE (Pierce). The blot was subsequently stained with India Ink overnight at room temperature as described [23].

*Peptide mass fingerprinting analysis.* The two-dimensional gel electrophoresed in a parallel experiment with far-Western blotting was stained with Coomassie Brilliant Blue (CBB) using Quick CBB (Wako Pure Chemical Industries). The portion of the gel containing a protein spot of interest was excised. The CBB-stained spot was washed with 400 µl of $H_2O$ for 1 hr at room temperature, and then destained by vigorous washing with 200 µl of 200 mM $NH_4HCO_3$ containing 50% acetonitrile for 10 min; destaining was then repeated twice more. After the gel piece was washed with 200 µl of 100% acetonitrile, it was then dehydrated in a SpeedVac concentrator. The dried gel piece was rehydrated for 45 min on ice with *Achromobacter* protease I solution (25 µg/ml) in 10 mM Tris-HCl (pH 8.8), followed by overnight incubation at 37 °C with additional 10 µl of 10 mM Tris-HCl (pH 8.8). The reaction was quenched with 30% acetonitrile/5% formic acid, and then the gel piece was washed by vortexing for 5 min; the washing was pooled and the step repeated once more. Next, 50% acetonitrile/5% formic acid was added to the gel piece, and it was washed by vortexing for 5 min; the washing was again pooled and the step repeated once more. All the pooled washings were combined, and concentrated to about 5 µl with a SpeedVac concentrator. The sample was then absorbed onto a Zip-TipC18 (Millipore). The desalted peptides were eluted according to the manufacturer's instructions, and analyzed by MALDI-TOF mass spectrometry using a REFLEX III mass spectrometer (Brucker Daltonics). The peptide masses were used to search the NCBI Non-redundant Protein Database using the

Mascot program [25].

*Measurements of polarized fluorescence.* His6-tagged CaMKP was labeled with Fluorescein Amine Labeling Kit (PanVera) to prepare fluorescein-labeled CaMKP (F-CaMKP) essentially according to the manufacturer's instructions. Fluorescence polarization assay was carried out at 30 °C in a reaction mixture comprising 50 mM Hepes-NaOH (pH 8.0), 0.1 mM EGTA, 0.01% Tween 20, 1 mM dithiothreitol, 6.3 nM F-CaMKP, and the indicated concentration of the protein. The mixture was incubated for 10 min in the dark, and then the fluorescence polarization of F-CaMKP was measured on a Beacon 2000 (PanVera). The ratio of the concentration of bound F-CaMKP to that of total F-CaMKP was calculated from the increase in the fluorescence polarization upon binding according to Eq.1,

$$\frac{[\text{bound F-CaMKP}]}{[\text{total F-CaMKP}]} = \frac{A - A_f}{A_b - A_f} \qquad \text{(Eq. 1)}$$

where A is the measured anisotropy in a mixture of free and bound F-CaMKP with anisotropies of $A_f$ and $A_b$, respectively [26]. The ratios were plotted against concentrations of the binding proteins. Kd values were determined from a direct fit of the data to Eq. 2 [26] using a nonlinear regression program (DeltaGrapgh 4.5, Delta Point).

$$\frac{[\text{bound F-CaMKP}]}{[\text{total F-CaMKP}]} = \frac{[\text{CaMKP-binding protein}]}{Kd + [\text{CaMKP-binding protein}]} \qquad \text{(Eq. 2)}$$

*Immunoprecipitation.* A 100,000 x g supernatant fraction of the crude extract of rat brain stem was prepared as described [8], except that a homogenization buffer containing neither EDTA nor EGTA was used. Either anti-CaMKP antibody or control normal rabbit serum (DAKO) was added to the extract, followed by incubation on ice for 1 hr. Protein G-Sepharose gel suspension (50% v/v) (Amersham Biosciences) was added to the mixture, which was shaken for 1.5 hr at 4 °C. The immunoprecipitated complex was washed four times with 50 mM sodium phosphate buffer (pH 7.1) containing 1% Triton X-100, 0.5 M NaCl, and 0.05% Tween 40. The complex was solubilized by Laemmli's sample buffer [22] consisting of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 130 mM 2-mercaptoethanol. The sample was then subjected to electrophoresis on a

10% SDS-polyacrylamide gel, followed by Western blotting analysis using anti-GAPDH monoclonal antibody.

*Preparation of CaMKP deletion mutants.* The vector pET-23a(+) encoding a rat CaMKP deletion mutant (1-414) tagged with $His_6$ at the COOH-terminus was prepared as described below. The following primers were used for PCR: UCP10/Nhe (5'-AAA GCT AGC GGA GCC CCA CAG AAC AGC AG-3') with an *Nhe*I site (underlined) and LCP1216/Xho (AA CTC GAG GGG GTC CCT AAG GAA AAC CAC) with an *Xho*I site (underlined). PCR was performed in a GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles (each consisting of denaturation for 10 s at 96 ℃, annealing for 10 s at 60 ℃, and extension for 2 min at 72 ℃) using Pyrobest DNA polymerase (Takara Shuzo) and a rat brain RACE-ready cDNA library as a template. After gel purification, the amplified product was digested with *Nhe*I and *Xho*I, and cloned into pET-23a(+) (Novagen), which was designated as pETrCaMKP(1-414). The N-terminal truncation mutants with GST at N-terminus were constructed by PCR as follows. PCRs were performed with a 5' primer containing an *Eco*RI site (underlined) (5'-A GAA TTC ATG GCC TCT GGA GCC CCA CAG AA-3', for G1-414H; 5'-GGG GAA TTC CTC TCT GAA TTT AAG AGG TTG CCT-3', for G91-414H; 5'-AAA GAA TTC GAT GCC AAA GGC CTG TCC CGG-3', for G117-414H; 5'-TTT GAA TTC TAC TTC GCT GTG TTT GAT GGT CAT-3', for G189-414H) and a 3' primer containing a *Sal*I site (underlined): (5'-TTT GTC GAC TCA GTG GTG GTG GTG GTG GTG GTG-3') using Pyrobest DNA polymerase and pETrCaMKP(1-414) as a template. PCR fragments were digested with *Eco*RI and *Sal*I, and subcloned into pGEX 4T-1 (Amersham Biosciences). To obtain a plasmid vector encoding GST-fused wild type and the C-terminal deletion mutants of CaMKP, DNA fragments were prepared by PCR using a 5' primer containing an *Eco*RI site (underlined) (5'-A GAA TTC ATG GCC TCT GGA GCC CCA CAG AA-3') and a 3' primer containing an *Xho*I site (underlined) (5'-AA CTC GAG GCT TCT CTG TGA GGT ATT GAT CTC AAG-3', for G-WT; 5'-AA CTC GAG GGG GTC CCT AAG GAA AAC CAC-3', for G1-414; 5'-TT CTC GAG GGC GG GTG CAC GGA GTC AGA-3', for G1-188; 5'- TT CTC GAG TAG GGT CGT GAG GAC TCT CTC TTC-3', for G1-116; 5'-TT CTC GAG AGG CAA CCT

CTT AAA TTC AGA GAG-3', for G1-98). These fragments were digested with *Eco*RI and *Xho*I, and subcloned into pGEX 4T-1. The sequences of these constructs were verified with a DNA sequencer (Applied Biosystems; model 3100).

The constructs were transformed into *E. coli* strain BL21(DE3). The transformed bacteria, except those transformed with the expression plasmids for G1-414H, G91-414H, G117-414H, and G189-414H mutants, were grown in LB medium containing 100 μg/ml ampicillin, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. After the recombinant proteins were sufficiently induced, the bacteria were harvested by centrifugation and the harvested cell pellets suspended in 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (TBS), and then disrupted by sonication. The extract was cleared by centrifugation, and the supernatants used for the following pull-down binding assays. Since G91-414H, G117-414H, and G189-414H mutants were exclusively expressed in the insoluble fraction, the bacterial cells expressed G1-414H, G91-414H, G117-414H, and G189-414H mutants, which were grown and induced as above, were then suspended in 50 mM Tris-HCl (pH 7.0) containing 8 M urea and 20 mM 2-mercaptoethanol followed by sonication to solubilize the mutant proteins. After the extract was cleared by centrifugation, the supernatants were dialyzed against dialysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.05% Tween 20, 20 mM 2-mercaptoethanol, and 4 M urea for 2 hr at 4 ℃. The dialysis buffer was diluted with an equal volume of the dialysis buffer without urea, and dialysis was continued for 16 hr. The dialysis buffer was then changed with the dialysis buffer without urea and the dialysis continued for further 2 hr. The dialysate thus obtained was cleared by centrifugation, and the supernatant used for the following pull-down binding assays.

*Pull-down binding assay.* GST pull-down binding assay was carried out as follows. Glutathione Sepharose 4B resin (Amersham Biosciences) was incubated for 60 min at 4 ℃ with the cell lysate prepared from mock transfected *E. coli* with gentle shaking. The resin was washed three times with TBS and suspended into TBS to make a 50% v/v suspension. The cell lysate (100 μl) prepared from the transformed *E. coli* as described above was incubated with GAPDH (4 μg) for 60 min at 4 ℃ with gentle shaking. The washed glutathione Sepharose 4B resin (10 μl) suspension was added to the mixture and incubated for

a further 60 min at 4°C with gentle shaking. Then, the resin was washed three times with TBS containing 0.05% Tween 40 (TBST), and incubated with 20 μl of 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione for 30 min at 4 °C with gentle shaking to elute the binding proteins from the resin. The eluted proteins were analyzed by Western blotting using anti-GAPDH monoclonal antibody.

GAPDH pull-down binding assay was carried out as follows. GAPDH (3.2 mg) was immobilized on CNBr-activated Sepharose 4B (0.3 g, Amersham Biosciences) according to the manufacturer's instructions. The immobilized GAPDH resin (10 μl) was treated with the cell lysate (100 μl) prepared from mock transfected *E. coli* as described above, and incubated for 60 min at 4 °C with the cell lysate (100 μl) prepared from the transformed *E. coli* with gentle shaking. The resin was washed three times with TBST. Laemmli's sample buffer (20 μl) was added to the washed resin and the suspension boiled for 2 min to solubilize the binding proteins. The solubilized proteins were analyzed by Western blotting using anti-GST antibody conjugated with horseradish peroxidase (Amersham Biosciences).

*Phosphorylation of GAPDH.* The purified recombinant rat CaMKI and CaMKIV were activated by incubation with CaMK kinase α as described previously [27]. GAPDH was phosphorylated at 30 °C by the indicated concentrations of the activated CaMKs in the presence or absence of CaMKP in a reaction mixture containing 40 mM Hepes-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 1 μM calmodulin, 0.1 mM EGTA, 0.5 mM $CaCl_2$, 0.01% Tween 20, and 50 μM [$\gamma$-$^{32}$P]ATP. After incubation for the indicated times, the incorporation of [$^{32}$P]phosphate into GAPDH was determined by the 3MM filter paper method of Corbin and Reinmann [28], except that the filter papers were washed with 10% trichloroacetic acid containing 1% sodium pyrophosphate. In some cases, the phosphorylation reaction was terminated by adding an equal volume of the 2 x Laemmli's sample buffer. The mixture was then boiled for 2 min, and an aliquot of the supernatant subjected to SDS-PAGE on a 10% polyacrylamide gel. The gel was dried and visualized by autoradiography.

*Dephosphorylation of CaMKI, CaMKIV, and GAPDH.* CaMKI and CaMKIV were phosphorylated by CaMK kinase for 10 min at 30 °C under the same conditions described above for the phosphorylation of GAPDH. GAPDH was phosphorylated by the activated

CaMKI or CaMKIV for 60 min at 30 °C as described above; at the end of the reaction, 10 mM EDTA was added and the solution kept on ice until the following dephosphorylation reaction was started. The mixture was then diluted 10 times with a solution composed of the same components as those of the phosphorylation mixture except that CaMKP (2.3 μM) or EDTA (25 mM) was included and that cold ATP was used instead of hot ATP. The dephosphorylation reaction was allowed to proceed at 30 °C. At the indicated times, aliquots were withdrawn, combined with an equal volume of the 2 x Laemmli's sample buffer, and subjected to SDS-PAGE on a 10% polyacrylamide gel. The gel was then dried and visualized by autoradiography.

*Assay of CaMKP activity.* $His_6$-tagged CaMKP was assayed using pp39 as a substrate as described previously [29] except that 0.07-2.8 μM GAPDH or aldolase was added instead of poly-L-lysine. CaMKP activity was also assessed as described [17] except that 0.07-0.7 μM GAPDH or aldolase was added instead of poly-L-lysine.

*Assay of GAPDH activity.* The glycolytic activity assay of GAPDH was carried out at 25 °C as described [30]. When the effect of CaMKP was examined, GAPDH activity was assessed after incubation of 8.7 nM GAPDH with 1-2 μM $His_6$-tagged CaMKP for 1.5 min. When the effect of phosphorylation was examined, GAPDH activity was assessed immediately after the phosphorylation reaction by CaMKI or CaMKIV for 15 min as above.

*Other analytical procedures.* Protein concentrations were determined by the method of Lowry et al. [31], as modified by Peterson [32], with BSA as a standard. Western blotting analysis was performed as described by Harlow and Lane [23]. The Western blot was developed by an enhanced chemiluminescence procedure (Super Signal BLAZE, Pierce). Unless otherwise specified, molecular weights for rabbit GAPDH and aldolase were calculated as tetramers, and were taken as 143,312 and 157,372, respectively. The molecular weight for $His_6$-tagged rat CaMKP was taken as 51,328.

## Results

*Identification of CaMKP-binding proteins in rat brain extract*

To identify CaMKP binding proteins in brain, we carried out two-dimensional far-Western blotting analysis of rat brain extract using DIG-CaMKP as a probe. Proteins in the extract of rat brain stem were resolved by two-dimensional electrophoresis followed by blotting onto a PVDF membrane. DIG-CaMKP was overlaid onto the membrane, and the DIG-CaMKP-binding spots visualized as described in "Materials and methods." As shown in Fig. 1A, four spots (spots 1-4) were discernible on the blot, which was then stained with India Ink, and the far-Western positive protein spots localized on the blot. In a parallel experiment, the same amount of extract was subjected to two-dimensional electrophoresis under the same conditions as those for the far-Western blotting experiments, followed by CBB staining. We then compared the protein spots on the CBB-stained gel with those on the India-ink stained PVDF membrane to isolate the CBB-stained protein spots corresponding to the far-Western positive spots (Fig. 1B, spots 1-4). These protein spots were excised from the CBB-stained gel and subjected to peptide mass fingerprinting analysis as described in "Materials and methods." A database search using the Mascot search engine suggested that spots 1 and 2 were aldolase and that spots 3 and 4 were GAPDH (Table 1).

To confirm the identification, we examined whether or not DIG-CaMKP could bind to commercially available GAPDH and aldolase, which had been purified from rabbit muscle, by far-Western blotting. Indeed, DIG-CaMKP markedly bound to purified GAPDH and aldolase blotted onto a PVDF membrane (Fig. 2A). Since sequence identity between the rabbit and rat proteins are 94.9% and 96.7%, for aldolase and GAPDH, respectively, it is strongly suggested that aldolase and GAPDH are CaMKP-binding proteins.

*Binding of GAPDH and aldolase with CaMKP in solution*

Next, we examined whether CaMKP could bind to GAPDH and aldolase in solution. For this we carried out fluorescence polarization binding assay using F-CaMKP.

Fluorescence polarization gives a direct measure of the bound/free ratio for protein-protein interaction, and if viscosity and temperature of the reaction mixture are held constant, an increase in the polarization value indicates binding of the labeled protein to its binding counterparts [33, 34]. As shown in Table 2, a significant increase in the fluorescence polarization of F-CaMKP was observed when GAPDH or aldolase was added to the mixture containing F-CaMKP, whereas none was observed when BSA or α-casein was added. Therefore, it is suggested that GAPDH and aldolase interact with CaMKP in solution.

To estimate binding affinities of GAPDH and aldolase for CaMKP, we examined the effect of increasing concentrations of GAPDH and aldolase on their binding to F-CaMKP, as shown in Fig. 3. Nonlinear regression analysis of the binding data yielded Kd values of 320 ± 26 nM and 1014 ± 338 nM for GAPDH and aldolase, respectively (n =3).

Next, we examined whether CaMKP activity was affected by binding to GAPDH or aldolase. When CaMKP activity was assessed as described in "Materials and methods," no significant activation or inhibition of CaMKP by GAPDH or aldolase was detected (data not shown). We also examined whether GAPDH activity was affected by binding to CaMKP. The glycolytic activity of GAPDH was not significantly altered in the presence or absence of CaMKP (data not shown).

*CaMKP-GAPDH complex in rat brain*

To ascertain whether GAPDH and/or aldolase would form a complex in brain, we carried out immunoprecipitation of CaMKP from rat brain extract using CaMKP-specific antibody. This antibody specifically detected CaMKP in rat brain extract by Western blotting [10], and could efficiently precipitated CaMKP (data not shown). As shown in Fig. 4, CaMKP-specific antibody coimmunoprecipitated GAPDH, which was detected by Western blotting using a specific anti-GAPDH antibody from rat brain stem extract, whereas control rabbit antiserum did not coprecipitate GAPDH. Thus, CaMKP is, at least in part, likely to form a complex with GAPDH in brain. On the other hand, no significant amount of aldolase was detected in the immunoprecipitate by Western blotting using a specific anti-aldolase antibody (data not shown).

*The GAPDH-binding region of CaMKP*

Since the formation of CaMKP-GAPDH complex was observed in brain extract, we carried out further analysis focused on the CaMKP-GAPDH complex. To identify the binding region within CaMKP that interacts with GAPDH, we constructed various deletion mutants of CaMKP fused with GST (Fig. 5A, B), and examined their binding capacity to GAPDH by GST pull-down assay. As shown in Fig. 5C, significant binding of GAPDH to wild type CaMKP fused with GST (G-WT) was shown in the pull-down assay, whereas GST alone did not (GST); again confirming that CaMKP can bind to GAPDH in solution. All of the C-terminal deletion mutants (G1-414, G1-188, G1-116, G1-98) and the mutant devoid of the Glu cluster in the N-terminal domain of CaMKP (G-ΔE) bound to GAPDH. Even the residues 1 to 98 of CaMKP fused with GST (G1-98) could bind to GAPDH, suggesting that the binding region for GAPDH is located within the N-terminal region of CaMKP, the residues 1 to 98. To further confirm these observations, we carried out another pull-down assay using GAPDH immobilized on Sepharose resin (GAPDH pull-down assay). In this assay, the resin should pull down GST-fusion proteins that could bind to GAPDH. When the precipitated proteins were analyzed by Western blotting using anti-GST antibody, specific binding of GAPDH to wild type CaMKP and to all of the C-terminal deletion mutants of CaMKP were shown (Fig. 5D); again confirming the binding ability of GAPDH to CaMKP. Of the N-terminal deletion mutants of CaMKP obtained (G1-414H, G91-414H, G117-414H, G189-414H), only G1-414H was pulled down in the GAPDH pull-down assay (Fig. 5E). Thus, it is strongly suggested that the N-terminal region, residues 1 to 98, of CaMKP is responsible for the binding of GAPDH.

*Effects of CaMKP on the phosphorylation of GAPDH by CaMKs*

It is reported that GAPDH is efficiently phosphorylated by CaMKII [35, 36], and as shown in Fig. 6, GAPDH was also phosphorylated by CaMKI and CaMKIV. Although the rate of phosphorylation by these CaMKs was somewhat slower than that of CaMKII, the stoichiometry of the phosphorylation by CaMKI (0.7 mol/mol) and CaMKIV (0.8 mol/mol)

was nearly comparable to that of CaMKII (0.9-1.0 mol/mol). When GAPDH was phosphorylated by CaMKI and CaMKIV in the presence of CaMKP, phosphorylation of GAPDH by both kinases was markedly attenuated (Fig. 7A). To see whether the attenuation of GAPDH phosphorylation was due to deactivation of the kinases by dephosphorylation or due to dephosphorylation of the phosphorylated GAPDH itself, CaMKI and CaMKIV that had been previously phosphorylated by CaMK kinase were incubated with CaMKP under the same conditions as those for GAPDH phosphorylation. As shown in Fig. 7B, CaMKI was rapidly dephosphorylated within 10 min. CaMKIV was also significantly dephosphorylated by CaMKP within 20 min, though it was somewhat refractory to the dephosphorylation compared to CaMKI; probably due to the subsequent autophosphorylation of serine residues of CaMKIV [37], which is refractory to dephosphorylation by CaMKP [29]. Since $Mn^{2+}$ is absolutely required for CaMKP activity [8], it is interesting that CaMKP dephosphorylated phosho-CaMKI and phospho-CaMKIV in the presence of $Mg^{2+}$ instead of $Mn^{2+}$. $Mg^{2+}$-dependent CaMKP activity, which was too low to detect under the conventional assay conditions, could be detected under those where the CaMKP concentration was higher than the substrate. In contrast, neither GAPDH phosphorylated by CaMKI nor GAPDH phosphorylated by CaMKIV was significantly dephosphorylated by CaMKP under the same conditions (Fig. 7C). Therefore, deactivation of the kinases through dephosphorylation by CaMKP is responsible for the attenuation of the GAPDH phosphorylation. The results suggest that CaMKP forms a complex with GAPDH to dephosphorylate/deactivate CaMKs, thereby keeping GAPDH phosphorylation at a low level.

## Discussion

Recent studies on protein phosphatases have revealed that they associate with various proteins to form stable complexes [38-40]. Protein phosphatases in these complexes play pivotal roles in the regulation of cellular signaling through dephosphorylation of the target proteins in the complexes. CaMKP, which belongs to the PPM family of protein phosphatases, was first identified and purified as a novel protein phosphatase that

dephosphorylates and regulates multifunctional CaMKs [5, 8, 9]. Although a possible involvement of CaMKP in apoptosis [13] and regulation of cellular morphology [14] is suggested, the physiological functions of the phosphatase remain to be clarified.

Using the two-dimensional far-Western blotting technique with DIG-CaMKP as a probe, in conjunction with peptide mass fingerprinting analysis, we identified GAPDH and aldolase as major binding partners of CaMKP in a soluble fraction of rat brain. The binding of these partners to CaMKP was also shown by fluorescence polarization assay. These results support the concept proposed by Pasquali et al. that two-dimensional far-Western blotting approach can be generally applied to identify possible protein-protein interactions [15]. Binding assay using F-CaMKP showed that Kd for the F-CaMKP-GAPDH interaction and for the F-CaMKP-aldolase interaction were 320 nM and 1014 nM, respectively. Since *in vivo* concentrations of GAPDH and aldolase were estimated to be 1-5 μM [41] and > 6 μM [42], respectively, it is highly likely that the CaMKP-GAPDH and CaMKP-aldolase interactions occur *in vivo*. The observation that a specific antibody against CaMKP coimmunoprecipitated GAPDH from brain extracts also supports the existence of CaMKP-GAPDH complex *in vivo*. Since the Kd value for aldolase is somewhat larger than that for GAPDH, the inability to detect the coimmunoprecipitation of aldolase with anti CaMKP antibody may be due to the low affinity of aldolase to CaMKP; not enough to detect the complex by the immunoprecipitation assay.

Sim et al. suggested that association with numerous proteins regulates the PPM family phosphatases, as with the PPP family phosphatases [40]. Indeed, it is reported that other PPM family protein phosphatases such as PP2Cβ and PP2Cε associate with various proteins to regulate cellular signaling [43-45]. Here, we report that CaMKP associates with GAPDH and aldolase, but that CaMKP activity was not significantly affected in the presence of GAPDH or aldolase (data not shown). CaMKP and GAPDH are cytosolic enzymes, and GAPDH is known to regulate glycolysis by interacting with various proteins including aldolase [46]. Although the glycolytic activity of GAPDH itself was not significantly altered by CaMKP binding (data not shown), binding of CaMKP to GAPDH might affect such multi-protein interaction to regulate glycolysis.

CaMKP and CaMKP-N have unique N-terminal domains that are not shared by other PPM family phosphatases; the functions of these domains remain to be elucidated [5]. In an earlier report, we showed that the Glu cluster 101-109 of CaMKP is a binding site for polycations, and is responsible for polycation-stimulated activation of CaMKP [17]. In this report, we have shown that GAPDH binds to the N-terminal region, residues 1-98, of CaMKP as revealed by GST- and GAPDH pull-down binding assays (Fig. 5). Since the mutant CaMKP devoid of the Glu cluster 101-109 (G-ΔE) bound GAPDH as the wild type enzyme did (Fig. 5, C &D), the Glu cluster is shown to not be essential for binding to GAPDH. It is also suggested that the N-terminal region 65-191 of human CaMKP is responsible for the binding of PIX [14]. Thus, one of the functions of the N-terminal domain of CaMKP appears to be interaction with its binding counterparts. Very recently, it was reported that human CaMKP directly interacts with CaMKII through multiple regions of CaMKP, of which the N-terminal region is not essential for the binding of human CaMKP to CaMKII [11]. Since autophosphorylated CaMKII is a good substrate for CaMKP, the CaMKII-binding region may be different from those of other CaMKP-binding proteins that do not serve as CaMKP substrates such as GAPDH.

GAPDH is reported to be phosphorylated by several protein kinases including CaMKII [35, 36], protein kinase C (PKC) [47], and epidermal growth factor receptor kinase [48]. Phosphorylation of GAPDH by PKC is suggested to be involved in cellular motility and energy metabolism [49]. Another report revealed that phosphorylation of GAPDH occurs in cells and that it is involved in viral infection, though the responsible protein kinases were not identified [50]. It has also been reported that phosphorylation of GAPDH by PKC ι/λ influences microtubule dynamics in the early secretory pathway [51]. Again very recently, Singh et al. have shown that CaMKIIβM can directly phosphorylate and activate GAPDH *in vitro* [36]. They suggested that the activation of CaMKIIβM would serve to modulate GAPDH and thereby ATP and NADH levels at the sarcoplasmic reticulum membrane. However, the physiological significance of the phosphorylation of GAPDH by other protein kinases has not yet been clarified. Based on computational analysis of 3-dimensional structures, Torshin suggested that phosphorylation of glycolytic enzymes

including GAPDH by various protein kinases is of physiological significance through influencing "activating oligomerization" mechanisms of the enzymes [52]. But these predictions need to be confirmed by biochemical studies.

In this paper, we reported that CaMKI and CaMKIV phosphorylated GAPDH *in vitro*. Since the phosphorylation did not significantly affect the glycolytic activity of GAPDH (data not shown), the physiological significance of the phosphorylation of GAPDH by CaMKI and CaMKIV is unclear. Nevertheless, the data presented here suggest that CaMKP regulates the phosphorylation level of GAPDH in the CaMKP-GAPDH complex by deactivating activated CaMKs that are responsible for the phosphorylation of GAPDH. Since the phosphorylated GAPDH was not dephosphorylated by CaMKP, such regulation is not due to the direct dephosphorylation of the phosphorylated binding proteins, so far well documented, but due to the abrogation of the phosphorylation through deactivation of the responsible protein kinases by dephosphorylation. By forming a complex with GAPDH, CaMKP might keep the phosphorylation of GAPDH at a low level. It has been reported that human CaMKP can regulate phosphorylation level of vimentin, which is a CaMKII substrate, through deactivation of CaMKII, though it is not clear whether or not vimentin and CaMKP form a complex [11].

Growing evidence suggests that GAPDH is a multifunctional protein not only with glycolytic activity but also with various other activities such as DNA repair, RNA binding and tubulin bundling [53]. CaMKP might affect these newly ascertained activities of GAPDH by direct binding to it or by the regulation of its phosphorylation level through dephosphorylation/deactivation of CaMKs. Especially, it is noteworthy that GAPDH is reported to be a general mediator of cell death [54, 55]. Since Tan et al. reported that overexpression of CaMKP can induce apoptosis [13], it will be interesting to clarify the relationship between CaMKP and GAPDH as a proapoptotic protein. They also reported that the N-terminal deletion mutants of human CaMKP are more potent than the wild type in inducing apoptosis. This might be due to the disruption of interactions with some proteins such as GAPDH that are responsible for regulation of apoptotic pathways through the CaMKP's N-terminal domain, which was shown in this study to be responsible for the

GAPDH-binding. Thus, further identification and analysis of binding proteins for CaMKP in various cells and tissues are likely to be valuable not only for exploring the physiological significance of CaMKP, but also for elucidation of molecular mechanisms by which CaMKP induces apoptosis.

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**Footnotes** (for the title page)

[1]Abbreviations used: aldolase, fructose bisphosphate aldolase; BSA, bovine serum albumin; CaMK, $Ca^{2+}$/calmodulin-dependent protein kinase; CaMKP, $Ca^{2+}$/calmodulin-dependent protein kinase phosphatase; CBB, Coomassie Brilliant Blue; DIG-CaMKP, digoxigenin-labeled CaMKP; F-CaMKP, fluorescein-labeled CaMKP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase.

**Figure legends**

Fig. 1. Detection of CaMKP-binding proteins by two-dimensional far-Western blotting analysis. (A) A crude extract prepared from rat brain stem (125 μg) was analyzed by two-dimensional far-Western blotting with DIG-CaMKP as a probe as described in "Materials and methods." (B) In a parallel experiment, the same amount of the extract was subjected to two-dimensional electrophoresis under the same conditions as above, followed by CBB staining. The protein spots on the CBB-stained gel were compared with those on the India-ink stained PVDF membrane to isolate the CBB-stained protein spots corresponding to the far-Western positive spots as described in the text. The far-Western positive spots and the corresponding CBB-stained protein spots are indicated by arrows (spots 1-4).

Fig. 2. Binding of DIG-CaMKP to GAPDH and aldolase blotted onto PVDF membrane. (A) The indicated amount of rabbit muscle GAPDH (lanes 2-6) and rabbit muscle aldolase (lanes 7-11) together with a crude extract prepared from rat brain stem (44 μg, lane 1) were subjected to SDS-PAGE on a 10% polyacrylamide gel, followed by far-Western blotting analysis using DIG-CaMKP as a probe. As a negative control, the same amount of BSA was mixed with GAPDH and aldolase. (B) The same blot was stained with India Ink overnight at room temperature.

Fig. 3. Effect of varying concentrations of GAPDH and aldolase on the fluorescence polarization of F-CaMKP. (A) Fluorescence polarization of F-CaMKP was measured in the presence of varying concentrations of GAPDH as described in "Materials and methods." The ratios of the concentration of bound F-CaMKP to that of total F-CaMKP were calculated and plotted against the concentrations of GAPDH. (B) Fluorescence polarization of F-CaMKP was measured in the presence of the varying concentrations of aldolase. The ratios of the concentration of bound F-CaMKP to that of total F-CaMKP were calculated and plotted against the concentrations of aldolase. Data are means ± SD values from three independent determinations.

Fig. 4. Coimmunoprecipitation of CaMKP and GAPDH from rat brain extract using CaMKP-specific antibody. A crude extract from rat brain stem was subjected to immunoprecipitation using an anti-CaMKP antibody (lane 2) or control normal rabbit serum (lane 3) as described in "Materials and methods." The immunoprecipitates were analyzed by Western blotting with anti-GAPDH antibody. Purified GAPDH from rabbit muscle was also loaded as positive control (lane 1). The data presented are representative of at least five independent experiments with similar results.

Fig. 5. Binding of GAPDH to CaMKP and its deletion mutants. (A) A schematic illustration of CaMKP-GST fusion proteins used in this study. Closed bars show GST, and open bars show CaMKP with the amino acid residue numbers of CaMKP being indicated. G-WT depicts the full length CaMKP coding region fused to GST. G-ΔE depicts a CaMKP deletion mutant, which is devoid of a Glu cluster 101-109, fused with GST. Gx-y depicts a fusion protein composed of GST and amino acid residues x-y of CaMKP as indicated. H indicates $His_6$ tag attached to the C-terminal portion of the fusion protein. The expression plasmids for these mutants were constructed and expressed as described in "Materials and methods." (B) CaMKP-GST fusion proteins expressed in *E. coli* were analyzed by SDS-PAGE on a 10% polyacrylamide gel. The gel was stained with CBB. (C) GST pull-down binding assay was carried out using the indicated CaMKP mutants as baits as described in "Materials and methods." Total CaMKP mutants used in the assay (Total) and bound GAPDH (GST pull-down) were analyzed by Western blotting with anti-GST antibody and anti-GAPDH antibody, respectively. (D) GAPDH pull-down binding assay was carried out using the indicated CaMKP mutants, in which the C-terminal portion was deleted, as preys as described in "Materials and methods." Total (Total) and bound (GAPDH pull-down) CaMKP mutants were analyzed by Western blotting with anti-GST antibody. (E) GAPDH pull-down binding assay was carried out using the indicated CaMKP mutants, in which the N-terminal portion was deleted, as preys as described in "Materials and methods." Total (Total) and bound (GAPDH pull-down)

CaMKP mutants were analyzed by Western blotting with anti-GST antibody.

Fig. 6. Phosphorylation of GAPDH by CaMKI and CaMKIV. GAPDH (0.57 μM) was phosphorylated at 30 °C by recombinant rat CaMKI (0.5 μg/ml, open circle) or CaMKIV (0.5 μg/ml, closed circle), which had been activated by CaMK kinase. The incorporation of [$^{32}$P]phosphate into GAPDH was determined by the 3MM filter paper method as described in "Materials and method."

Fig. 7. Effect of CaMKP on the phosphorylation of GAPDH by CaMKI and CaMKIV. (A) GAPDH (0.57 μM) was phosphorylated for 10 min at 30 °C by activated CaMKI (0.5 μg/ml, lanes 1-4) or activated CaMKIV (0.5 μg/ml, lanes 5-8) in the presence or absence of CaMKP (2.3 μM). After the reaction was terminated, the mixture was subjected to SDS-PAGE, followed by autoradiography as described in "Materials and methods." (B) Dephosphorylation of activated CaMKI and activated CaMKIV by CaMKP under the phosphorylating conditions. CaMKI (1 μg/ml, lanes 1-5) or CaMKIV (1 μg/ml, lanes 6-10), which had been phosphorylated by CaMK kinase for 10 min at 30 °C, was incubated in the presence of EDTA (25 mM, lanes 2, 3, 7, 8) or CaMKP (2.3 μM, lanes 4, 5, 9, 10) as described in "Materials and methods." After the reaction was terminated, the mixture was subjected to SDS-PAGE, followed by autoradiography. (C) Inability of CaMKP to dephosphorylate GAPDH phosphorylated by CaMKI and CaMKIV under the phosphorylating conditions. GAPDH (0.59 μM) was phosphorylated by activated CaMKI (lanes 1-5) or activated CaMKIV (lanes 6-10) for 60 min at 30 °C. The mixture was further incubated in the presence of EDTA (10 mM, lanes 2, 3, 7, 8) or CaMKP (2.3 μM, lanes 4, 5, 9, 10) as described in "Materials and methods." After the reaction was terminated, the mixture was subjected to SDS-PAGE, followed by autoradiography.

**Table**

Table 1.

Identification of far-Western positive spots by peptide mass fingerprinting analysis.

### A: spot 1

Index

| | Accession | Mass | Score | Description |
|---|---|---|---|---|
| **1.** | **gi\|6978487** | **39327** | **101** | **aldolase A; aldolase A, fructose-bisphosphate [Rattus norvegicus]** |
| 2. | gi\|68186 | 39235 | 101 | fructose-bisphosphate aldolase (EC 4.1.2.13) A - rat |
| 3. | gi\|6671539 | 39331 | 101 | aldolase 1, A isoform [Mus musculus] |
| 4. | gi\|7548322 | 39526 | 101 | aldolase A [Mus musculus] |
| 5. | gi\|229506 | 38993 | 61 | aldolase C |

Results List

**1. gi|6978487 Mass: 39327 Score: 101 aldolase A; aldolase A, fructose-bisphosphate [Rattus norvegicus]**

| *Observed* | Mr(expt) | Mr(calc) | Delta | Start | End | Miss | Peptide |
|---|---|---|---|---|---|---|---|
| *861.49* | 860.48 | 860.47 | 0.01 | 48 | 153 | 0 | WRCVLK Carbamidomethyl (C) |
| *1288.67* | 1287.66 | 1287.67 | -0.01 | 331 | 342 | 0 | RALANSLACQGK Carbamidomethyl (C) |
| *1342.70* | 1341.69 | 1341.70 | -0.01 | 88 | 99 | 0 | ADDGRPFPQVIK |
| *1377.70* | 1376.69 | 1376.71 | -0.02 | 2 | 13 | 0 | PHPYPALTPEQK |
| *1489.82* | 1488.81 | 1488.84 | -0.03 | 15 | 28 | 0 | ELADIAHRIVAPGK |
| *2258.05* | 2257.04 | 2257.03 | 0.01 | 343 | 364 | 0 | YTPSGQSGAAASESLFISNHAY |

No match to: 1197.66, 1475.71, 1505.78, 1695.87

### B: spot 2

Index

| | Accession | Mass | Score | Description |
|---|---|---|---|---|
| **1.** | **gi\|6978487** | **39327** | **77** | **aldolase A; aldolase A, fructose-bisphosphate [Rattus norvegicus]** |
| 2. | gi\|68186 | 39235 | 77 | fructose-bisphosphate aldolase (EC 4.1.2.13) A - rat |
| 3. | gi\|6671539 | 39331 | 58 | aldolase 1, A isoform [Mus musculus] |
| 4. | gi\|7548322 | 39526 | 58 | aldolase A [Mus musculus] |
| 5. | gi\|30147453 | 16395 | 52 | hypothetical protein XP_302088 [Homo sapiens] |

Results List

**1. gi|6978487 Mass: 39327 Score: 77 aldolase A; aldolase A, fructose-bisphosphate [Rattus norvegicus]**

| *Observed* | Mr(expt) | Mr(calc) | Delta | Start | End | Miss | Peptide |
|---|---|---|---|---|---|---|---|
| *861.55* | 860.54 | 860.47 | 0.07 | 148 | 153 | 0 | WRCVLK Carbamidomethyl (C) |
| *1342.79* | 1341.78 | 1341.70 | 0.08 | 88 | 99 | 0 | ADDGRPFPQVIK |
| *1377.78* | 1376.77 | 1376.71 | 0.06 | 2 | 13 | 0 | PHPYPALTPEQK |
| *1489.91* | 1488.90 | 1488.84 | 0.06 | 15 | 28 | 0 | ELADIAHRIVAPGK |
| *2258.18* | 2257.17 | 2257.03 | 0.14 | 343 | 364 | 0 | YTPSGQSGAAASESLFISNHAY |

No match to: 1058.72, 1108.70, 1197.74, 1482.80, 1695.95, 2650.59

### C: spot 3

Index

| | Accession | Mass | Score | Description |
|---|---|---|---|---|
| 1. | gi\|9838358 | 18510 | 98 | glyceraldehyde 3-phosphate dehydrogenase GAPDH [Marmota monax] |
| **2.** | **gi\|8393418** | **35805** | **81** | **glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus]** |
| 3. | gi\|120707 | 35813 | 81 | Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa BFA-dependent ADP-ribosylation substrate) (BARS-38) |
| 4. | gi\|27661099 | 35760 | 80 | similar to glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus] |
| 5. | gi\|13702286 | 34662 | 65 | glyceraldehyde 3-phosphate dehydrogenase [Cavia porcellus] |

Results List

**2. gi|8393418 Mass: 35805 Score: 81 glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus]**

| *Observed* | Mr(expt) | Mr(calc) | Delta | Start | End | Miss | Peptide |
|---|---|---|---|---|---|---|---|
| *1819.92* | 1818.91 | 1818.90 | 0.02 | 144 | 160 | 0 | IVSNASCTTNCLAPLAK 2 Carbamidomethyl (C) |
| *2153.23* | 2152.22 | 2152.15 | 0.07 | 193 | 213 | 0 | LWRDGRGAAQNIIPASTGAAK |
| *2277.13* | 2276.12 | 2276.03 | 0.09 | 85 | 105 | 0 | WGDAGAEYVVESTGVFTTMEK |
| *2293.13* | 2292.12 | 2292.03 | 0.10 | 85 | 105 | 0 | WGDAGAEYVVESTGVFTTMEK Oxidation (M) |
| *2369.30* | 2368.29 | 2368.20 | 0.09 | 116 | 137 | 0 | RVIISAPSADAPMFVMGVNHEK |
| *2380.36* | 2379.35 | 2379.26 | 0.09 | 4 | 25 | 0 | VGVNGFGRIGRLVTRAAFSCDK Carbamidomethyl (C) |
| *2385.32* | 2384.31 | 2384.20 | 0.11 | 116 | 137 | 0 | RVIISAPSADAPMFVMGVNHEK Oxidation (M) |
| *2401.32* | 2400.31 | 2400.19 | 0.12 | 116 | 137 | 0 | RVIISAPSADAPMFVMGVNHEK 2 Oxidation (M) |
| *2595.48* | 2594.47 | 2594.35 | 0.12 | 161 | 184 | 0 | VIHDNFGIVEGLMTTVHAITATQK |
| *2611.49* | 2610.48 | 2610.35 | 0.14 | 161 | 184 | 0 | VIHDNFGIVEGLMTTVHAITATQK Oxidation (M) |

No match to: 1077.63, 1342.76, 1489.88, 1641.92, 2137.30, 2248.33, 2267.36, 2329.07, 2345.09, 2703.56, 2719.57

### D: spot 4

Index

| | Accession | Mass | Score | Description |
|---|---|---|---|---|
| 1. | Mixture 1 | | 178 | gi\|8393418 + gi\|387422 |
| **2.** | **gi\|8393418** | **35805** | **94** | **glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus]** |
| 3. | gi\|27356782 | 35746 | 76 | glyceraldehyde-3-phosphate dehydrogenase [Meriones unguiculatus] |
| 4. | gi\|120707 | 35813 | 76 | Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa BFA-dependent ADP-ribosylation substrate) (BARS-38) |
| 5. | gi\|387422 | 35588 | 74 | malate dehydrogenase |

Results List

**2. gi|8393418 Mass: 35805 Score: 94 glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus]**

| *Observed* | Mr(expt) | Mr(calc) | Delta | Start | End | Miss | Peptide |
|---|---|---|---|---|---|---|---|
| *1819.97* | 1818.96 | 1818.90 | 0.07 | 144 | 160 | 0 | IVSNASCTTNCLAPLAK 2 Carbamidomethyl (C) |
| *2153.28* | 2152.27 | 2152.15 | 0.12 | 193 | 213 | 0 | LWRDGRGAAQNIIPASTGAAK |
| *2266.43* | 2265.42 | 2265.28 | 0.14 | 65 | 84 | 0 | LVINGKPITIFQERDPANIK |
| *2277.19* | 2276.18 | 2276.03 | 0.15 | 85 | 105 | 0 | WGDAGAEYVVESTGVFTTMEK |
| *2293.19* | 2292.18 | 2292.03 | 0.16 | 85 | 105 | 0 | WGDAGAEYVVESTGVFTTMEK Oxidation (M) |
| *2369.37* | 2368.36 | 2368.20 | 0.16 | 116 | 137 | 0 | RVIISAPSADAPMFVMGVNHEK |
| *2380.43* | 2379.42 | 2379.26 | 0.16 | 4 | 25 | 0 | VGVNGFGRIGRLVTRAAFSCDK Carbamidomethyl (C) |
| *2385.38* | 2384.37 | 2384.20 | 0.17 | 116 | 137 | 0 | RVIISAPSADAPMFVMGVNHEK Oxidation (M) |
| *2401.38* | 2400.37 | 2400.19 | 0.18 | 116 | 137 | 0 | RVIISAPSADAPMFVMGVNHEK 2 Oxidation (M) |
| *2595.54* | 2594.53 | 2594.35 | 0.18 | 161 | 184 | 0 | VIHDNFGIVEGLMTTVHAITATQK |

No match to: 1077.67, 1342.80, 1370.85, 1489.82, 1641.97, 1757.05, 1793.15, 2137.35, 2207.36, 2248.40, 2329.14, 2610.56, 2615.56

*(Notes for Table 1)*

*Notes*: Far-Western positive spots were excised, digested, and analyzed by MALDI mass spectrometry as described in "Materials and methods." The peptide masses obtained by the mass spectrometry were used for database searches with the Mascot search engine. (A), (B), (C), and (D) show the results of Mascot searches for spots 1, 2, 3, and 4, respectively. The top 5 of the results are indicated for each spot ("Index"). "Results List" shows the assignment of the observed masses to the deduced peptide fragments generated by the *Achromobacter* protease I digestion. "Mr (expt)" and "Mr (calc)" show molecular masses obtained from the observed data and those calculated from the indicated peptide sequence, respectively. Score is -10 x Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 74 are significant ($p < 0.05$) [25].

**Table**

Table 2.

Effect of GAPDH and aldolase on the fluorescence polarization of F-CaMKP.

| Addition | Polarization |
|---|---|
| none | 1 |
| BSA (2 μM) | 1.019 ± 0.001 |
| α-casein (2 μM) | 1.010 ± 0.003 |
| GAPDH (2 μM) | 1.350 ± 0.002 |
| Aldolase (2 μM) | 1.194 ± 0.004 |

*Notes:* Fluorescence polarization of F-CaMKP was measured in the presence or absence of the indicated protein as described in "Materials and methods." To compare with BSA and casein, concentrations of GAPDH and aldolase were expressed as monomers in this experiment. Data are means ± SD values from three independent determinations.

**Figure**

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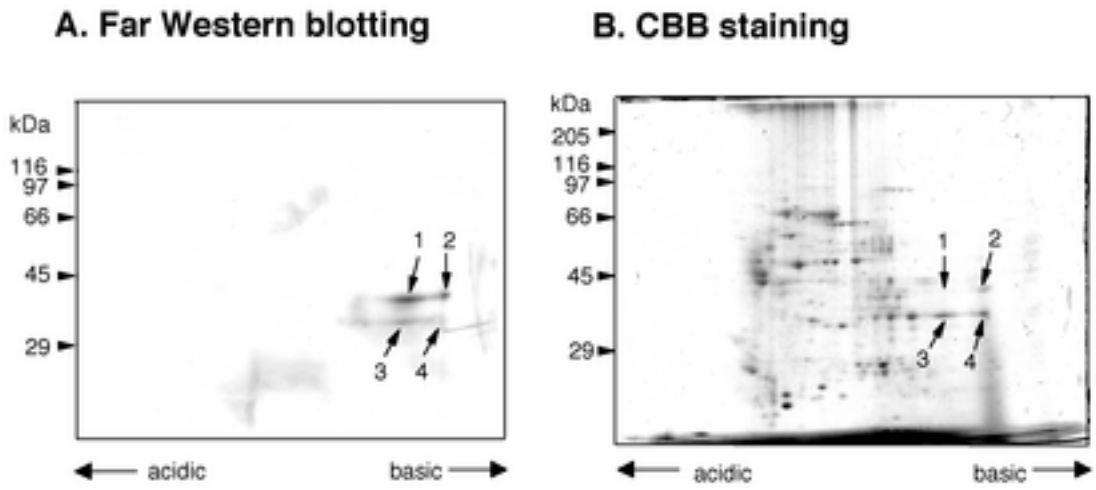
A. Ishida et al. Fig. 1



**Figure**

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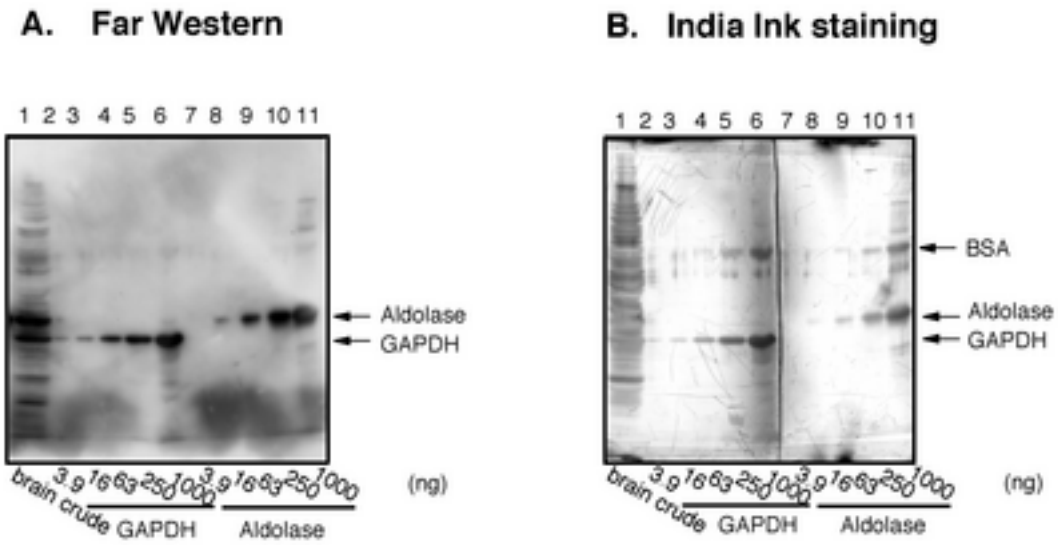
**A. Ishida et al. Fig. 2**



**Figure**

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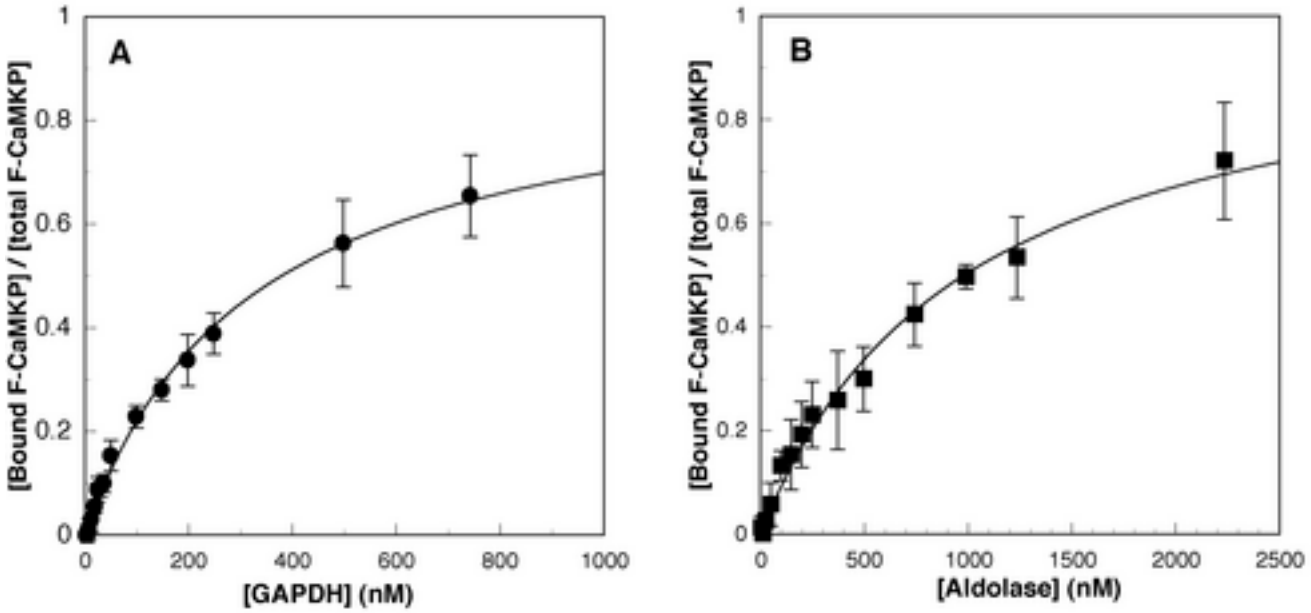
A. Ishida et al. Fig. 3



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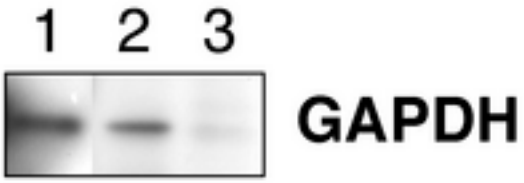
A. Ishida et al. Fig. 4



**Figure**

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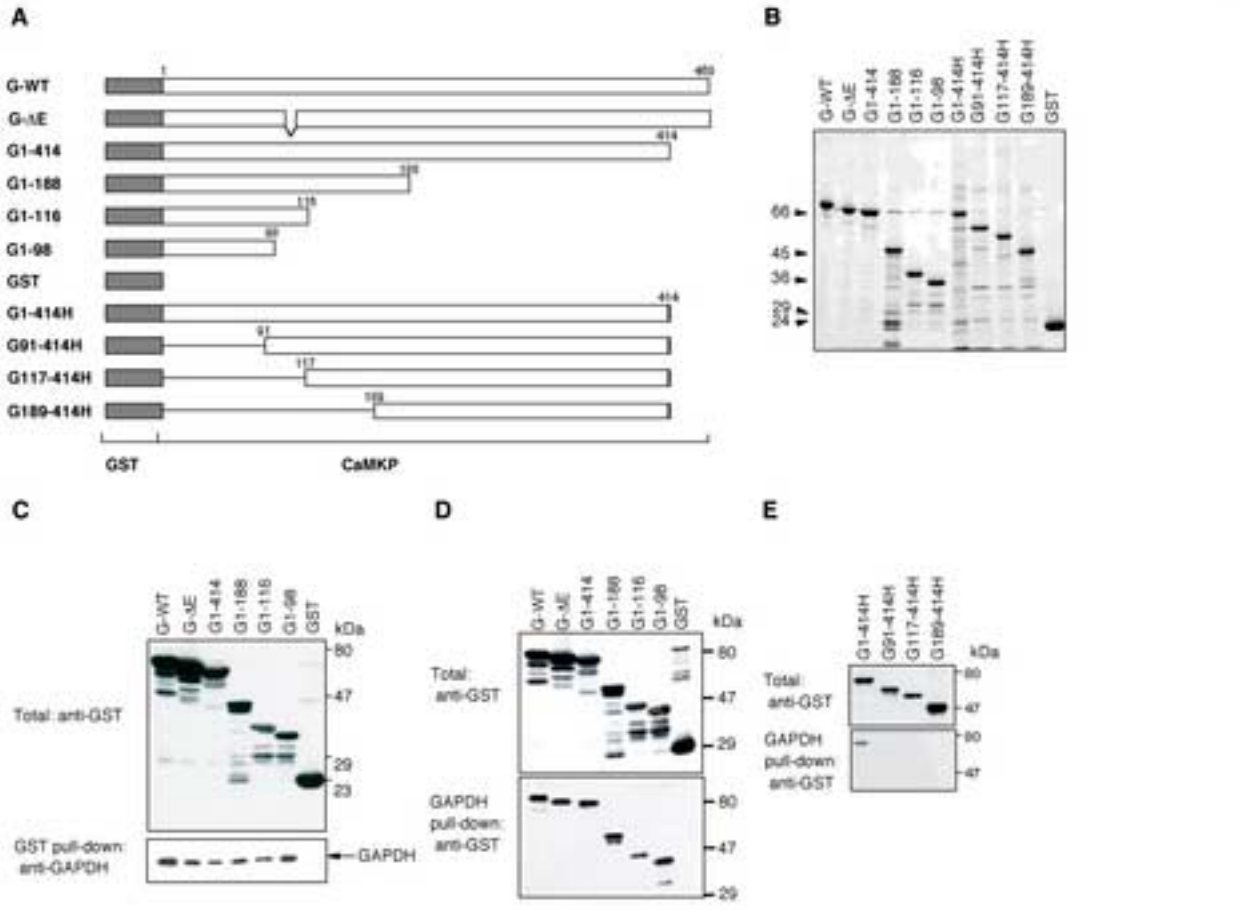
A. Ishida et al. Fig. 5



**Figure**

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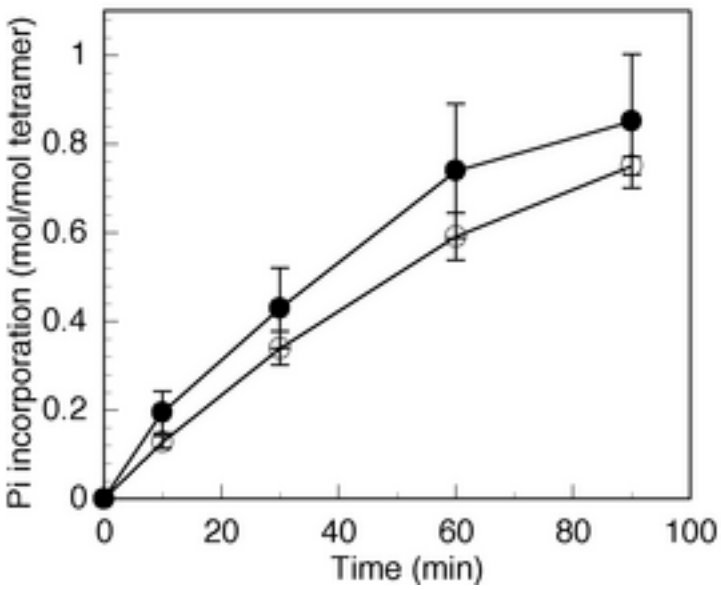
**A. Ishida et al. Fig. 6**



**Figure**

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A. Ishida et al. Fig. 7