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Phosphorylation and activation of nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) by Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI)

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ABSTRACT

Nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) is an enzyme that dephosphorylates and downregulates multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) as well as AMP-dependent protein kinase. In our previous study, we found that zebrafish CaMKP-N (zCaMKP-N) underwent proteolytic processing and translocated to cytosol in a proteasome inhibitor-sensitive manner. In the present study, we found that zCaMKP-N is regulated by phosphorylation at Ser-480. When zCaMKP-N was incubated with the activated CaMKI, time-dependent phosphorylation of the enzyme was observed. This phosphorylation was significantly reduced when Ser-480 was replaced by Ala, suggesting that CaMKI phosphorylates Ser-480 of zCaMKP-N. Phosphorylation-mimic mutants, S480D and S480E, showed higher phosphatase activities than those of wild type and S480A mutant in solution-based phosphatase assay using various substrates. Furthermore, autophosphorylation of CaMKII after ionomycin treatment was more severely attenuated in Neuro2a cells when CaMKII was cotransfected with the phosphorylation-mimic mutant of zCaMKP-N than with the wild-type or non-phosphorylatable zCaMKP-N. These results strongly suggest that phosphorylation of zCaMKP-N at Ser-480 by CaMKI activates CaMKP-N catalytic activity and thereby downregulates multifunctional CaMKs in the cytosol.

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1. Introduction

Nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) and its cytosolic homolog CaMKP/PPM1F are unique protein phosphatases that specifically dephosphorylate and regulate multifunctional CaMKs [1–5]. Recently, CaMKP-N and CaMKP were reported to dephosphorylate AMP-dependent protein kinase (AMPK) as well as CaMKs [6]. Therefore, these phosphatases may play key roles as signal regulators not only in neuronal systems but also in metabolic pathways responsible for energy homeostasis. CaMKP was found to be activated by phosphorylation with CaMKII [7], and stimulated by the addition of polycations such as poly-L-lysine [8]. However, detailed mechanisms for regulation of CaMKP-N activity *in vivo* still remain to be elucidated.

Recently, using human and zebrafish CaMKP-N expressed in Neuro2a cells as a model system, we found that the proteolytic processing occurred in the C-terminal region of CaMKP-N, and that proteasome inhibitors markedly suppressed the processing of CaMKP-N in Neuro2a cells [9]. Using MG-132, we found that the proteolytic processing changed the subcellular localization of CaMKP-N from the nucleus to the cytosol. In addition to our previous report that CaMKII phosphorylates and activates CaMKP [7], the observation that the proteolytic processing of CaMKP-N regulates its subcellular localization led us to the notion that the truncated form of CaMKP-N may be phosphorylated and regulated by cytosolic CaMKs.

In the present study, we found that Ser-480 of zebrafish CaMKP-N (zCaMKP-N) was phosphorylated by CaMKI. Phosphatase activities of S480D and S480E mutants, in which Ser-480 was replaced with Asp or Glu to mimic phosphorylated state, showed higher phosphatase activities than those of wild type and S480A mutant both *in vitro* and *in vivo*. We provide the evidence to show that specific phosphorylation of zCaMKP-N at Ser-480 by CaMKI upregulates its phosphatase activity and may cause downregulation of multifunctional CaMKs in the cytosol.

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKP-N, nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase; MUP, 4-methylumbelliferyl phosphate; NLS, nuclear localization signal.

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2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (111 TBq/mmol) was purchased from PerkinElmer. ATP, anti-actin antibody, Cy3-labeled anti-mouse IgG and 4-methylumbelliferyl phosphate (MUP) were obtained from Sigma Chemicals. Goat anti-mouse IgG and Goat anti-rabbit IgG conjugated with horseradish peroxidase were obtained from Pierce. MG-132 and Epoxomicin were purchased from PEPTIDE Institute. Recombinant rat CaM [10], rat CaMKIα [11], mouse CaMKKα [12], zebrafish CaMKP-N and its mutants [9,13] were expressed in *Escherichia coli*, and purified as described. Anti-GFP and anti-His_6 antibodies were from Roche and Wako Pure Chemical Industries, respectively. Anti-phospho-CaMKI [14] and anti-CaMKP-N [9] antibodies were obtained according to the methods in the cited reports. Anti-phospho-CaMKII(Thr-286) was purchased from Thermo SCIENTIFIC.

2.2. Construction of plasmids

Expression vectors for full-length of wild-type or phosphatase-dead CaMKP-N, pETzCaMKP-N(WT) or pETzCaMKP-N(D188A), were generated as described previously [9]. Point mutants of putative phosphorylation sites (S38A, T245A, S366A, S480A) and phosphorylation-mimic mutants on Ser-480 (S480D and S480E) were generated by inverse PCR method [15] using pETzCaMKP-N(1-502) (WT or D188A) [9] as a template. All of the cDNAs described above were subcloned into the multicloning site of pET23a to generate His_6-fused protein at their C-terminal ends.

For expression of zCaMKP-N and its mutants in mammalian cells, we constructed pcDNA-zCaMKP-N(WT) [9], pcDNA-zCaMKP-N(D188A) [9], pEGFP-zCaMKP-N [9] and pcDNA-CaMKI [16] as described. An expression vector for rat CaMKIIα (pcDNA-CaMKII) was generated by PCR using rat brain cDNA library as a template. pcDNA-zCaMKP-N(1-502) and its mutants on Ser-480 (S480A, S480D, S480E) were prepared by inverse PCR [15] using pcDNA-zCaMKP-N(WT) and pcDNA-zCaMKP-N(1-502) as templates, respectively.

2.3. Cell culture and transfection

Mouse neuroblastoma Neuro2a cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% heat-inactivated fetal calf serum (FCS) at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere. Transfection of Neuro2a cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Treatment of Neuro2a cells with proteasome inhibitors

Transfected cells were cultured for 12 h and then treated with 0.1% DMSO (negative control), 10 μM MG-132 or 1 μM Epoxomicin in DMEM containing 10% FCS for 12 h. The treated cells were washed with PBS, lysed with SDS–PAGE sample buffer and analyzed by Western blotting.

2.5. SDS–PAGE and Western blotting

SDS–PAGE was carried out essentially according to the method of Laemmli [17] on a slab gel consisting of a 10% separation gel and a 3% stacking gel. Western blotting was carried out as described previously [18].

2.6. Phosphorylation of CaMKP-N by CaMKI

For preparation of activated CaMKI, 100 ng of CaMKI was incubated in the reaction mixture (10 μl) consisting of 50 mM Hepes-NaOH (pH 7.5), 10 mM $Mg(CH_3COOH)_2$, 0.1 mM EGTA, 5 ng CaMKK, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP at 30 °C for 30 min, and the reaction was stopped by the addition of 1 μl of 20 mM EGTA. Recombinant CaMKP-N(1-502)(D188A) (200 ng) were incubated with or without CaMKI (2 ng) or phosphorylated CaMKI by CaMKK (2 ng) in the standard phosphorylation mixture (10 μl) containing 100 μM [γ-^{32}P]ATP. After incubation at 30 °C for the indicated time, equal volume of 2× SDS sample buffer was added to the phosphorylation mixture to stop the reaction. Phosphorylated proteins were then resolved on SDS–PAGE and visualized by autoradiography. [^{32}P]phosphate incorporation into CaMKP-N was quantified by a liquid scintillation counter.

2.7. Protein phosphatase assay using MUP as a substrate

The protein phosphatase assay was carried out at 30 °C for 10 min in a reaction mixture (100 μl) containing 50 mM Tris–HCl (pH 8.0), 10 mM $MnCl_2$, 20 mM dithiothreitol, 0.1 mM EGTA, 0.01% Tween 20, 25 μM MUP and 3 μg of CaMKP-N. The reaction was started by the addition of CaMKP-N and terminated by the addition of 25 μl of 500 mM EDTA. The 4-methylumbelliferone present in the reaction mixture was measured in a CytoFluor 4000TC fluorescence microplate reader (ABI) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

2.8. Protein phosphatase assay using phosphopeptide as a substrate

Protein phosphatase assay was carried out using phosphopeptide pp10 (YGGMHRQETpVDC), which contains an amino acid sequence around the autophosphorylation site of CaMKII, as a substrate [11]. Phosphatase assay was carried out in the reaction mixture (50 μl) containing 50 mM Tris–HCl (pH 8.0), 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 20 μM pp10 and an appropriate amount of CaMKP-N. The reaction was started by adding CaMKP-N and incubated at 30 °C for 6 min. The inorganic phosphate released in the mixture was determined by malachite green assay [19].

2.9. Protein phosphatase assay using phosphoprotein as a substrate

Protein phosphatase assay was carried out using CaMKI as a phosphoprotein substrate. Recombinant rat CaMKIα(K49R) (1 μg) was phosphorylated by CaMKK (50 ng) at 30 °C for 30 min in a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 1 μM CaM, 1 mM $CaCl_2$ and 100 μM ATP, and the reaction stopped by adding EGTA to the final concentration of 2 mM. Dephosphorylation of phospho-CaMKI was carried out at 30 °C for 10 min in a reaction mixture containing 50 mM Tris–HCl (pH 8.0), 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 1 mM dithiothreitol, 100 ng phospho-CaMKI and 2 ng CaMKP-N. The reaction was started by the addition of CaMKP-N and terminated by the addition of an equal volume of 2× SDS–PAGE sample buffer. The sample was then subjected to SDS–PAGE and analyzed by Western blotting using phospho-CaMKI antibody.

2.10. Immunocytochemistry

Transfected cells were cultured on cover glasses and treated with 3.7% formaldehyde in PBS for 20 min. After rinsing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After treatment with 1% bovine serum albumin in PBS, the cells were incubated with an anti-Myc antibody diluted 1:1000 in PBS containing 1% bovine serum albumin at 4 °C for

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