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Novel Ca^{2+}/calmodulin-dependent protein kinase expressed in actively growing mycelia of the basidiomycetous mushroom *Coprinus cinereus*

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ABSTRACT

We isolated cDNA clones for novel protein kinases by expression screening of a cDNA library from the basidiomycetous mushroom *Coprinus cinereus*. One of the isolated clones was found to encode a calmodulin (CaM)-binding protein consisting of 488 amino acid residues with a predicted molecular weight of 53,906, which we designated CoPK12. The amino acid sequence of the catalytic domain of CoPK12 showed 46% identity with those of rat Ca^{2+}/CaM-dependent protein kinase (CaMK) I and CaMKIV. However, a striking difference between these kinases is that the critical Thr residue in the activating phosphorylation site of CaMKI/IV is replaced by a Glu residue at the identical position in CoPK12. As predicted from its primary sequence, CoPK12 was found to behave like an activated form of CaMKI phosphorylated by an upstream CaMK kinase, indicating that CoPK12 is a unique CaMK with different properties from those of the well-characterized CaMKI, II, and IV. CoPK12 was abundantly expressed in actively growing mycelia and phosphorylated various proteins, including endogenous substrates, in the presence of Ca^{2+}/CaM. Treatment of mycelia of *C. cinereus* with KN-93, which was found to inhibit CoPK12, resulted in a significant reduction in growth rate of mycelia. These results suggest that CoPK12 is a new type of multifunctional CaMK expressed in *C. cinereus*, and that it may play an important role in the mycelial growth.

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are known to play pivotal roles in Ca^{2+}signaling pathways [1–4]. CaMKI, CaMKII and CaMKIV have broad substrate specificities, and are classified as multifunctional CaMKs. CaMKII undergoes autophosphorylation in the presence of Ca^{2+}/CaM, and becomes an autonomous enzyme. CaMKI and CaMKIV are phosphorylated by an upstream kinase, CaMK kinase (CaMKK), and become their active forms [5–8]. Although the multifunctional CaMKs in animal cells have been extensively characterized to date, CaMKs in plants and fungi remain elusive.

Coprinus cinereus, a typical mushroom, is one of the model organisms that has been commonly used for the studies of life cycle and developmental stages in the basidiomycetous fungi [9,10]. However, little is known about the cellular mechanisms of regulation of developmental processes through protein phosphorylation in mushrooms. In a previous study, we detected various protein kinases in the basidiomycetous mushroom *C. cinereus* by Western blotting analysis using Multi-PK antibodies [11]. These Multi-PK antibodies were produced in order to detect a wide variety of Ser/Thr protein kinases [12], and have been used for the detection of various novel protein kinases not only in animals [12,13] but also in plants [14]. We also reported that multifunctional CaMKs were expressed in the growing mycelia of *C. cinereus* and that they may play crucial roles in the early stages of development [11]. However, the identities and physiological functions of CaMKs in this mushroom are still unknown.

In the present study, we aimed to isolate CaMKs from *C. cinereus* by carrying out immunological screening of protein kinases using a mycelial λZAPII cDNA library. We obtained one cDNA clone that encoded a novel protein kinase with a CaM-binding capacity, and designated it CoPK12. CoPK12 was expressed in *Escherichia coli*, purified to homogeneity and characterized. In this paper, we report that CoPK12 is a unique multifunctional CaMK showing quite different catalytic properties form those of CaMKI, II, and IV, and that it may be correlated with the mycelial growth of *C. cinereus*.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (5000 Ci/mmol) was purchased from GE Healthcare Bio-Sciences. ATP, myelin basic protein (MBP), α-casein and histone type IIA were obtained from Sigma Chemicals. Goat anti-mouse IgA+G+M conjugated with horseradish peroxidase was from ICN Pharmaceuticals. Anti-His_6 antibody and anti-digoxigenin antibody conjugated

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; MBP, myelin basic protein

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with horseradish peroxidase were obtained from Roche Diagnostics. Water-soluble KN-93 was from Wako Chemicals. Myosin light chain was prepared as described previously [15]. Recombinant rat CaM [16], rat CaMKIα [17] and mouse CaMKKα [13] were expressed in *E. coli*, and purified as described in the cited reports.

2.2. Preparation of crude extracts and CaM-binding protein fraction of *C. cinereus*

The *C. cinereus* homokaryotic fruiting strain 326 [18] was kindly provided by Dr. T. Kamada of Okayama University. Mycelia of *C. cinereus* were inoculated and grown on MYG agar [1% malt extract, 0.4% yeast extract, 0.4% glucose, and 2% agar] in 9-cm glass dishes under 12-h light/dark cycle at 28 °C. Fruiting bodies and mycelia were harvested and frozen in liquid nitrogen and ground into fine powder with appropriate amounts of sea sands using mortar and pestle. These samples were homogenized with 3 volumes of homogenizing buffer [0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 100 mM KCl, 50 mM EDTA, 40 mM dithiothreitol and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A (Peptide Institute)]. The homogenates were centrifuged at 20,000 ×*g* for 20 min at 2 °C, and the supernatants thus obtained were used as crude extracts. CaM-binding protein fraction was prepared from mycelial extract by using CaM-Sepharose 4B essentially as described previously [11].

2.3. Expression cloning of protein kinases from mycelia of *C. cinereus*

A cDNA library was constructed with mRNA isolated from the mycelia of *C. cinereus* using a λZAPII cDNA Synthesis Kit (Stratagene). Screening of protein kinases was carried out using Multi-PK antibodies directed against highly conserved regions in Ser/Thr protein kinases [12]. Cloning, sequencing and data analyses of the cDNA clones corresponding to various protein kinases were carried out as described previously [14]. A SMART RACE cDNA Amplification Kit (Clontech) was used to obtain a full-length coding sequence for CoPK12 using primers based on a cDNA clone (CMZ012). The 5′-RACE first-strand cDNA was synthesized from mRNA isolated from the mycelia of *C. cinereus* with Superscript II reverse transcriptase using a SMARTII oligonucleotide and a 5′-RACE cDNA synthesis primer. The 5′-end of the cDNA was amplified by PCR with a gene-specific primer (AS871: 5′-CGT CAG CAG GGT TAG CAA CAA AGT GAG-3′) and a universal primer mix with the 5′-RACE first-strand cDNA as a template using an Advantage 2 PCR Kit (Clontech). The nucleotide sequences of the 5′-RACE and CMZ012 products were overlapped and aligned using the DNASIS computer program developed by Hitachi Software Engineering. An open reading frame of 1464 nucleotides was generated. A sense primer (5′-ACT CGC TTC CTC ACT TTC CCC C-3′) and an antisense primer (5′-GAT TGA CGA CAG CCC GAG GAT AA-3′) were designed from the outside sequences of the open reading frame, respectively. A full-length cDNA was prepared by PCR using these primers with a 5′-RACE ready cDNA library as a template using Pyrobest DNA polymerase (TaKaRa Bio). The PCR product was cloned into a pGEM-T Easy vector, and ten independent clones (pGEM-CoPK12-1 through-10) were sequenced.

2.4. Plasmid construction, expression and purification of CoPK12

To generate the plasmid pETCoPK12, the following primers were used for PCR with pGEMCoPK12-1 as a template: 5′-upstream primer (5′-GCT AGC ATG GGC TCT GCT CAA AGT AAA CAA-3′) and 3′-downstream primer (5′-CTC GAG ACC CTG GAT GGT GGC GTT G-3′). The NheI (underlined)–XhoI (double-underlined) fragment was inserted into the NheI–XhoI sites of pET-23a (+) (Novagen).

E. coli BL21(DE3) cells transformed with pETCoPK12 were grown at 37 °C to an A_{600} of 0.6–0.8, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. After 4 h at 37 °C, the bacteria were harvested by centrifugation and suspended in buffer A [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 40] containing 1 mM phenylmethylsulfonyl fluoride. After sonication, cell debris was removed by centrifugation at 20,000 ×*g* at 4 °C for 10 min, and the supernatant was loaded onto a HiTrap Chelating HP column (GE Healthcare Bio-Sciences) pre-equilibrated with buffer A. The column was sequentially washed with buffer A, buffer A containing 20 mM imidazole and buffer A containing 50 mM imidazole, before being eluted with buffer A containing 200 mM imidazole. The purified fractions were pooled, dialyzed against buffer A containing 1 mM dithiothreitol and stored in aliquots at −80 °C until use.

2.5. Production of antibodies

Antibody against CoPK12 was produced by immunizing BALB/c mice with purified CoPK12. Immunization was carried out essentially as described previously [19]. Multi-PK antibodies M1C and M8C were obtained from two hybridoma cell lines established as described previously [12].

2.6. SDS-PAGE, Western blotting analysis and CaM-overlay assay

SDS-PAGE was carried out essentially according to the method of Laemmli [20] in slab gels consisting of a 10% or 15% acrylamide separating gel and a 3% stacking gel. Western blotting analyses and CaM-overlay assays were carried out as described previously [13].

2.7. Degradation experiment of CoPK12

Mycelial extract was prepared as above except that homogenizing buffer without protease inhibitors was used. Purified CoPK12 (125 ng) was incubated with the aforementioned mycelial extract (12.5 ng) in buffer A in a final volume of 25 μl. After an appropriate time of incubation at 30 °C, equal volume of 2× SDS sample buffer was added to the reaction mixture, and subjected to SDS-PAGE. Degradation process of CoPK12 was monitored either by Western blotting with anti-CoPK12 antibody, Multi-PK antibody, and anti-His_6 antibody, or by CaM-overlay assay.

2.8. Protein kinase assay

Autophosphorylation of CoPK12 was carried out using a standard reaction mixture (10 μl) consisting of 40 mM Hepes–NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 2 mM dithiothreitol, 100 μM [γ-^{32}P]ATP and 200 ng CoPK12 in the presence or absence of 0.5 mM $CaCl_2$ and 1 μM CaM ($CaCl_2$/CaM). Phosphorylation of CaMKI (200 ng) was carried out by the addition of 5 ng of CaMKK in the standard phosphorylation mixture in the presence of $CaCl_2$/CaM. The reaction was started by the addition of CoPK12 or CaMKK and incubated at 30 °C for 30 min. For preparation of activated CaMKI, 200 ng of CaMKI was incubated in the aforementioned reaction mixture (10 μl) containing 5 ng CaMKK, 100 μM ATP and $CaCl_2$/CaM at 30 °C for 30 min, and the reaction was stopped by the addition of 10 μl of 20 mM EDTA. Protein kinase activity of CoPK12 and CaMKI was determined using syntide-2 as a substrate essentially according to the method described previously [13].

Phosphorylation of protein substrates (1 μg) was carried out using the standard reaction mixture (10 μl) containing 20 ng of CoPK12, autophosphorylated CoPK12, CaMKI or activated CaMKI in the presence or absence of $CaCl_2$/CaM. To prepare endogenous substrates for CoPK12, the mycelial extract was heat-treated at 55 °C for 60 min to inactivate endogenous protein kinases. The heat-treated extract (20 μg) was then incubated with autophosphorylated CoPK12 (35 ng) or previously activated CaMKI (35 ng) in the standard reaction mixture (20 μl) in the presence of $CaCl_2$/CaM. After incubation at 30 °C for 60 min, an equal volume of 2× SDS

sample buffer was added to the phosphorylation mixture to stop the reaction. Phosphorylated proteins were resolved on SDS-PAGE and analyzed by autoradiography.

In-gel protein kinase assay was carried out using 10% polyacrylamide separation gels containing 0.1 mg/ml MBP as a substrate essentially as described previously [21,22].

3. Results

3.1. Cloning of CoPK12

In our previous study, we found that at least two CaMKs were highly expressed in the actively growing mycelia of *C. cinereus* [11]. To characterize the CaMKs in *C. cinereus*, we attempted to isolate cDNA clones for CaMKs in the present study. Since CaMKs appeared to be expressed abundantly in actively growing mycelia, we prepared a λZAPII cDNA library from mycelia of *C. cinereus*. cDNA clones for protein kinases were immunologically screened using Multi-PK antibodies and subcloned as described previously [12]. Starting from 8×10^4 plaques, we obtained 14 positive clones after second screening. When the protein products obtained from these cDNA clones were analyzed by CaM-overlay assay, one of the clones, designated CMZ012, was found to encode a CaM-binding protein (data not shown). Therefore, the full-length cDNA containing the entire coding region of CMZ012 was obtained by the RACE PCR method and its entire nucleotide sequence was determined. We found that the gene encodes a protein of 488 amino acids with a molecular weight of 53,906 (Fig. 1A). Since this protein contains highly conserved subdomains specific to protein kinases, we designated it CoPK12. The sequence data for CoPK12 have been submitted to GenBank™/EBI Data Bank under Accession No. AB294221. When homology searches were carried out on the basis of the CoPK12 sequence, multifunctional CaMKs were found to show the highest homology with CoPK12. As shown in Fig. 1B, the amino acid sequences of both rat CaMKI and rat CaMKIV showed 46% identity with that of CoPK12 in their catalytic domains. A structural feature of CoPK12 is that its N-terminal sequence is longer than those of CaMKI and CaMKIV.

Mammalian CaMKI and CaMKIV are upregulated through phosphorylation by the upstream kinase CaMKK. The site phosphorylated by CaMKK is localized between subdomains VII and VIII (so-called activation loop) and the Thr residue in the primary sequence is highly conserved in all CaMKI and CaMKIV isoforms in animals, the yeast *Schizosaccharomyces pombe* and the fungus *Aspergillus nidulans* (Fig. 1C). One striking feature of CoPK12, however, is that the amino acid residue corresponding to the phosphorylatable Thr residue in the activation loop of the other CaMKs is replaced by a Glu residue in CoPK12 (Fig. 1C, arrowhead).

3.2. Expression and purification of CoPK12

When the expression plasmid of CoPK12, pETCoPK12, was transfected into *E. coli* BL21(DE3), His_6-tagged CoPK12 was expressed in a soluble form. Recombinant CoPK12 was purified from *E. coli* lysate by chromatography on a HiTrap Chelating column. Purified

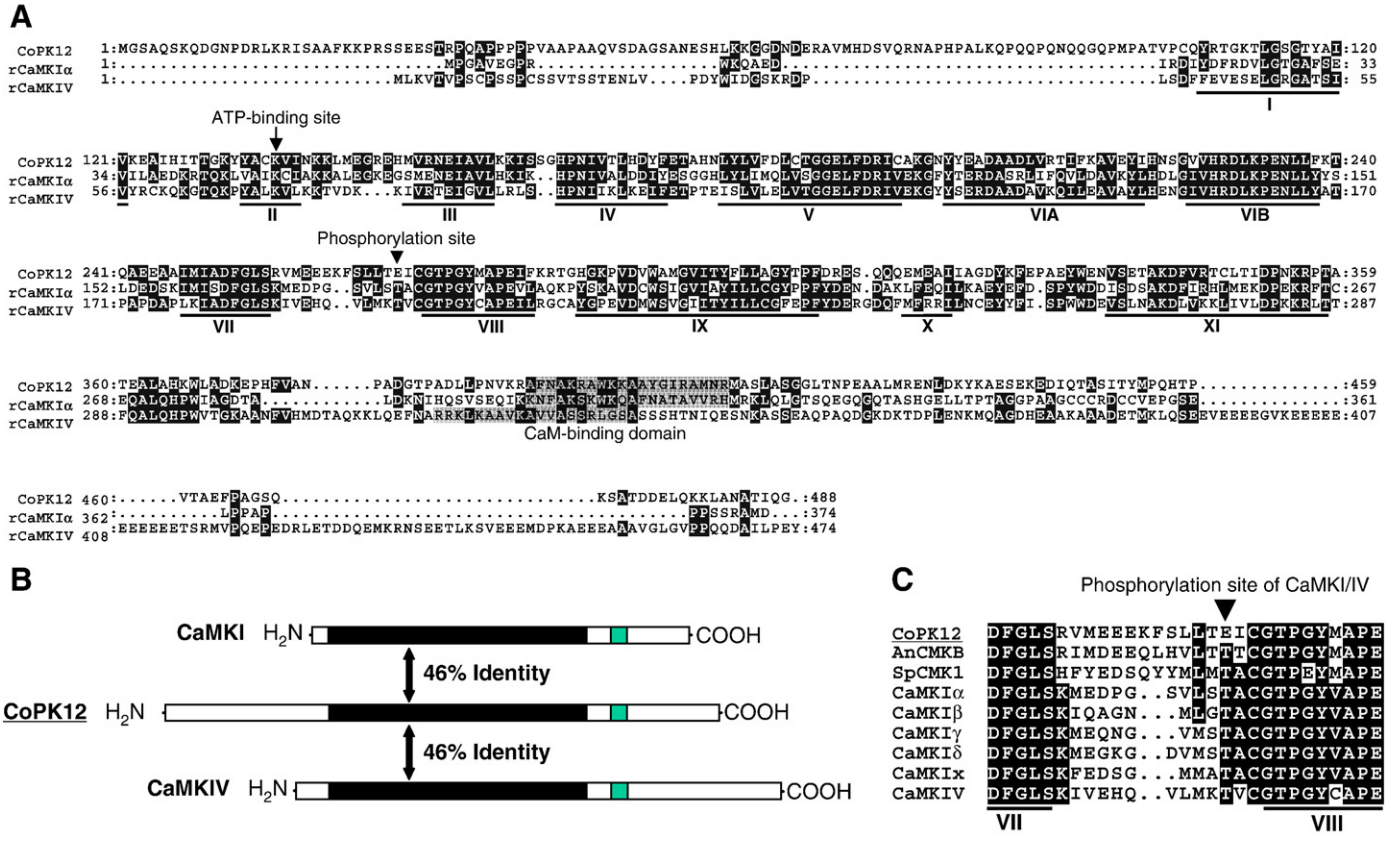

Fig. 1. Primary structure of CoPK12. (A) The amino acid sequence of CoPK12 was aligned with those of rat CaMKIα and rat CaMKIV using CLUSTAL W. Identical amino acids are shaded in black and gaps inserted into the sequences are indicated by dots. Twelve subdomains specific to protein kinases [29] are underlined. The ATP-binding site and the CaMKK phosphorylation site in CaMKI/IV are shown by the arrow and arrowhead, respectively. The CaM-binding domains are predicted on the basis of sequence motifs for CaM-binding [30] and are shaded in gray. (B) Schematic illustrations of the domain structures of CoPK12 and CaMKs. The catalytic domain and putative CaM-binding domain are shown by black and grey boxes, respectively. (C) Comparison of the amino acid sequences in the activation loops of CaMKs. The amino acid sequence between subdomains VII and VIII of CoPK12 was aligned with those of *A. nidulans* CMKB (Accession No.: AF156027), *S. pombe* CMK1 (Accession No.: AF073893), rat CaMKIα (Accession No.: NP_598687), CaMKIβ2 (Accession No.: NP_036170), CaMKIγ (Accession No.: NP_659066), CaMKIδ (Accession No.: NP_796317), *Xenopus laevis* CaMKIx (Accession No.: AB098710), and rat CaMKIV (Accession No.: NP_036859). The arrowhead indicates the activating phosphorylation site for the upstream kinase.

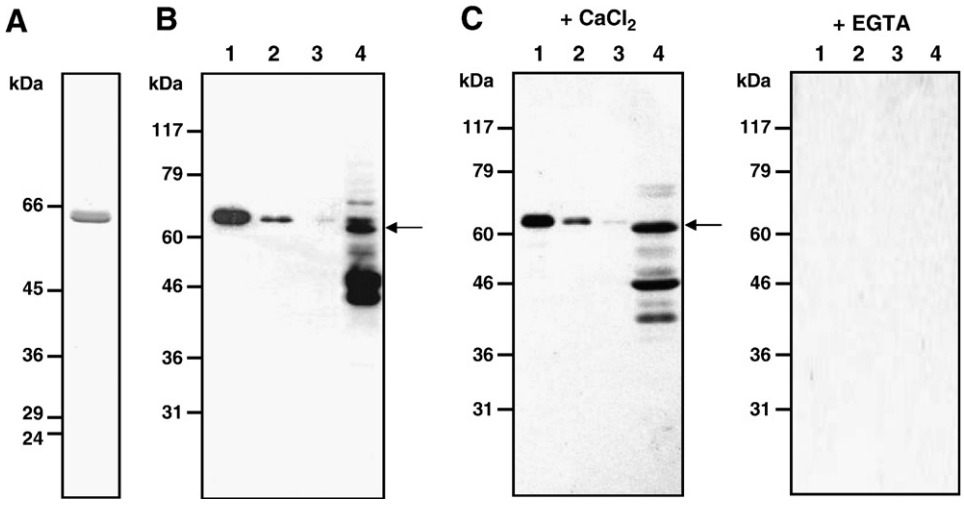

Fig. 2. Electrophoretic analyses of CoPK12. (A) Protein staining of recombinant CoPK12. Purified CoPK12 (0.5 μg) was subjected to 10% SDS-PAGE and stained with Coomassie brilliant blue. (B) Immunodetection of CoPK12 and other mycelial protein kinases. Purified CoPK12 (lane 1: 10 ng; lane 2: 2 ng; lane 3: 0.4 ng) and the *C. cinereus* mycelial extract (lane 4: 20 μg) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and detected by Western blotting analysis with Multi-PK antibodies. The arrow indicates the position of endogenous CoPK12. (C) CaM-overlay assay of CoPK12. Recombinant CoPK12 (lane 1: 10 ng; lane 2: 2 ng; lane 3: 0.4 ng) and the mycelial extract (lane 4: 20 μg) were subjected to SDS-PAGE, and CaM-overlay assay was carried out either in the presence (+$CaCl_2$) or absence (+EGTA) of $CaCl_2$.

CoPK12 showed a 65-kDa protein band by protein staining after SDS-PAGE (Fig. 2A). Purified CoPK12 and mycelial extract of *C. cinereus* were analyzed by Western blotting with Multi-PK antibodies and by CaM-overlay assay. Multi-PK antibody detected as low as 0.4 ng of CoPK12 as shown in Fig. 2B. Among multiple protein bands detected by the Multi-PK antibodies, a positive band corresponding to CoPK12

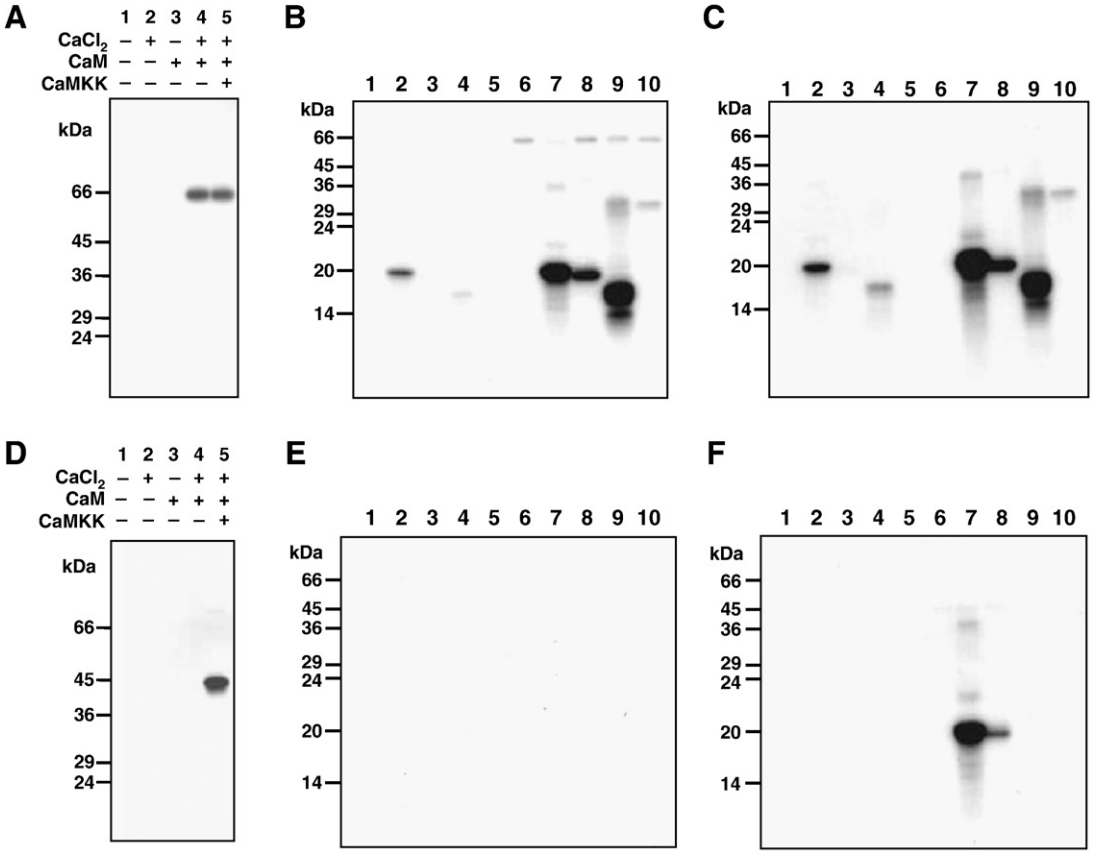

Fig. 3. Catalytic properties of CoPK12 and CaMKI. Approximately 200 ng of CoPK12 (A) or CaMKI (D) was incubated with 100 μM [γ-^{32}P]ATP in the presence or absence of 0.5 mM $CaCl_2$, 1 μM CaM and 5 ng of CaMKK as indicated. After a 30-min incubation at 30 °C, the reaction was stopped by the addition of 2× SDS sample buffer, and the mixtures were subjected to 10% SDS-PAGE and analyzed by autoradiography. Phosphorylation of protein substrates by CoPK12 (B), autophosphorylated CoPK12 (C), unphosphorylated CaMKI (E) or CaMKI phosphorylated by CaMKK (F) was shown. Protein kinases were incubated with 1 μg of MBP (lanes 2 and 7), myosin light chain (lanes 3 and 8), histone (lanes 4 and 9), casein (lanes 5 and 10) or in the absence of substrate (lanes 1 and 6) under phosphorylating conditions in the absence (lanes 1–5) or presence (lanes 6–10) of Ca^{2+}/CaM. Phosphorylated proteins were resolved on 15% SDS-PAGE and analyzed by autoradiography.

was observed in the mycelial extract (Fig. 2B, arrow). Since recombinant CoPK12 was expressed as His_6-tagged protein on its C-terminal end, it showed somewhat slower migration on SDS-PAGE as compared to the intact CoPK012. By CaM-overlay experiment, CaM was found to bind CoPK12 in the presence of $CaCl_2$ but not in its absence (Fig. 2C). These results suggest that CoPK12 is a typical CaM-binding protein. CaM-overlay assay also indicated that one of the major CaM-binding protein of 65 kDa detected in the mycelial extract was CoPK12 (Fig. 2C, lane 4).

3.3. Catalytic properties of CoPK12

One of the most characteristic features of CoPK12 is that the phosphorylatable Thr residue required for activation of other CaMKs is replaced by a Glu residue in CoPK12 (Fig. 1C). The occurrence of an acidic amino acid such as Glu at this position in CoPK12 may be expected to mimic the phosphorylated forms of CaMKI/IV. To examine this hypothesis, the catalytic properties of CoPK12 were compared with those of CaMKI. Although CoPK12 underwent autophosphorylation following the addition of Ca^{2+}/CaM, further addition of CaMKK did not cause any additional phosphorylation of CoPK12 (Fig. 3A). In contrast, CaMKI was not autophosphorylated in either the presence or absence of Ca^{2+}/CaM, but was significantly phosphorylated by CaMKK in the presence of Ca^{2+}/CaM (Fig. 3D).

Unphosphorylated CaMKI exhibited no kinase activity in either the presence or absence of Ca^{2+}/CaM, whereas CaMKI phosphorylated by CaMKK could phosphorylate MBP and myosin light chain in the presence of Ca^{2+}/CaM (Fig. 3E and F). In contrast to CaMKI, CoPK12 strongly phosphorylated MBP, myosin light chain and histone in the presence of Ca^{2+}/CaM. Protein kinase activity of CoPK12 was found to be slightly activated upon autophosphorylation, but marked induction of autonomous activity was not observed (Fig. 3B and C). These results indicate that CoPK12 is a unique multifunctional CaMK that does not require an upstream kinase for its activation.

3.4. Expression of CoPK12 during the life cycle of C. cinereus

Expression of CoPK12 during the life cycle was investigated using highly specific antibody raised against purified CoPK12. Crude extract was prepared from four different developmental stages of *C. cinereus*: mycelia, primordia, immature fruiting bobies, and mature fruiting bodies (Fig. 4A). When CoPK12 was detected by Western blotting with anti-CoPK12 antibody, it was strongly detected in mycelial extract and weakly detected in primordia. However, CoPK12 was scarcely detected either in the immature or mature fruiting bodies. In addition to 65-kDa protein band corresponding to full length CoPK12, 46-kDa immunoreactive band was also detected in mycelia, primordia, and slightly in the early fruiting bodies (Fig. 4B).

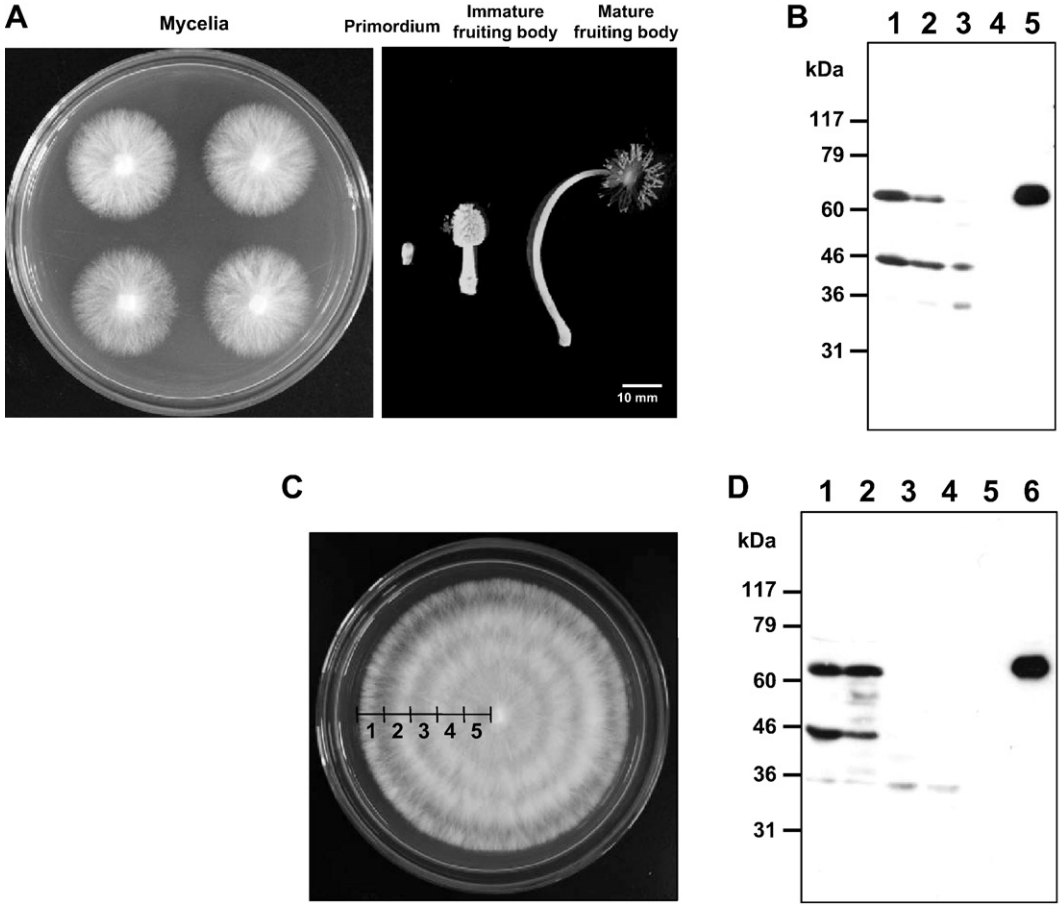

Fig. 4. Detection of CoPK12 expressed in mycelia and later developmental stages of *C. cinereus*. *C. cinereus* was photographed at the indicated stages after inoculation (A). Approximately 10 μg of crude extracts from mycelia (lane 1), primordia (lane 2), immature fruiting bodies (lane 3), and mature fruiting bodies (lane 4) were electrophoresed on SDS-polyacrylamide gel and detected by Western blotting using anti-CoPK12 antibody. Recombinant CoPK12 (5 ng, lane 5) was applied as a positive control (B). Growing mycelia (approximately 8 cm in diameter) of *C. cinereus* were divided into five zones from the outer region (My1) to the central region (My5), and harvested separately (C). Approximately 10 μg of crude extract of My1 (lane 1), My2 (lane 2), My3 (lane 3), My4 (lane 4), and My5 (lane 5) were electrophoresed on SDS-polyacrylamide gel and analyzed by Western blotting with anti-CoPK12 antibody. Purified CoPK12 (5 ng, lane 6) was also applied as a positive control (D).

Since CoPK12 was found to be highly expressed in mycelia, expression profile of CoPK12 in growing mycelia was investigated. Mycelial colony, which had been fully grown in agar plates, was divided into five zones from outer edge to the central zone, and harvested separately (Fig. 4C). When these samples were analyzed by Western blotting with anti-CoPK12 antibody, both 65-kDa and 46-kDa immunoreactive bands were clearly observed in the outer regions (Fig. 4D, lanes 1 and 2). In contrast, these immunoreactive bands were not detected in the central non-growing regions (Fig. 4D, lanes 3–5). These results suggest that CoPK12 is abundantly expressed in the actively growing region of mycelia, but not in the resting mycelia or the fruiting bodies in the later stages.

3.5. Proteolytic digestion of CoPK12 by endogenous protease

Two immunoreactive bands corresponding to 65 kDa and 46 kDa were detected when mycelial extract was analyzed with anti-CoPK12 antibody (Fig. 4). To investigate whether or not the 46-kDa protein is a proteolytic product of 65-kDa CoPK12, recombinant CoPK12 was incubated with mycelial extract. When CoPK12 was incubated with the extract, degradation products appeared just below the 65-kDa protein band, gradually shifted to smaller species, and finally converted to 46-kDa protein (Fig. 5A, B, and C). The 46-kDa protein was not detected by an antibody against C-terminal His_6-tag, suggesting that C-terminal end of CoPK12 was removed by proteolytic digestion (Fig. 5D). The 46-kDa protein, however, could be detected not only by CaM-overlay assay but also by Multi-PK antibody (Fig. 5B and C). These results suggest that 46-kDa protein is a proteolytic fragment of CoPK12 and it contains both catalytic and CaM-binding domains of CoPK12.

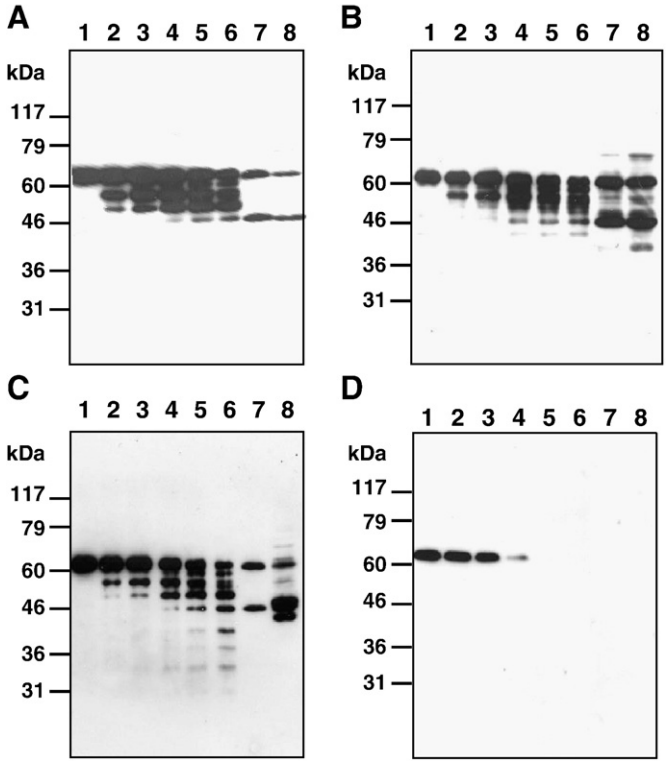

Fig. 5. Limited proteolysis of CoPK12 by endogenous protease in mycelial extract. Recombinant CoPK12 (125 ng) was incubated with mycelial extract (12.5 ng) of *C. cinereus* at 30 °C. After incubation for 5 min (lane 2), 10 min (lane 3), 30 min (lane 4), 60 min (lane 5), and 90 min (lane 6), reaction was stopped by the addition of 2× SDS sample buffer, and incubated samples (15 ng) were subjected to SDS-PAGE. Recombinant CoPK12 without incubation (15 ng, lane 1), CaM-binding protein fraction (60 ng, lane 7), and crude extract of mycelia (10 μg, lane 8) were also electrophoresed. Resolved proteins were electrophoretically transferred to nitrocellulose membrane and detected by anti-CoPK12 antibody (A), CaM-overlay assay (B), Multi-PK antibody (C), and anti-His_6 antibody (D), respectively.

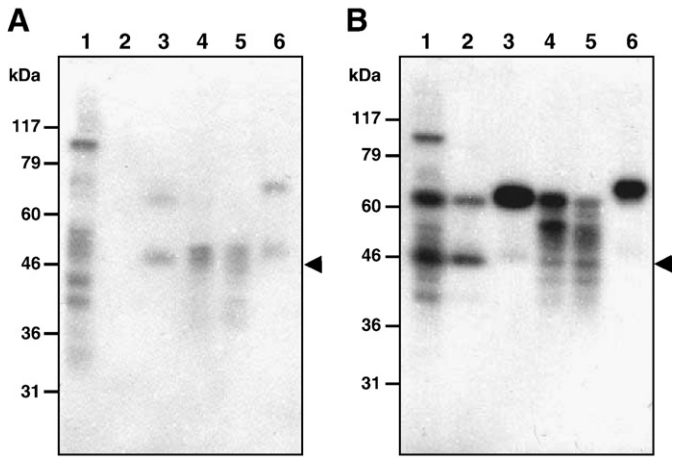

Fig. 6. Detection of protein kinase activities of 65-kDa CoPK12 and 46-kDa CoPK12 by in-gel protein kinase assay. Mycelial extract of *C. cinereus* (10 μg, lane 1), CaM-binding protein fraction (60 ng, lane 2), CoPK12 (15 ng, lane 3), CoPK12 pretreated with mycelial extract for 30 min (lane 4) and 90 min (lane 5), and autophosphorylated CoPK12 (15 ng, lane 6) were electrophoresed on SDS-polyacrylamide gels containing 0.1 mg/ml MBP. Proteins were renatured *in situ* and protein kinase activities detected in the absence (A) or presence (B) of Ca^{2+}/CaM. The arrowhead indicates the position of 46-kDa protein.

To examine whether the 46-kDa fragment is just a degradation intermediate of CoPK12 or it has some physiological function, catalytic activity of 46-kDa CoPK12 was compared with that of 65-kDa CoPK12 by in-gel protein kinase assay. Protein kinase activities were detected in gel containing MBP as a substrate [21]. Protein kinase activities of 65-kDa CoPK12 and 46-kDa protein could be detected only in the presence of Ca^{2+}/CaM (Fig. 6A and B, lanes 1 and 2), suggesting that both proteins have Ca^{2+}/CaM-dependent protein kinase activities. Incubation of recombinant CoPK12 with mycelial extract produced 46-kDa protein in addition to intermediate species detected in between 65-kDa CoPK12 and 46-kDa protein. Autophosphorylated CoPK12 did not exhibit autonomous activity, but showed Ca^{2+}/CaM-dependent protein kinase activity as in case of non-phosphorylated CoPK12 (Fig. 6A and B, lane 6). These results suggest that 46-kDa protein is derived from 65-kDa CoPK12 by proteolytic digestion with endogenous protease and it still retains Ca^{2+}/CaM-dependent protein kinase activity.

3.6. CoPK12 phosphorylates endogenous substrates

CoPK12 was found to be especially highly expressed in the mycelia of *C. cinereus* (Fig. 4B). Therefore, we examined whether CoPK12 was able to phosphorylate endogenous substrates in the mycelial extract. The mycelial extract was heat-treated at 55 °C for 60 min to inactivate endogenous protein kinases and then used for phosphorylation experiments. CoPK12 clearly phosphorylated some endogenous proteins, such as 72-kDa and 31-kDa proteins (Fig. 7A), as well as 65-kDa protein presumably corresponding to CoPK12 (Fig. 7A, lane 3). However, activated CaMKI mainly phosphorylated the 72-kDa protein (Fig. 7B, lane 3). These results suggest that the 72-kDa and 31-kDa proteins may serve as physiological substrates for CoPK12 in the mycelia of *C. cinereus*.

3.7. Effect of CaMK inhibitor on mycelial growth

Although KN-93 has been often used as a specific inhibitor for CaMKII [23], it was also reported to be an inhibitor for CaMKI/CaMKIV [24]. When protein kinase activity of CoPK12 was assayed in the presence or absence of KN-93, CoPK12 was significantly

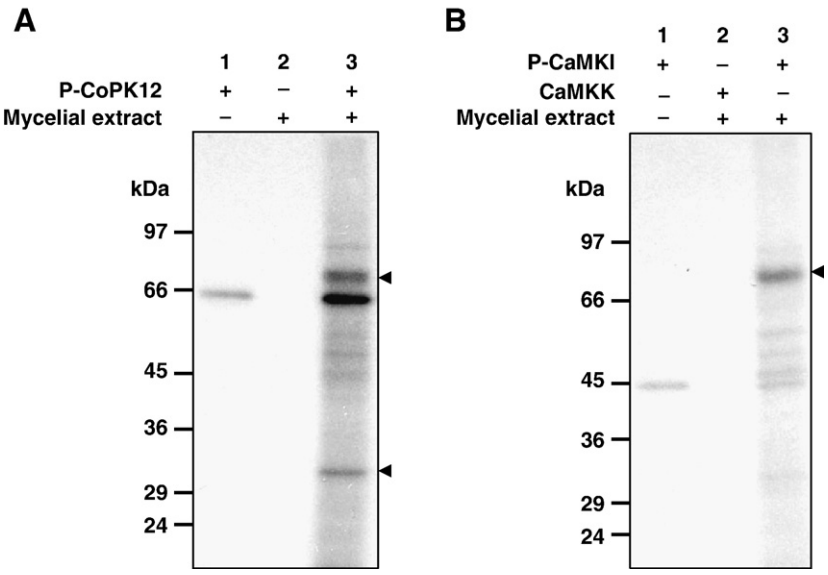

Fig. 7. Endogenous substrates of CoPK12. The mycelial extract was heat-treated at 55 °C for 60 min to inactivate endogenous protein kinases. (A) Phosphorylated CoPK12 (35 ng) and the heat-treated mycelial extract (20 μg) were incubated with [γ-^{32}P]ATP in the presence of Ca^{2+}/CaM at 30 °C for 60 min as indicated. Phosphorylated proteins were electrophoresed on 10% SDS-polyacrylamide gel and detected by autoradiography. (B) CaMKI (35 ng), which had been phosphorylated by CaMKK, was incubated with the mycelial extract (20 μg) as indicated and phosphorylated proteins were detected as described in (A). The arrowheads indicate endogenous substrates for CoPK12 and CaMKI.

inhibited in the presence of 100 μM KN-93 (Fig. 8A). In the next experiment, effect of KN-93 on the mycelial growth of *C. cinereus* was investigated. When mycelial plugs were pretreated with 1 mM of KN-93 for 24 h before inoculation, growth rate of mycelial colony was significantly reduced as compared to control mycelia without treatment (Fig. 8B and C). Significant inhibition of mycelial growth, however, was not observed when KN-93 in a concentration lower than 100 μM was used (data not shown). The reason why higher concentrations of KN-93 is required for inhibition of mycelial growth may be due to inefficient permeability into hyphal cells of *C. cinereus*.

4. Discussion

In the present study, we have cloned and characterized a CaM-binding protein kinase, designated CoPK12, from the mushroom *C. cinereus*. The amino acid sequence of the catalytic domain of CoPK12 showed 46% identity with those of mammalian CaMKI/IV. All eukaryotic CaMKI/IV homologues characterized to date possess a Thr residue at the same position in the activation loop of the catalytic domain that represents the site for activating phosphorylation. Interestingly, the phosphorylatable Thr residue at this position in the activation loop of CaMKI/IV is replaced by a Glu residue in CoPK12. Substitution of a phosphorylatable amino acid with a negatively charged amino acid may convert the kinase into a similar form to a phosphorylated CaMK. As predicted from its primary sequence, CoPK12 showed Ca^{2+}/CaM-dependent protein kinase activity toward various protein substrates, similar to the case for CaMKI phosphorylated by CaMKK. Unlike CaMKI, CoPK12 was found to undergo Ca^{2+}/CaM-dependent autophosphorylation, similar to the case for CaMKII. Although mammalian CaMKII undergoes autophosphorylation and thereby becomes an autonomous enzyme, autophosphorylation of

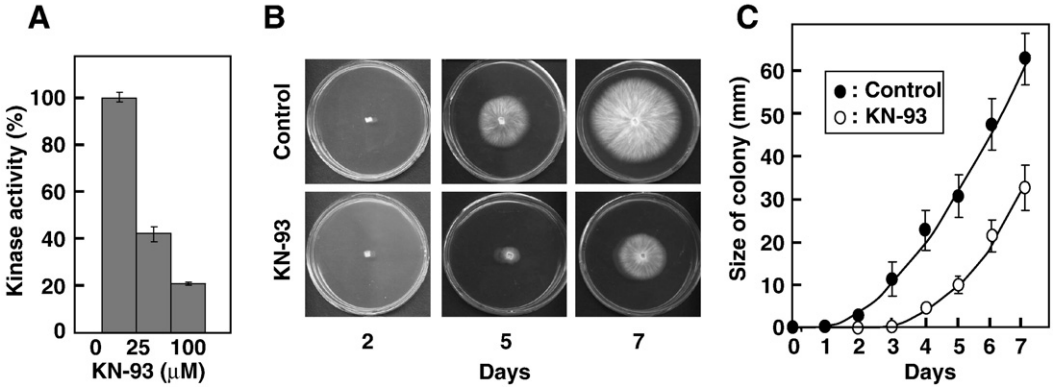

Fig. 8. Effects of KN-93 on CoPK12 activity and mycelial growth of *C. cinereus*. (A) Effect of KN-93 on CoPK12 activity. CaMK activities were assayed using 20 μM syntide-2 as a substrate in the presence (25 and 100 μM) or absence of KN-93. The reaction mixture (20 μl) contains 0.5 mM $CaCl_2$, 1 μM CaM, 20 μM syntide-2, 100 μM [γ-^{32}P]ATP, and CoPK12 (22 ng). After incubation at 30 °C for 20 min, [^{32}P]phosphate incorporated into syntide-2 was measured as described in "Materials and methods." (B) (C) Effect of KN-93 on the mycelial growth of *C. cinereus*. Mycelial plugs were pretreated with 1 mM KN-93 or H_2O (Control) for 24 h before inoculation. Mycelia were grown in 9-cm agar plates and cultured at 28 °C. Mycelial growth of all plates was photographed every day. Mycelial colonies of representative plates at 2, 5, and 7 days after inoculation are shown (B). Size of colonies in all plates was measured every day. Data are means ± SD values from three independent experiments (C).

CoPK12 showed no significant effect on its catalytic properties. These results indicate that CoPK12, the first CaMK isoform isolated from a mushroom, has different characteristic properties from those of mammalian CaMKI/IV and CaMKII.

Anti-CoPK12 antibody clearly detected two proteins of 65 kDa and 46 kDa in mycelial extract (Fig. 4). Since 65-kDa protein band corresponds to full length CoPK12, there may be two possible explanations for the occurrence of 46-kDa immunoreactive protein. It may be derived from 65-kDa CoPK12 by limited proteolysis, or it may be expressed as an alternative splicing valiant of CoPK12. When recombinant 65-kDa CoPK12 was incubated with mycelial extract, it was gradually converted to 46-kDa protein, suggesting that 46-kDa protein was produced from CoPK12 by an endogenous proteolytic enzyme (Fig. 5). Not only 65-kDa CoPK12 but also 46-kDa protein showed significant Ca^{2+}/CaM-dependent protein kinase activity as demonstrated by in-gel protein kinase assay (Fig. 6). Furthermore, considerable amount of 46-kDa CoPK12 appeared to be present in a stable form in mycelia. These results suggest that 46-kDa CoPK12 is another active form of multifunctional CaMK in *C. cinereus* and may exert some, still unknown, biological activities.

In a previous study, we found that various protein kinases were abundantly expressed in actively growing mycelia, and decreased during the maturation process of fruiting bodies [11]. CoPK12 was also highly expressed in mycelia and phosphorylatable proteins, possibly its physiological substrates, were present in the mycelial extract. Although the catalytic domain of CoPK12 is highly homologous to that of CaMKI, their N-terminal and C-terminal sequences are different (Fig. 1). These kinases exhibited somewhat different substrate specificities, probably due to the differences in their primary structures. Specifically, CoPK12 significantly phosphorylated histone as well as MBP and myosin light chain, while phosphorylated CaMKI significantly phosphorylated MBP, but not histone. An endogenous 72-kDa protein served as a common substrate for CoPK12 and CaMKI, while a 31-kDa protein served as an efficient substrate for CoPK12 but not for CaMKI. Therefore, the identities of the 31-kDa and 72-kDa substrate proteins will be the next issues to be solved for clarification of the physiological functions of CoPK12 in growing mycelia of *C. cinereus*.

Lu et al. [25] created a strain of the genetically tractable filamentous fungus, *A. nidulans*, that is conditional for CaM expression. They demonstrated that *A. nidulans* could not grow when expression of CaM was repressed, but that the fungi grew faster when CaM was overexpressed. Recently, Wang et al. reported that CaM is highly concentrated in the apex of growing hyphae of *A. nidulans* as visualized by the CaM fusion protein with green-fluorescent protein in a transformed strain [26]. These findings, therefore, suggest that CaM may play an important role in apical organization, morphogenesis, and growth of hyphae. In our study, we found that CaM-dependent protein kinase in *C. cinereus*, CoPK12, was highly concentrated in the actively growing region of mycelial colony (Fig. 4C and D). When mycelia were treated with KN-93, a specific inhibitor of CaMKs including CoPK12, mycelial growth was significantly inhibited (Fig. 8B and C). These results, taken together, suggest that Ca^{2+}-signaling pathway consisting of Ca^{2+}/CaM and CoPK12 may be involved in the mycelial growth probably at the actively growing hyphal tip of *C. cinereus*.

In the present paper, we have reported a unique CaMK in the mushroom *C. cinereus*. Since the amino acid sequence in the activation loop of CoPK12 appears to mimic the phosphorylated form of CaMKI/IV, CoPK12 behaved like a phosphorylated form of CaMKI. The Thr residue in the activation loop of CMKB, a CaMKI/IV homologue in *A. nidulans*, is phosphorylated by CMKC, a homologue of CaMKK in *A. nidulans* [27]. CMK1, a homologue of CaMKI in *S. pombe* is also regulated through phosphorylation at the identical Thr residue [28]. In contrast to the cases of mammals and *A. nidulans*, we could not find any genes for CaMKK homologues in the database of *C. cinereus*, suggesting that CaMKs in this mushroom are not regulated by an activating kinase. When we checked the sequence database, we found a putative Ser/Thr kinase (Accession No.: AB218428) corresponding to a multifunctional CaMK in *Schizophyllum commune*, another well-known mushroom. In this case, the amino acid sequence in the activation loop is highly homologous to that of CoPK12 and a Glu residue is present at the identical position in the activating loop. These results suggest that the CaMK cascade in mushrooms differs from those in other organisms, and that CaMK may not be regulated by an upstream kinase corresponding to CaMKK. Extensive studies are still required to elucidate the regulatory mechanisms of the Ca^{2+}signaling pathways in mushrooms.

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