Phosphorylation of the C2 subunit of the proteasome in rice (*Oryza sativa* L.)

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Abstract Proteasomes function mainly in the ATP-dependent degradation of proteins that have been conjugated with ubiquitin. To demonstrate the phosphorylation of proteasomes in plants, we conducted an enzymatic dephosphorylation experiment with a crude extract of rice cultured cells. The results indicated that the C2 subunit of the 20S proteasome is phosphorylated in vivo in cultured cells. An in-gel kinase assay and analysis of phosphoamino acids revealed that the C2 subunit is phosphorylated by a 40-kDa serine/threonine protein kinase, the activity of which is inhibited by heparin, a specific inhibitor of casein kinase II. The catalytic subunit of casein kinase II from *Arabidopsis* was also able to phosphorylate the C2 subunit. These results suggest that the C2 subunit in rice is probably phosphorylated by casein kinase II. Our demonstration of the phosphorylation of proteasomes in plants suggests that phosphorylation might be involved in the general regulation of the functions of proteasomes.

Key words: Proteasome; Ubiquitin; Casein kinase II; Phosphorylation; (Rice)

1. Introduction

A proteasome is a multicatalytic proteinase complex that has been identified in archaebacteria and in eukaryotes from yeast to humans [1–4]. There are two types of eukaryotic proteasome, and they have sedimentation coefficients of 20S and 26S, respectively [5]. Energy derived from ATP is required for the formation of the 26S complex (with a molecular mass of more than 1500 kDa), which contains the 20S complex (≈700 kDa) as well as a characteristic set of other protein components (35–110 kDa) [6–9]. The 20S complex is composed of 13–15 different subunits. The 26S complex seems to be an active form, and it catalyzes the ATP-dependent degradation of various proteins that have been conjugated with ubiquitin.

Proteasomes have been found in several plants, such as tobacco [10], potato and mung bean [11], pea [12], and wheat [13]. Proteasomes of 20S and 26S have been purified from pea [14] and spinach [15,16]. A gene homologous to the gene for the largest subunit (32–35 kDa) of proteasomes in human, rat and *Drosophila* has been identified in *Arabidopsis* [17]. Several other subunits have also been isolated from *Arabidopsis* [18–20] and rice [21]. We recently isolated a rice cDNA clone for a protein that was homologous to the largest subunit of the 20S complex, the C2 subunit (DDBJ/EMBL/Genbank database accession number: D37886). We found that antibodies against the C2 subunit cross-reacted with a 35-kDa protein that was part of both the 20S and the 26S complexes (submitted).

Pereira and Wilk [22] reported that at least two of the subunits of proteasomes were phosphorylated by a cAMP-dependent protein kinase that had copurified with a complex isolated from bovine pituitaries. Since then, several reports have described the phosphorylation in vivo or in vitro of animal proteasomes [23–25]. However, the phosphorylation of plant proteasomes has not yet been described.

In the present study, we showed that the C2 subunit from rice is phosphorylated by a serine/threonine protein kinase that has a molecular mass of 40 kDa. The catalytic subunit of casein kinase II from *Arabidopsis* also phosphorylated the C2 subunit in vitro. This report includes a brief discussion of the possible general involvement of the phosphorylation of proteasomes in their regulatory functions.

2. Materials and methods

2.1. Plant material and growth conditions

Suspension cultured cells of rice (*Oryza sativa* L. cv. Yamahoushi) were maintained in liquid AA medium [26] on a gyratory shaker (80 rpm) at 25°C, and they were subcultured at weekly intervals.

2.2. Preparation of polyclonal antibodies

A *Hin*dIII–*Eco*RI fragment of the cDNA that included the entire protein-coding region was blunt-ended with Klenow fragment and cloned into the *Sma*I site of the pGEX-2T vector (Pharmacia). A plasmid carrying the cDNA insert in the same frame as the gene for glutathione-S-transferase (GST) was isolated, and the GST-fusion protein was produced in *E. coli* cells and purified with Glutathione Sepharose 4B (Pharmacia) as specified by the manufacturer. Antibodies against the C2 subunit were raised in a rabbit with the purified GST-fusion protein as the antigen. The antibodies were partially purified by ammonium sulfate precipitation.

2.3. Treatment of rice proteins with phosphatase

Total protein (15 μg) from rice cultured cells was treated with calf intestine alkaline phosphatase (Takara Shuzo Co.) in an alkaline buffer (50 mM glycine–NaOH (pH 10), 1 mM $MgCl_2$, 1 μg/ml heparin) for 1 h at 37°C. As a control, total protein was incubated in the same buffer without alkaline phosphatase. These samples were fractionated by SDS-PAGE on a 17% polyacrylamide gel that contained a low concentration of *N,N'*-methylenebisacrylamide (0.135%), and then proteins were subjected to Western blotting with antibodies against the C2 subunit.

2.4. In-gel kinase assay

An in-gel kinase assay was performed as described by Geahlen et al. [27]. One hundred micrograms of total protein, isolated from cultured cells, was subjected to SDS-PAGE in a 12% polyacrylamide gel that contained 0.5 mg/ml GST-C2 fusion protein.

2.5. Analysis of phosphoamino acids

The GST-C2 fusion protein that had been phosphorylated by rice endogenous kinase(s) was eluted from the gel in elution solution (40 mM NH_4HCO_3 and 200 μg/ml TPCK-treated trypsin) and hydrolyzed in 6 N HCl for 1.5 h at 110°C. The hydrolysate was then evaporated

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to dryness and the residue was suspended in an aqueous solution of standard phosphoamino acids (3.3 mg/ml each, phosphoserine, phosphothreonine and phosphotyrosine; Sigma). Samples were fractionated by thin-layer chromatography with electrophoresis in a solution of 0.5% pyridine in 5% acetic acid. Amino acids were identified by the ninhydrin reaction, and phosphoamino acids were detected by autoradiography.

2.6. Phosphorylation by the catalytic subunit of casein kinase II from Arabidopsis

The catalytic subunit of casein kinase II (ATCKA1) from *Arabidopsis* was expressed as a GST–fusion protein in *E. coli* cells that harbored the plasmid pGEX-ATCKA1 [28], and a cell extract was collected by centrifugation after sonication. As a control, a cell extract from *E. coli* that contained GST encoded by the pGEX4T-2 vector was also prepared. GST or the GST-C2 fusion protein was bound to beads of Glutathione Sepharose (Pharmacia) in an Eppendorf tube, and the Sepharose was washed 3 times with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and then incubated with cell extracts, prepared as described above, for 2 h at 4°C. After extensive washing of the beads 3 times with PBS and once with kinase buffer (50 mM Tris-HCl (pH 7.8), 15 mM $MgCl_2$, 5 mM EGTA and 1 mM dithiothreitol (DTT)), the kinase reaction was started as described below for the assay of phosphorylation in vitro. After electrophoresis of the proteins in the reaction mixture, the gel was stained with Coomassie brilliant blue (CBB), and phosphorylated proteins were detected by autoradiography.

2.7. Fractionation of a crude lysate by glycerol density gradient centrifugation

Four-day-old rice cultured cells were harvested and frozen in liquid nitrogen. Frozen cells were suspended in buffer A (20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 1 mM DTT). The suspension was centrifuged at 15000×*g* for 30 min at 4°C for preparation of the crude lysate. About 1 ml of crude lysate containing 2 mg protein was loaded on a 12-ml linear gradient of 10–40% (v/v) glycerol in buffer A. The gradient was centrifuged at 27000 rpm for 18 h at 4°C in an SW40 rotor (Beckman), and 18 fractions were collected from the bottom. Proteins in 100 μl of each fraction were precipitated with acetone and subjected to electrophoresis and Western blotting analysis.

2.8. Assay of phosphorylation in vitro

For the assay of phosphorylation in vitro, 300 μl of each fraction from the above-mentioned gradient were incubated for 2 h at 4°C with the GST-C2 fusion protein that had been bound to Glutathione Sepharose. The Sepharose was extensively washed 3 times with 50 mM Tris-HCl (pH 7.8) and once with kinase buffer (50 mM Tris-HCl (pH 7.8), 15 mM $MgCl_2$, 5 mM EGTA and 1 mM DTT). The Sepharose was then suspended in kinase buffer that contained 5.3 MBq/ml [γ-^{32}P]ATP (ICN Biomedicals) plus 10 nM cold ATP and incubated for 15 min at room temperature. The reaction was stopped by addition of sample buffer for SDS-PAGE and boiling for 5 min, and the clear supernatant after centrifugation was loaded onto a 12% polyacrylamide gel for SDS-PAGE. After electrophoresis, the gel was stained with CBB, dried and subjected to autoradiography.

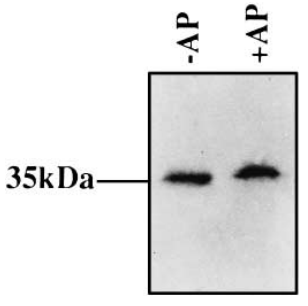


Fig. 1. Dephosphorylation of proteins in a crude extract of rice cells by alkaline phosphatase. Total protein from rice cultured cells was treated with alkaline phosphatase, subjected to SDS-PAGE in a 17% gel that contained a low concentration of *N,N'*-methylenebisacrylamide (0.135%), and then subjected to Western blotting with antibodies against the C2 subunit (+AP). As a control, total protein was incubated in alkaline buffer without phosphatase (-AP).

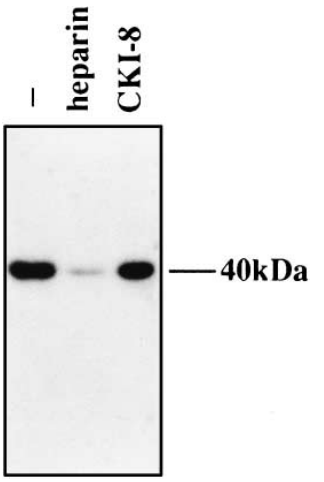


Fig. 2. In-gel kinase assay. A crude extract from rice cultured cells was subjected to electrophoresis in a gel that contained the GST-C2 subunit fusion protein. The kinase reaction was conducted in the gel without any inhibitor (–) or in the presence of either 0.5 nM heparin or 10 μM CKI-8, as indicated.

2.9. Assay of proteolytic activity

Equal volumes of individual fractions after glycerol density gradient centrifugation were used in an assay of the hydrolysis of the fluorogenic substrate *N*-succinyl–Leu–Leu–Val–Tyr 7-amido-4-methylcoumarin (Sigma). The substrate was incubated with each fraction for 30 min at 37°C in the presence of 0.04% SDS in 100 mM Tris-HCl (pH 8.0) as described previously [29]. The reaction was stopped by addition of 100 μl of 10% SDS and 2 ml of 0.1 M Tris-HCl (pH 9.0), and the fluorescence of the reaction products was then measured [29]. Proteolytic activity was calculated by reference to the fluorescence of 7-amido-4-methylcoumarin (Nacalai Tesque) as the standard.

3. Results and discussion

3.1. Evidence for phosphorylation of the C2 subunit in rice

To demonstrate the phosphorylation in vivo of plant proteasomes, we dephosphorylated proteins in an extract of rice cultured cells with alkaline phosphatase. When antibodies against the C2 subunit were used for immunoblotting, the band of the C2 subunit shifted to a lower mobility than that in the control preparation in which total protein had been incubated in alkaline buffer without phosphatase (Fig. 1). This result was reproducible. When the dephosphorylation reaction was conducted in the presence of sodium phosphate, which inhibits protein phosphatases, we did not detect a shift in mobility (data not shown). Dephosphorylation of a protein often alters its mobility during SDS-PAGE. For example, phosphatase digestion of the KIN28 subunit of holo-TFIIH from yeast produced a decrease in mobility of the protein [30]. Therefore, an effect of phosphatase digestion on electrophoretic mobility suggests prior phosphorylation. Our result indicated that the C2 subunit was phosphorylated in rice cultured cells in vivo.

3.2. Characterization of the protein kinase that phosphorylates the C2 subunit in rice

To characterize the endogenous kinase that phosphorylates the C2 subunit, we fractionated total protein from rice cultured cells in a gel that contained the GST-C2 subunit fusion

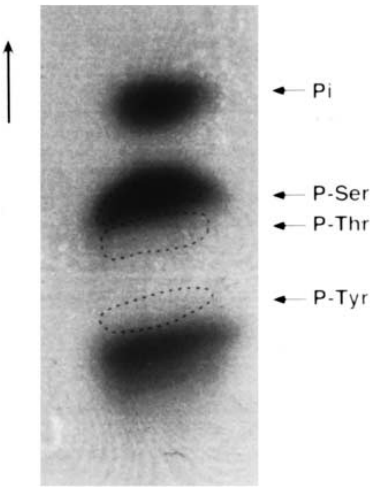


Fig. 3. Analysis of phosphoamino acids in the C2 subunit. The GST-C2 fusion protein that had been phosphorylated by the endogenous kinase in rice cells was hydrolyzed in 6 N HCl. Phosphoamino acids were separated by thin-layer chromatography and visualized by autoradiography. The arrow indicates the direction of electrophoresis. The positions of the phosphoamine acid standards, as detected by the ninhydrin reaction, are shown on the autoradiogram by dotted circles. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; Pi, inorganic phosphate.

protein and performed an in-gel kinase assay. As shown in Fig. 2, a single band of 40 kDa was detected after autoradiography, suggesting that a 40-kDa protein was the catalytic subunit of the C2 kinase. When 0.5 nM heparin was added to the solution for the kinase reaction, the intensity of the signal decreased dramatically. Heparin is known to be a specific inhibitor of casein kinase II. However, no inhibition was observed in the presence of 10 μM CKI-7 or CKI-8, which are inhibitors of casein kinase I. GST was not phosphorylated by the 40-kDa kinase in the in-gel kinase assay (data not shown).

We subjected the phosphorylated C2 subunit to phosphoamino acid analysis. After thin-layer chromatography and autoradiography, we observed a strong signal with the same mobility as the standard sample of phosphoserine (Fig. 3). No signals were observed at the positions of phosphothreonine and phosphotyrosine. We also detected another signal with lower mobility (Fig. 3), which we assumed was due to incompletely hydrolyzed peptides. These results indicated that a serine residue in the C2 subunit was phosphorylated by a 40-kDa serine/threonine protein kinase.

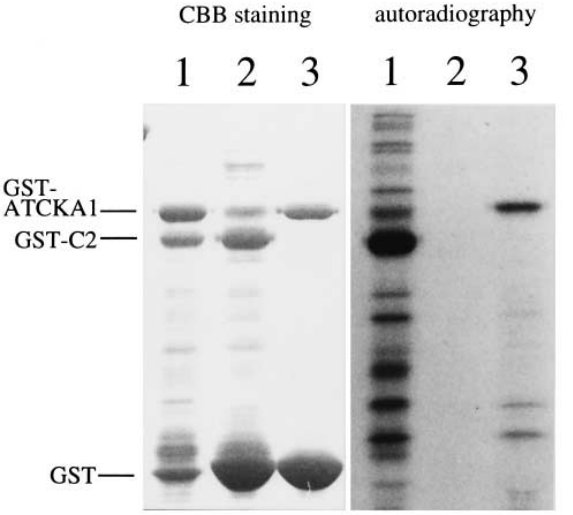
3.3. *Casein kinase II from Arabidopsis phosphorylates the C2 subunit in vitro*

Since a low concentration of heparin inhibited the activity of the C2 kinase, as described above, we examined the interaction in vitro between the C2 subunit and ATCKA1, one of the catalytic subunits of casein kinase II from *Arabidopsis* [28]. ATCKA1 expressed in *E. coli* had a molecular mass of 40 kDa, the same as that of the C2 kinase [28]. When the GST-C2 fusion protein was bound to Glutathione Sepharose and incubated with an extract of *E. coli* cells that contained GST-ATCKA1 and subjected to the kinase reaction, a signal was detected after electrophoresis and autoradiography at the position of the 57-kDa GST-C2 protein (Fig. 4, lane 1). However, a cell extract containing only GST instead of the fusion protein did not phosphorylate any protein (Fig. 4, lane 2). When GST was bound to Glutathione Sepharose and incubated with a cell extract that contained GST-ATCKA1, GST was not phosphorylated (Fig. 4, lane 3). We detected a 67-kDa protein that corresponded to the autophosphorylated GST-ATCKA1. Such autophosphorylation was also observed by Mizoguchi et al. [28]. These results indicated that the C2 subunit interacted with ATCKA1 in the cell extract and was phosphorylated in vitro. When purified GST-C2 and GST-ATCKA1 fusion proteins were mixed and subjected to the conditions of the kinase reaction, the C2 subunit was phosphorylated. This kinase activity was completely inhibited by 0.5 nM heparin (data not shown).

3.4. *Behavior of the C2 kinase during fractionation by glycerol density gradient centrifugation*

A human 30-kDa subunit which was copurified with the 20S proteasome from erythrocytes was phosphorylated by casein kinase II [24]. Castaño et al. [23] identified two subunits of 29 kDa, C8 and C9, that were phosphorylated by casein kinase II in the proteasome complex from the rat kidney. Arribas et al. [31] reported evidences for post-translational modification, like to be phosphorylation, for rat C2 and C9 subunits. Protein kinases that phosphorylated proteasome subunits were copurified with the 20S complex from animal cells [22,25]. Therefore, we fractionated the crude extract of rice cells by glycerol density gradient centrifugation in an attempt to identify fractions with C2 kinase activity.

The level of the C2 subunit and the latent proteolytic activity of the 20S complex were maximum in the same fraction,



Lane	1	2	3
Extract	GST-ATCKA1	GST	GST-ATCKA1
Sepharose	GST-C2	GST-C2	GST

Fig. 4. Phosphorylation of the C2 subunit by the catalytic subunit (ATCKA1) of casein kinase II from *Arabidopsis*. GST-C2 (lanes 1 and 2) and GST (lane 3) were bound to Glutathione Sepharose beads and incubated with an extract of *E. coli* cells that contained GST-ATCKA1 (lanes 1 and 3) or GST (lane 2). After extensive washing, the beads were subjected to the kinase reaction, and phosphoproteins were separated by SDS-PAGE with subsequent staining with CBB and autoradiography. The positions of GST-ATCKA1, GST-C2 and GST are indicated.

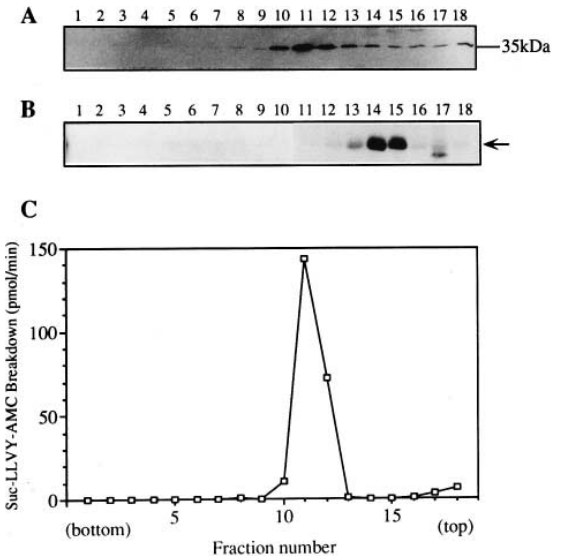


Fig. 5. Phosphorylation assay with each fraction after glycerol density gradient centrifugation. A crude lysate of rice cells was fractionated by glycerol density gradient centrifugation, and aliquots of individual fractions were subjected to (A) Western blotting, (B) the phosphorylation assay and (C) the assay of proteolytic activity. For the phosphorylation assay, the GST-C2 fusion protein was bound to Glutathione Sepharose, incubated with an aliquot of each fraction and then subjected to the kinase reaction. The arrow indicates the position of the GST-C2 fusion protein. Suc-LLVY-AMC, *N*-succinyl–Leu–Leu–Val–Tyr 7-amido-4-methylcoumarin.

fraction 11 (Fig. 5A,C). By contrast, when the GST-C2 fusion protein bound to Glutathione Sepharose was incubated with an equal volume of each fraction to detect the activity of the C2 kinase, maximum phosphorylation activity was detected in fractions 14 and 15, and almost no activity was detected in fraction 11 (Fig. 5B). This result suggests that the protein kinase that interacts with and phosphorylates the C2 subunit might not be part of the 20S complex. However, we cannot exclude the possibility that, while the C2 kinase is associated with the proteasome complex, it is difficult for the enzyme to phosphorylate an exogenous C2 substrate. It is important now to determine whether the purified proteasome complex includes a protein kinase that phosphorylates the C2 subunit.

3.5. *Involvement of the phosphorylation of the proteasome in its functions*

Rihs et al. [32] proposed that casein kinase II might introduce functional determinants that modify the rate of transport of proteins to nuclei, in addition to nuclear localization signals (NLSs), by phosphorylating sites close to NLSs. In the carboxy-terminal part of the rice C2 subunit, we found a bipartite NLS [33] that consisted of two pairs of basic amino acids (Arg–Lys and Lys–Arg), separated by a spacer of 13 residues. Moreover, a sequence, SMQE, which corresponds to the consensus sequence for sites of phosphorylation by casein kinase II (Ser/Thr–X–X–acidic amino acid) [34] was also found in the carboxy-terminal region, which was rich in glutamyl residues. An acidic cluster near the site of phosphorylation is known to increase the efficiency of phosphorylation by casein kinase II [34]. Therefore, the combination of the potential NLS and the consensus site for phosphorylation by casein kinase II leads to the hypothesis that the rice proteasome might move to the nucleus under the control of phosphorylation. Although the sequence SMQE was not conserved in other C2 subunits from different organisms, we could find another consensus sequence in the acidic carboxy-terminal region of these subunits.

Proteasomes have been found in nuclei of human cells at high concentrations [13,35]. Haass et al. [36] also reported the tissue-dependent differential distribution of the C2 subunit between the nucleus and the cytoplasm of *Drosophila* cells. Phosphorylation of proteasomes might regulate other functions of the 20S and/or 26S complexes, such as catalytic activity, assembly, and protein turnover. Further studies of the phosphorylation of proteasomes may help to clarify the regulation of ubiquitin-dependent proteolytic activity in plants.

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