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Rapid Paper

The Ubiquitin–Proteasome Pathway is Involved in Rapid Degradation of Phosphoenolpyruvate Carboxylase Kinase for C4 Photosynthesis

Masakazu Agetsuma [1], Tsuyoshi Furumoto [1], Shuichi Yanagisawa [2] and Katsura Izui [1, 3]

[1] *Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502 Japan*
[2] *Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657 Japan*

In C4 photosynthesis, phosphoenolpyruvate carboxylase (PEPC) is the enzyme responsible for catalyzing the primary fixation of atmospheric CO_2. The activity of PEPC is regulated diurnally by reversible phosphorylation. PEPC kinase (PEPCk), a protein kinase involved in this phosphorylation, is highly specific for PEPC and consists of only the core domain of protein kinase. Owing to its extremely low abundance in cells, analysis of its regulatory mechanism at the protein level has been difficult. Here we employed a transient expression system using maize mesophyll protoplasts. The PEPCk protein with a FLAG tag could be expressed correctly and detected with high sensitivity. Rapid degradation of PEPCk protein was confirmed and shown to be blocked by MG132, a 26S proteasome inhibitor. Furthermore, MG132 enhanced accumulation of PEPCk with increased molecular sizes at about 8 kDa intervals. Using anti-ubiquitin antibody, this increase was shown to be due to ubiquitination. This is the first report to show the involvement of the ubiquitin–proteasome pathway in PEPCk turnover. The occurrence of PEPCks with higher molecular sizes, which was noted previously with cell extracts from various plants, was also suggested to be due to ubiquitination of native PEPCk.

Keywords: C4 photosynthesis — Phosphoenolpyruvate carboxylase kinase — 26S Proteasome — Protein degradation— Ubiquitination.

Abbreviations: CaMV, cauliflower mosaic virus; CHX, cycloheximide; Ctag-PEPCk, PEPCk conjugated with a FLAG tag at the C-terminus; FtPEPCk, *Flaveria trinervia* PEPCk; Ntag-sGFP, sGFP(S65T) conjugated with a FLAG tag at the N-terminus; Ntag-PEPCk, PEPCk conjugated with a FLAG tag at the N-terminus; PEPC, phosphoenolpyruvate carboxylase; PEPCk, PEPC kinase; PMSF, phenylmethylsulfonyl fluoride; PNP, phosphorylated peptide of the N-terminal region of PEPC; sGFP(S65T), synthetic green-fluorescent protein S65T mutant; ZmPEPC, *Zea mays* C4-type PEPC.

Introduction

In addition to the C3 photosynthetic pathway, C4 plants are endowed with the C4 cycle, through which they achieve high photosynthetic performance and high efficiencies of water and nutrient usage (Edwards et al. 2001). In the C4 cycle, phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) plays a key role by catalyzing the primary fixation reaction of atmospheric CO_2. This enzyme catalyzes irreversible carboxylation of phosphoenolpyruvate, yielding oxaloacetate and orthophosphate in the presence of Mg^{2+} (O'Leary 1982, Chollet et al. 1996, Izui et al. 2004). The activity of the PEPC isoform for C4 photosynthesis is regulated diurnally by reversible phosphorylation at the conserved serine residue near the N-terminus (Chollet et al. 1996, Vidal and Chollet 1997, Nimmo 2003a, Izui et al. 2004). Upon phosphorylation in the daytime, when C4 photosynthesis is active, PEPC diminishes its sensitivity to an allosteric feedback inhibitor, L-malate; however, at night, PEPC is dephosphorylated to recover its original sensitivity. This diurnal change in the phosphorylation state is regulated mainly by a protein kinase, since the protein kinase activity changes diurnally while that of protein phosphatase does not. On the other hand, in CAM plants, in which atmospheric CO_2 is captured at night by the action of PEPC, the diurnal change in phosphorylation is reversed compared with C4 plants (Nimmo 2003b). At present, all PEPCs of higher plants, including C3 plants, are thought to be involved in regulatory phosphorylation in response to a variety of metabolic demands (Izui et al. 2004).

Enzymological studies on the protein kinase involved in phosphorylation of PEPC have revealed that it is calcium independent and highly specific to PEPC (Chollet et al. 1996, Vidal and Chollet 1997, Nimmo 2003a, Izui et al. 2004); the enzyme was named PEPC kinase (PEPCk). Cloning and expression of cDNAs for PEPCk were accomplished using CAM plants, *Kalanchoe fedtschenkoi* (Hartwell et al. 1999) and *Mesembryanthemum crystallinum* (Taybi et al. 2000), a C4 plant, *Flaveria trinervia* (Tsuchida et al. 2001), and a root nodule of a C3 plant, *Lotus japonicus* (Nakagawa et al. 2003). In addition, further cloning studies and/or annotation of the DNA sequence database have been performed with C3 plants such as *Arabidopsis thaliana* (Fontaine et al. 2002), *Glycine max* (Xu et al.

[3] Corresponding author: E-mail, izui@kais.kyoto-u.ac.jp; Fax, +81–75–753–6470.

2003, Sullivan et al. 2004), *Lycopersicon esculentum* (Marsh et al. 2003) and *Solanum tuberosum* (Marsh et al. 2003). These studies revealed that the genes for PEPCks constitute a small gene family, and that PEPCks are generally made up of only the core domain of conventional protein kinases with an M_r of 30–33 kDa.

Analysis at the mRNA level of PEPCk isoforms in a C3 plant suggested the multiplicity of their regulatory mechanisms, since their expression patterns were different from one another (Sullivan et al. 2004). In C4 plants, the diurnal change in PEPCk activity was shown to be linked to a change in the mRNA level, suggesting the high turnover rate of PEPCk protein (Hartwell et al. 1996, Tsuchida et al. 2001). In corroborating this, rapid decay of PEPCk activity was observed in detached leaves and protoplasts fed with an inhibitor of protein synthesis (Jiao et al. 1991, Giglioli-Guivarc'h et al. 1996). Thus, the activity of PEPCk in vivo is thought to be regulated mainly at its protein level, which is determined by the relative rate of its synthesis to degradation. The tight linkage between activity and mRNA levels was also reported in CAM plants (Nimmo 2003b).

However, with regards to the regulatory mechanism of PEPCk at the protein level, much remains to be investigated. Despite the proposed occurrences of an endogenous protein that inhibits PEPCk (Nimmo et al. 2001) and possible thioredoxin-mediated redox regulation of PEPCk (Saze et al. 2001, Tsuchida et al. 2001), few reports have shown the mechanism of PEPCk degradation or other post-translational regulation. Direct analysis at the protein level has been hampered because of the extremely low abundance of PEPCk protein in vivo (Saze et al. 2001). In fact, detection of the protein with specific antibodies against PEPCk has proved difficult in several laboratories including ours.

On the other hand, an in-gel protein kinase assay revealed that there are at least two molecular species of PEPCk with sizes of about 30 and 37 kDa, respectively, in several plants such as maize, ice plants, tobacco and soybean (Li and Chollet 1993, Li and Chollet 1994, Li et al. 1996, Zhang and Chollet 1997). Although many *PEPCk* genes have been identified, all encode proteins of only about 30 kDa, not 37 kDa. The mRNA for PEPCk isoforms of tomato and potato are known to be processed by alternative splicing (Marsh et al. 2003), while the resulting transcripts cannot produce the 37 kDa PEPCk. Thus, it is possible that the 37 kDa PEPCk might be derived through covalent modification of the 30 kDa PEPCk. One plausible modification is conjugation with ubiquitin, but no experimental evidence has been presented so far (Nimmo 2003a).

In this study, to overcome the difficulties in detecting the scarce PEPCk protein and to investigate the regulation of PEPCk at the protein level, we utilized the transient expression system (Sheen 1991). A cDNA for PEPCk carrying a FLAG tag was expressed under the cauliflower mosaic virus (CaMV) 35S promoter in maize mesophyll protoplasts, and the expressed fusion protein was detected with a monoclonal anti-FLAG antibody with high sensitivity. Using this system, we were able to report for the first time that PEPCk protein is rapidly degraded in the protoplasts of C4 plants, and that this degradation is mediated through the ubiquitin–proteasome pathway. Although involvement of the ubiquitin–proteasome pathways has been reported for several plant enzymes and proteins (Smalle and Vierstra 2004), no report has shown this for plant protein kinases. Furthermore, this is the first example of participation of the ubiquitin–proteasome pathway in the regulation of C4 photosynthesis. We also suggest that the 37 kDa PEPCk previously reported in plants in vivo might be derived from mono-ubiquitination of intact 30 kDa PEPCk, mainly presenting an inhibited and latent state bound with some other protein(s).

Results

Transient expression of PEPCk in protoplasts

A cDNA encoding *Flaveria trinervia* PEPCk (FtPEPCk) for regulatory phosphorylation of C4-form PEPC (Tsuchida et al. 2001) was used in our experiments. Plasmids were constructed to produce fusion proteins of FtPEPCk (32 kDa) tagged with a FLAG (a peptide of eight amino acid residues, DYKDDDDK; about 1 kDa) on either the N- or C-terminus (termed Ntag-PEPCk and Ctag-PEPCk, respectively). Expression of these fused genes was driven by a CaMV 35S promoter.

The DNA constructs were transfected by electroporation into mesophyll protoplasts prepared from greening leaves of maize seedlings for transient expression. After incubation for 11 h under light, the protoplasts were harvested and the lysates were subjected to SDS–PAGE followed by immunoblot analysis with an anti-FLAG antibody. As shown in Fig. 1, immunoreactive bands of FLAG-tagged proteins of the expected molecular size (approximately 33 kDa) were clearly detected in lanes N and C, indicating successful transient expression of FLAG-tagged PEPCk proteins. The mobility of Ctag-PEPCk was slightly higher than that of Ntag-PEPCk, in spite of an identical molecular size predicted from the open reading

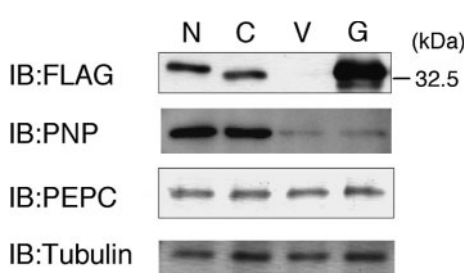

Fig. 1 Transient expression of PEPCk in maize mesophyll protoplasts. PEPCk protein conjugated to a FLAG tag at its N- (N) or C-terminus (C) was expressed in maize mesophyll protoplasts. The level of FLAG-tagged protein and the PEPC phosphorylation status were detected immunologically. A similar amount of endogenous PEPC (both phosphorylated and non-phosphorylated PEPC) in each sample was also verified. V, vector control; G, Ntag-sGFP; IB, immunoblotting.

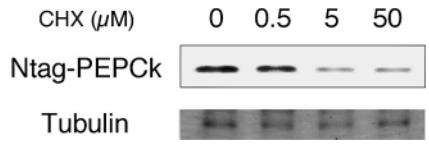

Fig. 2 Effect of varying concentrations of CHX on the PEPCk protein level. The maize mesophyll protoplasts expressing Ntag-PEPCk were treated with the indicated concentrations of CHX for 2 h. The protein level of Ntag-PEPCk was detected by immunoblot analysis with anti-FLAG antibody.

frames in the plasmids; the reason for this discrepancy is not clear at present. The levels of these proteins were not different from those of the samples incubated in the dark, indicating that accumulation of PEPCk was not affected by light/dark conditions in our experimental system (data not shown). A synthetic green fluorescent protein S65T mutant [sGFP(S65T), Chiu et al. 1996] conjugated with a FLAG tag at the N-terminus (named Ntag-sGFP) was also expressed as a control. Expression was highly efficient (lane G) and its molecular size estimated from mobility was well in accordance with the experiment of Chiu et al. (1996).

To examine whether the FtPEPCk conjugated to the FLAG tag was correctly folded to form an active enzyme in the protoplast, PEPCk activity was inferred from the extent of phosphorylation of endogenous PEPC. The phosphorylation state of PEPC was estimated by immunoblot analysis with an antibody specific to the phosphorylated peptide of the N-terminal region of PEPC (anti-PNP antibody) (Ueno et al. 2000). As shown in Fig. 1, the extent of PEPC phosphorylation was very low in both the control sample transfected with an empty plasmid (lane V) and in the protoplasts expressing Ntag-sGFP (lane G), while the extent of phosphorylation in both protoplasts expressing PEPCk with the FLAG tag was considerably higher (lanes N and C). These results indicate that the expressed PEPCk proteins, Ntag-PEPCk and Ctag-PEPCk, were correctly folded so as to exert enzyme activity in the protoplasts. Green fluorescence from Ntag-sGFP in the protoplasts was also detected normally using fluorescence microscopy, indicating correct folding of Ntag-sGFP protein (data not shown).

Rapid degradation of PEPCk

To investigate the stability of the PEPCk protein, we used cycloheximide (CHX), an inhibitor of de novo protein synthesis. After 11 h incubation, CHX was added to the incubation buffer of protoplasts expressing Ntag-PEPCk, and then the protoplasts were incubated further for 2 h before harvest. Extracts from the protoplasts were subjected to immunoblot analysis with an anti-FLAG antibody. The protein level of Ntag-PEPCk was decreased at higher concentrations of CHX (Fig. 2). The CHX chase experiments also showed that the protein level of Ntag-PEPCk decreased rapidly with time, whereas that of

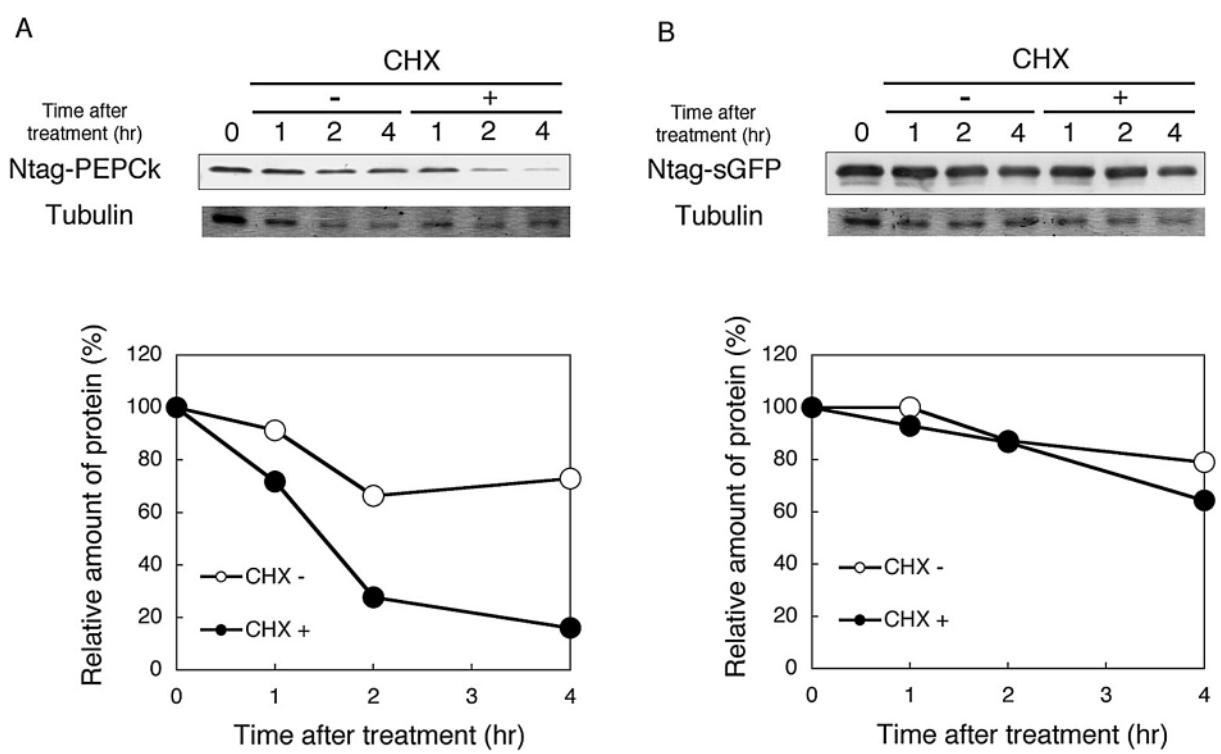

Fig. 3 The CHX chase experiments. Transfected protoplasts were incubated with or without 100 μM CHX and the protein levels were chased. Ntag-PEPCk (A) or Ntag-sGFP (B) was detected immunologically (upper panels). Each signal strength of FLAG-tagged protein in (A) and (B) was measured by Image Gauge and plotted in each lower panel as the relative amount of remaining protein.

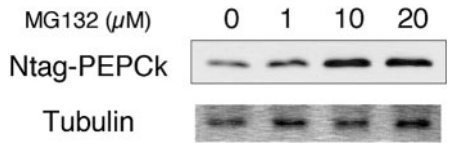

Fig. 4 Effect of varying concentrations of MG132 on the degradation of PEPCk protein. After pre-treatment with MG132 for 30 min at the indicated concentrations, protoplasts expressing Ntag-PEPCk were incubated with 50 μM CHX for 2 h. The Ntag-PEPCk levels were detected by immunoblot analysis with anti-FLAG antibody.

Ntag-sGFP was not so affected (Figs 3A and B, upper panels). When the relative amount of the remaining PEPCk at each time point was estimated from these data and plotted, Ntag-PEPCk was shown to be degraded with an approximate half-life of about 1–2 h in the presence of CHX (Fig. 3A, lower panel). In contrast, the degradation rate of Ntag-sGFP protein was considerably slower and the effect of CHX on the remaining Ntag-sGFP was not significant (Fig. 3B, lower panel). These observations suggested that the rapid degradation of Ntag-PEPCk was due neither to overexpression of proteins nor to conjugation with a FLAG tag, but to some specific mechanism for the degradation of Ntag-PEPCk.

PEPCk is ubiquitinated and degraded by the 26S proteasome

In cultured mammalian cells, the ubiquitin–proteasome pathway mediates rapid degradation of an extracellular signal-regulated kinase 3 (ERK3), a homolog of mitogen-activated protein (MAP) kinase (Coulombe et al. 2003). In analogy with the rapid degradation of ERK3, it can be envisaged that PEPCk degradation is also performed by the ubiquitin–proteasome pathway. To obtain a clue to this theory, we investigated the effect of MG132, a well-known 26S proteasome inhibitor (Lee and Goldberg 1998), on the degradation of PEPCk. When MG132 was fed to the protoplasts expressing Ntag-PEPCk 30 min before the addition of CHX, the accumulated Ntag-PEPCk protein was clearly observed (Fig. 4). The effect was more obvious with an increase in MG132 concentration. Therefore, MG132 seemed to inhibit the degradation of PEPCk when de novo synthesis of its protein was inhibited by CHX. This indicated the possible involvement of an ubiquitin–proteasome pathway in the degradation of PEPCk. MG132 similarly inhibited degradation of Ctag-PEPCk and Ntag-PEPCk, while degradation of Ntag-sGFP (more highly accumulated than PEPCks in protoplasts) was not significantly affected by MG132 (Fig. 5). From these results, it was apparent that the rapid degradation of PEPCk is triggered by neither FLAG tag conjugations nor overexpression in the protoplast, but rather by a mechanism specific to PEPCk.

Since MG132 is known to inhibit not only 26S proteasome in the ubiquitin–proteasome pathway but also other proteases including certain lysosomal cysteine proteases and calpains (Lee and Goldberg 1998), we conducted experiments to demonstrate the ubiquitination of PEPCk in vivo using an antibody against ubiquitin. First, we performed immunoblot analysis using anti-FLAG antibodies and lysates from Ntag-PEPCk-expressing protoplasts incubated in the presence or absence of MG132 for 4 h. As shown in Fig. 6A, MG132 enhanced an accumulation of higher molecular weight immunoreactive species. One of these (denoted by asterisk 2) was 7–8 kDa larger than the molecular size of intact Ntag-PEPCk (asterisk 1). Since there was no open reading frame for the protein with the FLAG tag other than for PEPCk in the transfected plasmid, the increase in the apparent M_r of Ntag-PEPCk was thought to be due to post-translational modification, most plausibly mono-ubiquitination; it has been predicted that the M_r of a single ubiquitin molecule in maize, which consists of 76 amino acid residues highly conserved among plants (Christensen et al. 1992), is about 8 kDa.

Secondly, to detect ubiquitination of Ntag-PEPCk, the extracts of protoplasts prepared in the same manner as above were immunoprecipitated with the anti-FLAG antibody covalently attached to agarose resin. Trapped proteins were then eluted specifically by 3× FLAG peptide and analyzed by immunoblot analysis with an anti-ubiquitin antibody. Compared with the samples not treated with MG132, the MG132-treated samples gave stronger bands of immunoreactive species (Fig. 6B). In addition to the discrete ladder-like bands indicated by asterisks 2 and 3, smeared positive signals were observed in the region corresponding to a molecular size ranging from 80 to 180 kDa. The ubiquitin antibody recognized the Ntag-PEPCks with molecular sizes greater than that of intact Ntag-PEPCk, but it did not recognize intact Ntag-PEPCk with an M_r of 33 kDa (indicated by asterisk 1 in Fig. 6A). The presence of intact Ntag-PEPCk in the samples was confirmed with parallel immunoblot analysis using an anti-FLAG antibody as shown in the inset of Fig. 6B. This certifies the specific recognition of an anti-ubiquitin antibody in our experiments. In similar experiments with lysates of MG132-treated protoplasts expressing Ntag-sGFP, accumulation of the FLAG-tagged protein able to react with anti-ubiquitin antibody was not observed (data not shown). These results strongly suggest that PEPCk is

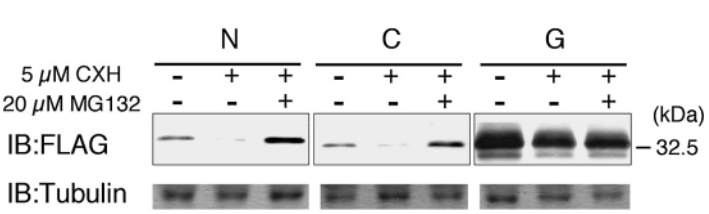

Fig. 5 Effects of MG132 on the stability of FLAG-tagged proteins. After pre-treatment with or without 20 μM MG132 for 30 min, protoplasts expressing FLAG-tagged proteins were incubated with or without 5 μM CHX for 4 h. The protein levels were determined by immunoblot analysis with anti-FLAG antibody. N, Ntag-PEPCk; C, Ctag-PEPCk; G, Ntag-sGFP; IB, immunoblotting.

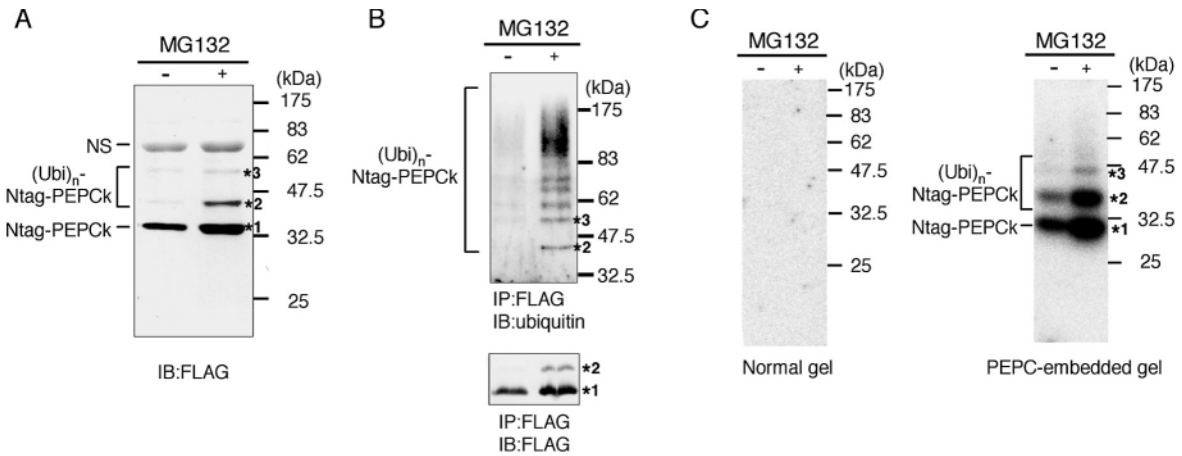

Fig. 6 Detection of ubiquitinated PEPCks and their activities. (A) Lysates were prepared from Ntag-PEPCk-expressing protoplasts treated with or without 20 μM MG132 for 4 h, separated on a 12.5% SDS–polyacrylamide gel, and subjected to immunoblot analysis with anti-FLAG antibody. NS, non-specific signal. (B) The same samples as in (A) were immunoprecipitated with anti-FLAG antibody, separated on a 7.5% SDS–polyacrylamide gel, and subjected to immunoblot analyses with anti-FLAG antibody (lower panel) or anti-ubiquitin antibody (upper panel). (C) In-gel protein kinase assay. The FLAG-immunoprecipitated proteins shown in (B) were separated on an SDS–polyacrylamide gel containing PEPC (right panel) or not-containing PEPC (left panel). Then the proteins were renatured and their PEPCk activities were detected as described in Materials and Methods. Radioactivities on the gels visualized by BAS 2500 Image Analysis System are shown. The bands with asterisks are discussed in the text. IP, immunoprecipitation; IB, immunoblotting.

specifically ubiquitinated and degraded through the ubiquitin–proteasome pathway. Thus, the molecular species with an M_r increased by about 8 kDa (indicated with asterisk 2 in Fig. 6A and B) is thought to be a mono-ubiquitinated Ntag-PEPCk, and the other higher molecular weight species might be ascribed to oligo- and poly-ubiquitinated Ntag-PEPCk.

PEPCk activity of ubiquitinated Ntag-PEPCk

To investigate whether the ubiquitinated PEPCk shows enzymatic activity, the proteins immunoprecipitated with the immobilized FLAG-tag antibody were subjected to an in-gel protein kinase assay. In this experiment, recombinant *Zea mays* C4-type PEPC (ZmPEPC) was embedded in SDS–polyacrylamide gels as a substrate for PEPCk. As a negative control, SDS–polyacrylamide gels not containing ZmPEPC were also prepared and processed in the same way. As shown in the right panel of Fig. 6C, at least three radioactive bands showing PEPCk activity were detected with the samples from MG132-teated protoplasts expressing Ntag-PEPCk. The band intensities of the sample without MG132 were much lower than those of the MG132-treated samples. Dependency of PEPCk activity on the presence of PEPC in the gel was evident, since no radioactive band was detected with control gels not containing PEPC (Fig. 6C, left panel). A difference in the molecular weights of positive bands in Fig. 6A and C was apparent. However, this difference was due to the ingredients of the polyacrylamide gels, since separate experiments showed that the mobility of a recombinant Ntag-PEPCk produced in *Escherichia coli* cells was also faster in PEPC-embedded gels than in usual gels, and was the same as the highest mobility band of Ntag-PEPCk expressed in the protoplasts (data not shown). Therefore, the activity band with the highest mobility (denoted by asterisk 1 in Fig. 6) was assigned to the intact Ntag-PEPCk, while the second (asterisk 2) and third (asterisk 3) bands were thought to correspond to mono- and di-ubiquitinated Ntag-PEPCk, respectively.

Endogenous PEPCks of larger sizes in maize leaves

Since ubiquitination of Ntag-PEPCk could be successfully shown using a transient expression system, an attempt was made to characterize endogenous maize leaf PEPCks with molecular weights higher than 30 kDa. Experiments were carried out with crude extracts from maize leaves, which were prepared in the presence of MG132 and other conventional protease inhibitors. The crude extracts were subjected to desorption chromatography. PEPCk absorbed to a small column of TOYOPEAL HW-55F resin in the presence of 1 M $(NH_4)_2SO_4$ was eluted with 0.7 M $(NH_4)_2SO_4$ as previously described (Ueno et al. 2000). Fig. 7A shows the protein stain for each fraction of eluate from the column. Most proteins were not absorbed and were recovered in the flow through fraction (FT), and proteins in fractions 5–9 were hardly detectable. When the PEPCk activity in each fraction was measured using recombinant ZmPEPC as a substrate in an ordinary PEPCk assay, two activity peaks (Nos. 4–5 and 8–9) were obtained, with about 2-fold higher activity in the latter peak than in the former (Fig. 7B). The peak fractions were then subjected to an in-gel protein kinase assay (Fig. 7C). Surprisingly, very strong activity (>10-fold higher than for Nos. 8–9) was found for fractions 4–5 with an apparent M_r of 38 kDa (denoted β), and weak activity was seen in fractions 8–9 with an apparent M_r of 30 kDa (denoted α) as shown in the upper panel of Fig. 7C. Dependency of the

activity bands for the eluates at 0.7 M $(NH_4)_2SO_4$ on gel embedded with PEPC was confirmed by running a gel not containing PEPC (lower panel, Fig. 7C). The data in Fig. 7B and C indicate that the activity of PEPCk in β is inhibited or mostly latent in the native state, while this suppressed activity seems to be released after electrophoresis under denaturing conditions, presumably due to separation from some inhibitory proteins. Close examination of Fig. 7C (upper panel) revealed that there are at least two more activity bands (denoted γ and δ) making up a ladder of α, β, γ and δ forms with intervals of approximately 8 kDa. PEPCk activity similar to the γ form in size was also mentioned previously by Li et al. (1996), but the δ form was not. These results strongly suggest that endogenous PEPCk is also ubiquitinated in maize leaves in the same manner as observed with the FLAG-tagged PEPCk in the protoplast.

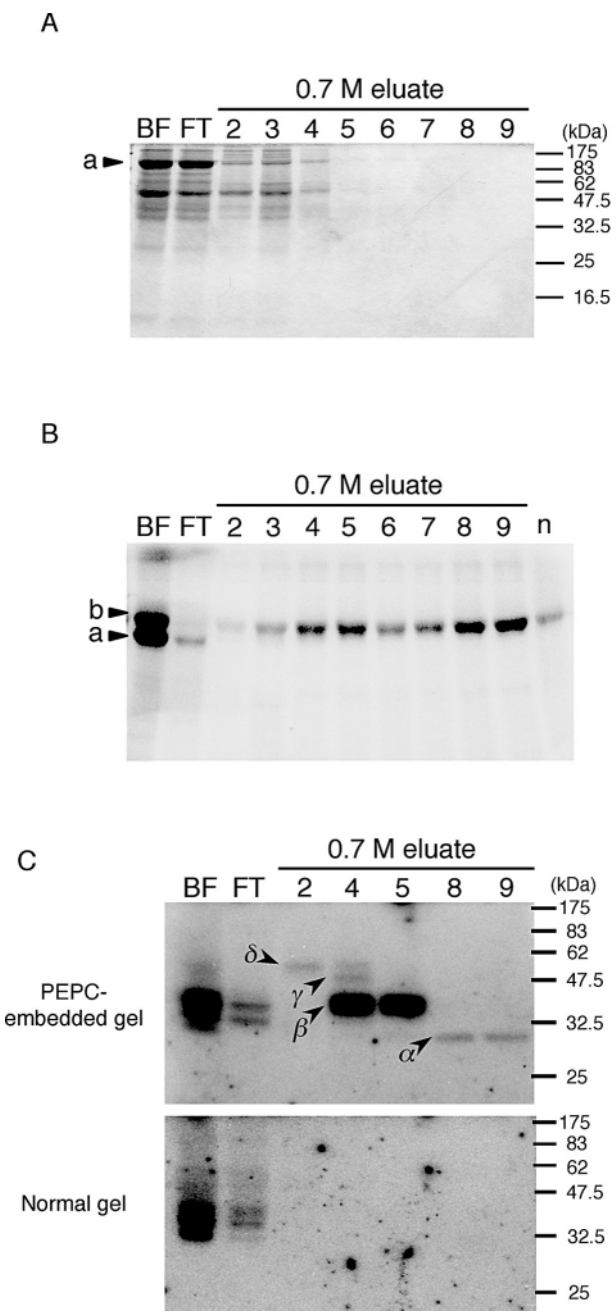

Fig. 7 Identification of multiple forms of maize endogenous PEPCks. Leaf crude extracts from maize were applied to a TOYOPEAL HW-55F column, and proteins bound to this column were eluted by decreasing the $(NH_4)_2SO_4$ concentration to 0.7 M. Numbers above the lanes represent the fraction number. The positions of the maize endogenous PEPC (a) and recombinant ZmPEPC synthesized in *E. coli* (b) are indicated in (A) and (B). BF, the protein extracts before chromatography; FT, flow through; n, negative control (recombinant PEPC only). (A) Protein staining. (B) PEPCk assay. Fractionated proteins were incubated with recombinant PEPC in the reaction mixture and then loaded onto a 7.5% SDS–polyacrylamide gel. Radioactivities on the gels were visualized by the BAS 2500 Image Analysis System. Signal intensity at the site of the recombinant PEPC corresponds to ^{32}P incorporation into PEPC, indicating the native kinase activity of each fraction. (C) In-gel protein kinase assay. Each fraction was separated in a PEPC-containing (upper panel) or non-containing (lower panel) SDS–polyacrylamide gel, and their PEPCk activities were detected as renatured PEPCk activity (upper panel) or background (lower panel), respectively, by in-gel protein kinase assay. Arrowheads indicate the bands reflecting PEPC-dependent phosphorylation. Radioactivities on the gels were visualized by the BAS 2500 Image Analysis System.

Discussion

In this report, we succeeded in following the fate of PEPCk protein using a maize protoplast transient expression system and FLAG-tagged PEPCk. In this system, a facet of the mechanism functioning in rapid turnover of PEPCk, the involvement of the ubiquitin–proteasome pathway, was elucidated. It must be kept in mind that our transient assay was carried out in a heterologous system using *PEPCk* cDNA from *F. trinervia* and mesophyll protoplasts from maize leaves. However, our experiments also strongly support the involvement of the ubiquitin–proteasome pathway in maize leaves, and the occurrence of PEPCks with higher molecular weights has been observed in various plants previously (Li and Chollet 1993, Li and Chollet 1994, Li et al. 1996, Zhang and Chollet 1997). Therefore, the ubiquitination of PEPCk is probably a conserved phenomenon in various plants. It might further substantiate the contribution of the ubiquitin–proteasome pathway in the regulation of PEPCk to investigate effects of 26S proteasome inhibitors on the molecular species and the fate of PEPCk in maize intact leaves.

To demonstrate directly the ubiquitination of endogenous PEPCk in leaves, preliminary experiments were also carried out. Fraction 5 in Fig. 7, containing predominantly the 37 kDa PEPCk, was reacted with an immobilized anti-ubiquitin antibody, and then the proteins supposedly bound to the resin were subjected to an in-gel protein kinase assay for PEPCk. However, the immunoprecipitation was unsuccessful. This seems

partly due to the weak binding activity of the antibody with ubiquitin and mainly to inaccessibility of the antibody to ubiquitin molecules on PEPCk. The latter possibility seems plausible, because most of the 37 kDa PEPCk was found in an inhibited and latent state. Presumably the ubiquitin moiety conjugated to PEPCk was masked by some protein(s) complexed with the ubiquitinated PEPCk in the native state. Further work is required to prepare larger amounts of PEPCks with higher molecular weights, freed from the inhibitor protein(s). By establishing a sensitive and versatile method for detecting ubiquitination, the general occurrence of ubiquitination of PEPCk and its biological significance will be elucidated.

The ubiquitin–proteasome pathway is now being recognized as an important regulatory system in plants, and the number of examples in which the role of this pathway has been established is increasing (reviewed by Smalle and Vierstra 2004). The pathway helps to remove disordered proteins that arise from cellular housekeeping. It is also known to be involved in various regulatory mechanisms of plants, such as light-responsive gene expression and hormonal signal transduction. Protein kinases are known to play key roles in various signal transduction systems, and some are also known to be degraded via ubiquitination in animals (Joazeiro et al. 1999, Oda et al. 1999, Coulombe et al. 2003, Aebersold et al. 2004, Giannini and Bijlmakers 2004); however, this is the first report to document protein kinase in plants. Recently, a few metabolic enzymes also regulated by this system were reported: pyruvate kinase, an enzyme of glycolysis (Tang et al. 2003), sucrose synthase, an essential enzyme for carbon partitioning accompanied by sink-source switching (Hardin and Huber 2004), and tryptophan decarboxylase for the synthesis of monoterpenoid indole alkaloids (Fernandez and De Luca 1994). The present study indicates that C4 photosynthetic metabolism is also under the control of the ubiquitin–proteasome pathway.

For the selected degradation of PEPCk, a specific E3 enzyme that specifically recognizes PEPCk protein must be present in cells of C4 plants. The ATP-dependent E1–E2–E3 conjugation cascade is known to correspond to attachment of free ubiquitins to specific intracellular targets. E3s are responsible for identifying the many proteins that should be ubiquitinated. Since the proteins interacting specifically with PEPCk are candidates for the specific E3 enzyme, the inhibitor protein of PEPCk reported by Nimmo et al. (2001) and the protein(s) postulated to interact with the ubiquitinated PEPCk in this study require molecular characterization.

Our study raised many intriguing and important questions that require clarification in the future. In particular, the molecular feature of PEPCk that serves as a signal for ubiquitination, and the sites of ubiquitination should be examined. Expression of mutant PEPCks that lack the target sites for ubiquitination in the experimental system developed here and purification of a complex containing FLAG-tagged PEPCk from transfected cells by FLAG-tag immunoprecipitation will, for example, be highly useful and efficient in investigating these issues.

Materials and Methods

Plasmid construction

Plasmids for the production of recombinant protein in *E. coli* were constructed in the pET-44a(+) plasmid (Novagen, Madison, WI, U.S.A.), which is designed for expression of a protein conjugated to huge multiple tags (His tags, a Nus tag and a S tag) at its N-terminus, as described by Tsuchida et al. (2001). FLAG-tagged PEPCks were designed to be separated from the huge tags by digestion with thrombin. Plasmids for transient expression were constructed in the pART7 plasmid, containing the CaMV 35S promoter, a multiple cloning site and the transcriptional termination region of the octopine synthase gene (Gleave 1992). To generate a construct for the recombinant Ntag-PEPCk in pET-44a(+), a coding region of a *FtPEPCk* cDNA fragment (Tsuchida et al. 2001) was amplified with a forward primer, 5′-CCCGGGGCAGCGGATCCATGAAGGAAACTCTGAACAACG-3′ (containing an artificial *Bam*HI site), and reverse primer, 5′-GCTCGAGCTTCTCGACTTAGGTTAGATCCG-3′ (containing an artificial *Xho*I site). In addition to this cDNA fragment, a double-stranded oligonucleotide, 51 bp in size, 5′-CCCCGGGGCAGCAAGCTTATGGACTACAAGGACGACGATGACAAAGGATCC-3′, and containing a *Sma*I site, nucleotide sequences for methionine and the FLAG peptide (DYKDDDDK), and a *Bam*HI site, in this order, was also prepared. Both fragments were combined at the *Bam*HI site. Then the combined fragment, with *Sma*I and *Xho*I sites at both ends, was digested with these enzymes and inserted between the *Sma*I and *Xho*I sites of pET-44a(+). To generate the Ntag-PEPCk construct in pART7, a fragment of *Ntag-PEPCk* was amplified from the *Ntag-PEPCk* sequence constructed in pET-44a(+), with a forward primer, 5′-CGCTCGAGATGGACTACAAGGACGACGATG-3′ (containing an artificial *Xho*I site), and reverse primer, 5′-GCGGATCCTTAGGTTAGATCCGCCATTGA-3′ (containing an artificial *Bam*HI site). The polymerase chain reaction (PCR) product was inserted into *Xho*I and *Bam*HI sites of pART7. In the case of the Ctag-PEPCk construction in pART7, a *Ctag-PEPCk* sequence was also constructed in pET-44a(+) and then transferred to pART7. The coding region of the *FtPEPCk* fragment was amplified with a forward primer, 5′-CCCGGGGCAGCGGATCCATGAAGGAAACTCTGAACAACG-3′ (containing an artificial *Sma*I site), and reverse primer, 5′-GAATTCGGTTAGATCCGCCATTGAT-3′ (containing an artificial *Eco*RI site). The amplified fragment was combined at the *Eco*RI site with the synthesized double-stranded oligonucleotide 5′-GAATTCGACTACAAGGACGATGACGACAAGTAACTCGAG-3′, containing an *Eco*RI site, FLAG sequence and *Xho*I site, in this order. The combined cDNA fragment was inserted into pET-44a(+) between the *Sma*I and *Xho*I sites. To generate the Ctag-PEPCk construct in pART7, the fragment amplified from the *Ctag-PEPCk* constructed in pET-44a(+) with a forward primer, 5′-GGCTCGAGATGAAGGAAACTCTGAACAACG-3′ (containing an artificial *Xho*I site), and reverse primer, 5′-GGAAGCTTTTACTTGTCGTCATCGTCCTTG-3′ (containing an artificial *Hin*dIII site), was inserted into a pART7 multiple cloning site at the *Xho*I and *Hin*dIII sites. To generate the Ntag-sGFP construct in pART7, a *sGFP(S65T)* cDNA fragment was derived from the plasmid constructed by Chiu et al. (1996) and amplified with a forward primer, 5′-CTGGATCCATGGTGAGCAAG-3′ (containing a *Bam*HI site), and reverse primer, 5′-GCTCTAGATTACTTGTACAGCTCGTCC-3′ (containing an artificial *Xba*I site). The PCR fragment was exchanged with the *FtPEPCk* sequence between the *Bam*HI and *Xba*I sites in the pART7-Ntag-PEPCk plasmid. As a vector control, the original pART7 plasmid was used.

Correct sequences of all these constructions were confirmed by DNA sequencing.

Production and purification of recombinant Ntag-PEPCk

The recombinant Ntag-PEPCk, derived from the pET44-Ntag-PEPCk plasmid, was produced in *E. coli* as a fusion protein to huge multiple tags at the N-terminus, and purified by a Hitrap Chelating HP column (Amersham Biosciences, Piscataway, NJ, U.S.A.), according to the method of Tsuchida et al. (2001). After purification, for specific removal of the huge tags except for the FLAG tag, the recombinant proteins were treated with thrombin for 2 h in phosphate-buffered saline at 16°C and the huge tags were removed by application to the Hitrap Chelating HP column.

Protoplast preparation, transient expression and chemical treatments

Protoplasts were isolated from the second true leaves of greening maize, grown in the dark for 10.5 days and illuminated for 16 h under 68 μE m^{-2} s^{-1} at 25°C, as described previously (Sheen 1990, Sheen 1991, Yanagisawa and Sheen 1998, Yanagisawa et al. 2003). Electroporation was performed with 2.5×10^5 protoplasts and 40 μg of each plasmid in 0.3 ml of electroporation buffer (4 mM MES-KOH, pH 5.7, 0.6 M mannitol and 20 mM KCl). The electroporated protoplasts were incubated under illumination (68 μE m^{-2} s^{-1} at 25°C) for 11 h in an incubation buffer (4 mM MES-KOH, pH 5.7, 0.6 M mannitol and 4 mM KCl). The chemical reagents were treated after 11 h incubation for the indicated time. A 26S proteasome inhibitor, MG132, was purchased from the Peptide Institute (Osaka, Japan) and dissolved in dimethyl sulfoxide. In each experiment, an aliquot derived from a mixture of several independent transfected lines was used to compensate an experimental error of transformation efficiency.

Immunoblot analysis and immunoprecipitation

Protoplasts were lysed with pipetting in buffer [0.1 M Tris–HCl, pH 7.5, 5 mM sodium phosphate, pH 7.5, 50 mM NaF, 1 mM EDTA, 14 mM 2-mercaptoethanol, 2 mM benzamidine-HCl, 10% (v/v) glycerol, 10% (v/v) ethylene glycol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and other protease inhibitors (antipain, chymostatin, and leupeptin, each at 1 μg ml^{-1})] and then the insoluble fraction was roughly removed by centrifugation at 15,000×*g* for 30 min at 4°C. For the immunoblot analysis, equal amounts of the supernatants were subjected to SDS–PAGE, and proteins in the gels were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). The membranes were incubated in Blocking One (Nacalai Tesque, Kyoto, Japan) and further incubated with primary antibodies at the concentrations noted below. The primary antibodies utilized were mouse anti-FLAG M2 monoclonal antibody (1 : 5,000) (Sigma, St. Louis, MO, U.S.A.), rabbit polyclonal anti-ubiquitin antibody (1 : 30) (Sigma), anti-PNP antibody (1 : 500) (Ueno et al. 2000), anti-PEPC antisera (1 : 1,000,000) (Ueno et al. 2000) and mouse monoclonal anti-α-tubulin clone DM1A (1 : 500) (Sigma). Tubulin was used as an internal control for each experiment. Secondary antibodies conjugated to alkaline phosphatase were used for the immunodetection of FLAG-tagged proteins, whole PEPC and tubulin. Ubiquitin and phosphorylated PEPC were detected with secondary antibody conjugated to peroxidase, with BM Chemiluminescence Western Blotting Substrate (Roche, Basel, Switzerland). To measure the amounts of the FLAG-tagged protein, the intensity of each signal was measured by Image Gauge (Fujifilm, Tokyo, Japan). Chemical luminescence was visualized and its strength was measured by LAS1000 plus (Fujifilm). Immunoprecipitation was performed with a FLAG-tagged protein immunoprecipitation kit (Sigma) according to the technical bulletin. Proteins reacting with anti-FLAG antibody were eluted with 3× FLAG peptide.

Partial purification of PEPCk from maize leaves

Leaf crude protein extracts were prepared as described previously (Ueno et al. 2000). Maize plants were grown in the field for >1 month then the mature leaves were harvested at 10 a.m. and immediately frozen with liquid nitrogen. The frozen leaves were homogenized with liquid nitrogen into a fine powder, and suspended in extraction buffer (0.1 M Tris–HCl, pH 7.5, 5 mM sodium phosphate, 50 mM NaF, 1 mM EDTA, 14 mM 2-mercaptoethanol, 2 mM benzamidine-HCl, 10% (v/v) glycerol, 10% (v/v) ethylene glycol, 5% (w/v) insoluble polyvinylpolypyrrolidone, 1 mM PMSF and other protease inhibitors (antipain, chymostatin, and leupeptin, each at 1 μg ml^{-1})] including 20 μM MG132. The homogenate was filtered through a layer of 100 μm nylon mesh and then centrifuged at 15,000×*g* for 30 min at 4°C. The supernatant fluid was fractionated with $(NH_4)_2SO_4$ (25–60% saturation) then the proteins precipitated at 60% saturation of $(NH_4)_2SO_4$ were collected by centrifugation (15,000×*g* for 30 min at 4°C) and the precipitate was dissolved in buffer A [20 mM Tris–HCl, pH 7.5, 5% (v/v) glycerol, 2 mM benzamidine-HCl, 14 mM 2-mercaptoethanol]. After the addition of $(NH_4)_2SO_4$ to a final concentration of 1 M, the sample was centrifuged at 100,000×*g* for 30 min. A portion of the supernatant fraction containing 12 mg of protein was applied to a column (5 ml bed volume) packed with TOYOPEARL HW-55F resin (TOSOH, Tokyo, Japan) pre-equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. The column was washed with 15 ml of buffer A containing 1 M $(NH_4)_2SO_4$, and PEPCk was eluted with 15 ml of buffer A containing 0.7 M $(NH_4)_2SO_4$. Each 1.5 ml fraction was collected, and serially numbered from 1 to 10. For detection of PEPCk activity in each fraction, $(NH_4)_2SO_4$ was removed by passing it through a Sephadex G-25 column (Amersham Biosciences).

PEPCk assay

PEPCk activity was measured by ^{32}P incorporation from [γ-^{32}P]ATP into the recombinant ZmPEPC produced in *E. coli* (Dong et al. 1997, Ueno et al. 1997). The kinase reaction was performed in a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 0.8 μM okadaic acid, 0.1 mM (1 μCi) [γ-^{32}P]ATP, 5 μg of recombinant PEPC and 9 μl of enzyme preparation, in a total volume of 15 μl. After 45 min incubation at 25°C, the reaction was terminated by the addition of 7.5 μl of 3× SDS–PAGE sample buffer, incubated at 95°C for 5 min, and then subjected to SDS–PAGE. The ^{32}P incorporation into recombinant ZmPEPC was detected with BAS 2500 Image Analysis System (Fujifilm).

In-gel protein kinase assay

The in-gel protein kinase assay was performed according to the method described previously (Kameshita and Fujisawa 1989, Wang and Chollet 1993). The phosphorylation substrate, recombinant ZmPEPC, was prepared as previously described (Dong et al. 1997, Ueno et al. 1997). Protein samples were separated by SDS–PAGE on 12.5% polyacrylamide gels containing 1 mg ml^{-1} of ZmPEPC for detection of PEPCk activity or on normal 12.5% polyacrylamide gels as a background. The proteins were then renatured in the gels, and incubated in assay buffer (40mM HEPES-NaOH, pH 8.0, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM EGTA and 1 μM ATP) containing 15 μCi/ml [γ-^{32}P]ATP for 1 h at 25°C. After incubation, the gel was washed with wash buffer containing 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate at 25°C to remove free [γ-^{32}P]ATP; it was changed several times over a total period of 3 h. The gel was dried under a vacuum then the radioactivity on the gels was detected by BAS 2500 Image Analysis System.

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