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Biochemical characterization of a 34 kDa ribonucleoprotein (p34) purified from the spinach chloroplast fraction as an effective phosphate acceptor for casein kinase II

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A 34 kDa ribonucleoprotein (p34) was purified to homogeneity from a 1.0 M KCl extract of spinach chloroplasts and characterized as an effective phosphate acceptor for casein kinase II (CK-II). The N-terminal 21 residues (W-V-A-Q-T-S-E-E-E-Q-E-G-S-T-N-A-V-L-E-G-E) of p34 were 95% identical with the sequence reported for 28RNP (plastid mRNA 3′ end processing factor in chloroplast). Moreover, the findings that DNAs as well as RNAs significantly stimulate the CK-II catalyzed phosphorylation of p34 in vitro and induce its conformational change, suggest that the physiological activity of p34-bound RNA or DNA in chloroplast post-transcriptional regulation is controlled by specific p34 phosphorylation by CK-II.

Ribonucleoprotein; Casein kinase II; Protein phosphorylation; Plastid mRNA 3′ end processing; Chloroplast; Spinach

1. INTRODUCTION

Casein kinase II (CK-II) is a cAMP- and Ca^{2+}-independent serine/threonine protein kinase [1,2] with important roles in the regulation of DNA replication and transcription: it specifically modifies DNA-binding proteins (DNA topoisomerase [3], DNA ligase [4] and SV-40 large T antigen [5]) and transcriptional factors (Sp1 [6], serum response factor [7] and G box binding protein [8]). Recently [9], we purified two distinct forms (α-monomeric (38 kDa) and oligomeric ($\alpha_2\beta_2$, 140 kDa) forms) of CK-II from cultured cells of liverwort, and characterized these biochemically as protein kinases similar to those purified from various animal cells [1,2]. During biochemical characterization of CK-IIs in plant cells, we recognized that (i) two forms of CK-II were also present in a 1.0 M KCl extract from spinach chloroplasts; (ii) a 34 kDa ribonucleoprotein (p34) in the 1.0 M KCl chloroplast extracts prepared from various plant cells was detected as a common polypeptide phosphorylated by CK-II in vitro; and (iii) some biochemical properties of p34, such as RNA- and DNA-binding abilities and molecular size, were similar to those of nuclear-encoded 28–33 kDa ribonucleoproteins (RNPs) required for plastid mRNA 3′ end processing and stability in chloroplasts [10–12]. However, the physiological role of these RNPs in the regulatory mechanisms involved in plastid mRNA 3′ end processing and their physiological correlation with CK-II in chloroplasts remain to be elucidated. Therefore, the present study was undertaken (i) to purify p34 from the 1.0 M KCl chloroplast fraction of spinach by means of successive DNA-cellulose and Mono Q (HPLC) column chromatographies as an effective phosphate acceptor for CK-II; and (ii) to biochemically characterize it. The physiological significance of specific phosphorylation of p34 by CK-II in post-transcriptional regulation in chloroplast is discussed.

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Abbreviations: p34, 34 kDa ribonucleoprotein; CK-II, casein kinase II; 28RNP, 28 kDa ribonucleoprotein; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; CD, circular dichroism.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP was obtained from Amersham Corp. (Arlington Heights, IL, UK); dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), *p*-aminobenzamidine, dsDNA (calf thymus), ssDNA (calf thymus) and tRNA (yeast) from Sigma Chemicals Corp. (St. Louis, MO, USA); trypsin inhibitor (soybean) from Wako Pure Chemical Ind. (Osaka, Japan); and poly (dA):poly (dT), poly (dG):poly (dC), poly (dA), poly (dC), poly (rA), poly (rU), poly (rG), poly (rC), poly (rI), Sephacryl S300, DNA-cellulose (dsDNA), Mono

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Q HR 5/5, Superdex 200 pg and Superdex 75 pg from Pharmacia Fine Chemicals (Uppsala, Sweden).

2.2. *Extraction of p34 from spinach chloroplasts*

Isolation of chloroplasts from spinach leaves followed the method described by Ohyama et al [13]. To extract p34, the isolated chloroplasts (about 1 kg wet weight) were homogenized with a glass homogenizer in 3 l of Buffer A (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM 2-ME, 0.1 mM Mg^{2+}, 0.1 mM PMSF and 10% glycerol) containing 1.0 M KCl, 5 μg/ml of trypsin-inhibitor and 0.5 mM *p*-aminobenzamidine, and then sonicated for 30 s in an ice bath. After centrifugation (33,000 × *g* for 30 min at 4°C), the supernatant was concentrated by ammonium sulfate (65% saturation), and then dialyzed against Buffer B (50 mM Tris-HCl pH 7.4, 0.2 mM EDTA, 10 mM 2-ME, 0.1 mM PMSF and 10% glycerol) containing 0.2 M KCl. The dialysate was used for the present study as a crude p34 extract.

2.3. *Partial purification of p34*

To remove CK-II, the crude p34 extract (about 1,200 mg protein) was passed through a column of heparin-agarose in Buffer A containing 0.2 M KCl [9]. The heparin-agarose eluant (about 1,050 mg protein) was applied on a column (5 × 30 cm) of DEAE-cellulose, previously equilibrated with Buffer A containing 0.1 M KCl. Elution was carried out in a step-wise manner with Buffer A containing 0.1 M, 0.6 M and 1.0 M KCl, successively. p34 in the 0.6 M KCl fraction (about 250 mg protein) was further purified by gel filtration on Sephacryl S300 (1.6 × 100 cm) in Buffer A containing 0.6 M KCl. p34 was eluted between apparent molecular sizes of 25 kDa and 45 kDa, as estimated from the marker proteins γ-globulin (150 kDa), BSA (67 kDa) and trypsin inhibitor (22 kDa). p34 in the Sephacryl S300 fraction (about 60 mg protein) was purified by DNA-cellulose column chromatography and p34 was eluted from the column with Buffer A containing 0.7 M KCl.

2.4. *Purification of CK-II*

Two forms (α-monomeric and oligomeric forms) of CK-II were purified separately from the crude 1.0 M KCl extract of spinach chloroplasts by the modified method using heparin-agarose column chromatography and gel filtration on Superdex 200 pg column (HPLC), as reported previously [9]. The activity of CK-II was determined using either p34 or dephosphorylated α-casein as a phosphate acceptor [9].

2.5. *SDS-PAGE and autoradiography*

Polypeptides phosphorylated by the two forms of CK-II were detected by SDS-PAGE followed by autoradiography [9,14] after incubation (30 min at 25°C) of the purified CK-IIs with the partially or purified p34 fractions, in the presence of 20 μM [γ-^{32}P]ATP (500 cpm/pmol) and 3 mM Mn^{2+}.

2.6. *Determination of CD spectra*

The CD spectra of p34 (0.2 mg protein), calf thymus dsDNA (40 μg/ml) and poly (rG) (40 μg/ml) alone, and p34 in the complexes with calf thymus dsDNA or poly (rG) were determined, with a Jasco 720A spectropolarimeter (Japan spectroscopic Co. Ltd, Tokyo) in 10 mM Tris-HCl pH 7.4 containing 0.2 M KCl. Vertical dimension of the obtained CD spectra was expressed in 10^{-3} deg.

3. RESULTS

3.1. *Detection of p34 as an effective phosphate acceptor for CK-II in the partially purified p34 fraction*

When the heparin-agarose eluant was incubated for 30 min at 25°C with α-monomeric CK-II and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of 3 mM Mn^{2+}, several polypeptides (molecular sizes 40, 38, 35, 34, 28, 25 and 20 kDa) were highly phosphorylated (Fig. 1). Phosphorylation of a 34 kDa polypeptide (designated p34) by CK-II was selectively stimulated when calf thymus dsDNA (between 0.3 μg/ml and 10 μg/ml) was present in the reaction mixtures. However, the CK-II catalyzed phosphorylation of other polypeptides in the fraction was inhibited at DNA concentrations higher than 1 μg/ml (Fig. 1). Under the same experimental conditions, a similar 34 kDa polypeptide was also detected in the heparin-agarose eluants prepared from liverwort and tobacco (data not shown). A similar stimulatory effect of calf thymus dsDNA on p34 phosphorylation was observed when oligomeric CK-II was used instead of the α-monomeric form. These results demonstrate that p34 functions as an effective DNA-facilitated phosphate acceptor for both α-monomeric and oligomeric forms of CK-II purified from spinach chloroplasts, and its presence in other species suggests that it is common to most green plants.

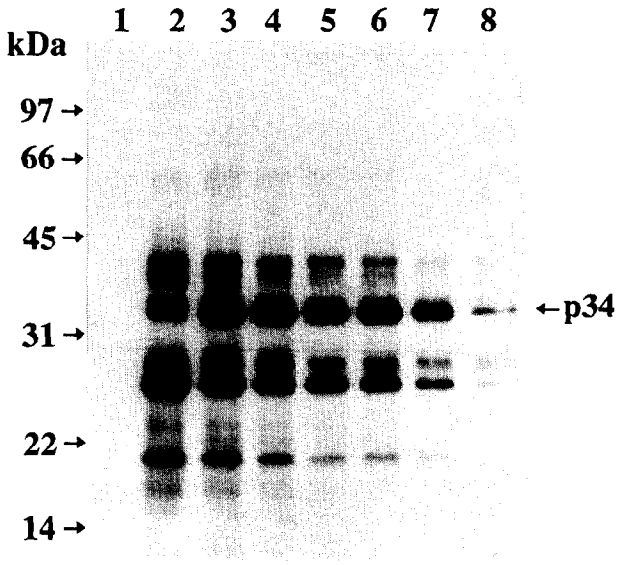

Fig. 1. Detection of p34 in the heparin-agarose eluant as an effective phosphate acceptor for CK-II and the stimulatory effect of calf thymus dsDNA on its phosphorylation. Partially purified p34 (heparin-agarose eluant, 10 μg protein) was boiled to inactivate non-specific protein kinases and then incubated with α-monomeric CK-II (0.1 μg, purified from the chloroplast fraction of spinach), 20 μM [γ-^{32}P]ATP (500 cpm/pmol) and 3 mM Mn^{2+} in the presence or absence of calf thymus dsDNA. The ^{32}P-labeled polypeptides in the reaction mixtures were detected directly by autoradiography after SDS-PAGE. Lane 1, the boiled heparin-agarose eluant (10 μg) alone; lane 2, the boiled fraction (10 μg) incubated for 30 min at 25°C with α-monomeric CK-II and [γ-^{32}P]ATP in the absence of calf thymus dsDNA; lanes 3–8, as lane 2 in the presence of calf thymus dsDNA: lane 3, 0.3 μg/ml; lane 4, 1 μg/ml; lane 5, 3 μg/ml; lane 6, 10 μg/ml; lane 7, 30 μg/ml; and lane 8, 100 μg/ml.

3.2. *Final purification of p34 and its partial sequence*

p34 in the DNA-cellulose fraction (about 2 mg protein) was purified by Mono Q column chromatography (HPLC), from which at least three distinct protein peaks (P-I, P-II and P-III) were obtained (Fig. 2A). The mo-

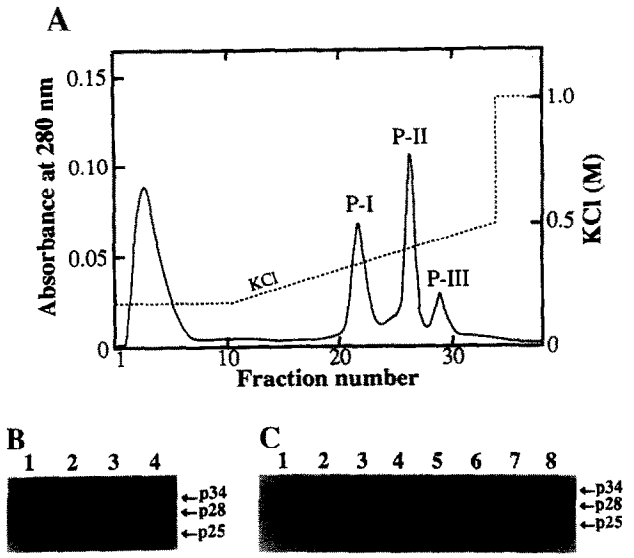

Fig. 2. Separation of three distinct fractions (P-I, P-II and P-III) from the DNA-cellulose fraction by Mono Q column chromatography. (A) p34 in the DNA-cellulose fraction (approximately 2 mg protein) was further purified by Mono Q column chromatography (HPLC). Elution was carried out with a linear gradient between 0.2 M and 0.5 M KCl, and 1.0 ml fractions were collected. (B) To determine the main polypeptide in the indicated Mono Q fractions (P-I, P-II and P-III), each fraction (10 μl each) was analyzed by SDS-PAGE and polypeptides on the gel were stained with Coomassie brilliant blue R-250. Lane 1, Pre-Mono Q fraction (DNA-cellulose fraction); lane 2, P-I; lane 3, P-II; and lane 4, P-III. (C) To detect p34, aliquots (20 μl) of these three fractions (P-I, P-II and P-III) were incubated separately for 30 min at 25°C with oligomeric CK-II (0.1 μg) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5 and 7) of calf thymus dsDNA (10 μg/ml) and 3 mM Mn^{2+}. Lanes 1 and 2, Pre-Mono Q fraction, lanes 3 and 4, P-I; lanes 5 and 6, P-II; and lanes 7 and 8, P-III.

lecular size of the main polypeptide in the P-I, P-II and P-III fractions was estimated as 25 kDa (designated p25), 28 kDa (designated p28) and 34 kDa, respectively (Fig. 2B). p34 in the P-III fraction was a homogeneous polypeptide corresponding to that (approximately 33 kDa) determined by gel filtration on Superdex 75 pg column (HPLC) in Buffer B containing 0.6 M KCl. As expected, p34 in the P-III fraction was phosphorylated by CK-II (lane 7, Fig. 2C) and pre-incubation of p34 with calf thymus dsDNA (10 μg/ml) resulted in great stimulation of its phosphorylation (lane 8, Fig. 2C). Similar stimulation was also observed when p25 (lane 4) and p28 (lane 6) were phosphorylated by CK-II after their pre-incubation with dsDNA (10 μg/ml) (Fig. 2C). In addition, the N-terminal 21 residues (W-V-A-Q-T-S-E-E-E-Q-E-G-S-T-N-A-V-L-E-G-E) of p34 were 95% identical with the corresponding sequence reported for 28RNP [10].

3.3. *The effects of DNAs and RNAs on the phosphorylation of p34 by CK-II in vitro*

The effects of various DNAs and RNAs on the CK-II catalyzed phosphorylation of p34 were examined in vitro using both α-monomeric and oligomeric forms of CK-II. It was found that pre-incubation of p34 with DNAs (dsDNA and ssDNA) induced significant stimulation of its phosphorylation by these two kinases (Fig. 3A), but no base-specificities of DNAs on protein phosphorylation were detected. Also, poly (rG), poly (rC) and poly (rI) significantly stimulated phosphorylation of p34 by CK-II (Fig. 3B), but no significant effects of yeast tRNA, poly (rA), poly (rU) and poly (rA):poly (rU) were detected (Fig. 3B). In the presence of 10 μg/ml, the CK-II catalyzed phosphorylation of p34 was stimulated about 12-fold by dsDNA and 4.2-fold by poly (rG), respectively, (Fig. 3C). However, no significant effect of these nucleic acids was detected when casein was used as a phosphate acceptor for these two kinases under the same experimental conditions (data not shown).

3.4. *Conformational changes of p34 induced by DNA and RNA*

Fig. 4 shows that the CD spectrum of p34, with a negative maximum at 210 nm, is shifted to 226 nm and 218 nm after incubation with calf thymus dsDNA (A) and poly (rG) (B), respectively. Under the same experimental conditions, similar shift of the CD spectrum was obtained when p34 in the complex with poly (rU) instead of poly (rG) was determined (data not shown). These results suggest that the conformational change of p34 is induced by calf thymus dsDNA and RNAs (poly (rG) and poly (rU)).

4. DISCUSSION

p34 purified from a 1.0 M KCl extract of spinach chloroplasts is an effective phosphate acceptor for CK-II and is probably a RNP since the N-terminal 21 residues (W-V-A-Q-T-S-E-E-E-Q-E-G-S-T-N-A-V-L-E-G-E) of p34 are 95% identical with the sequence reported for 28RNP [10]. p34 could be separated from other phosphorylating polypeptides (p25 and p28) in the DNA-cellulose fraction by Mono Q column chromatography (Fig. 2A). The substrate activity of these two polypeptides (p25 and p28) for CK-II and the stimulatory effect of dsDNA on their phosphorylation by the kinase in vitro were similar to those observed with p34 (Fig. 2C). Moreover, the following preliminary experimental evidence suggests that p25 and p28 are degradation products derived from p34 during protein purification: (i) the protein ratio of P-III (p34) to P-I (p25) and P-II (p28), in Mono Q column chromatography (Fig. 3A), significantly decreases when the heparin-agarose p34 eluant is incubated for a long time (more than 5 h at 25°C) in the absence of protease inhibitors (PMSF, *p*-aminobenzamidine and trypsin inhibitor); (ii) p25 is formed on limited proteolysis of purified p34 with modified trypsin in vitro; and (iii) the N-terminal 11 residues (A-V-L-E-G-E-S-D-P-E-G) of p28 (P-II fraction) are

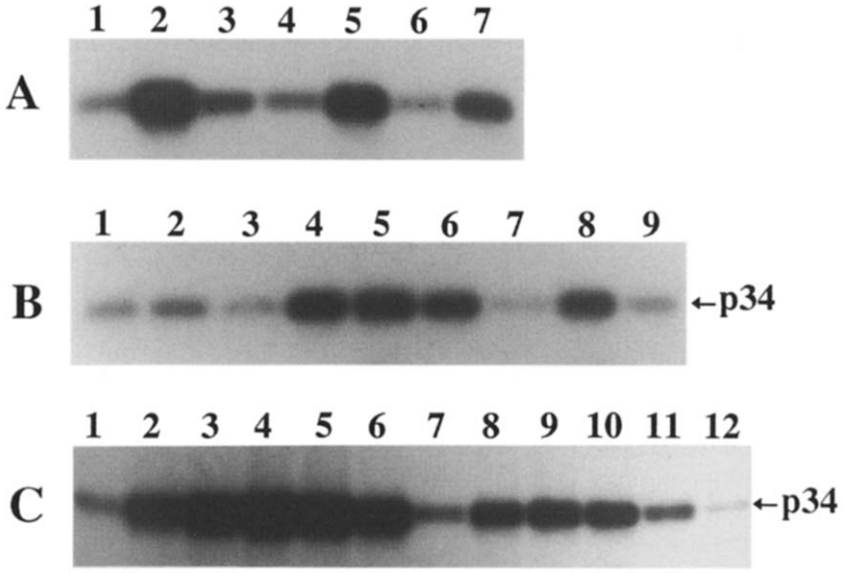

Fig. 3. The effects of (A) DNAs, (B) RNAs and (C) dosage of calf thymus dsDNA and poly (rG) on the CK-II driven phosphorylation of p34 in vitro. (A) p34 (1 μg) was incubated for 30 min at 25°C with oligomeric CK-II (0.1 μg) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol): lane 1, in the absence of DNA (10 μg/ml each); lane 2, + calf thymus dsDNA; lane 3, + poly (dA):poly (dT); lane 4, + poly (dG):poly (dC); lane 5, + calf thymus ssDNA; lane 6, + poly (dA); and lane 7, + poly (dC). (B) p34 (1 μg) was incubated for 30 min at 25°C with oligomeric CK-II and [γ-^{32}P]ATP: lane 1, in the absence of RNA (10 μg/ml each); lane 2, + poly (rA); lane 3, + poly (rU); lane 4, + poly (rG); lane 5, + poly (rC); lane 6, + poly (rI); lane 7, + poly (rA):poly (rU); lane 8, + poly (rG):poly (rC); and lane 9, + yeast tRNA. (C) Pre-incubation (30 min in an ice bath) of p34 with calf thymus dsDNA (lanes 2 through 6) or poly (rG) (lanes 8 through 12), and then further incubated (30 min at 25°C) with oligomeric CK-II (0.1 μg) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of 3 mM Mn^{2+}. Lanes 1 and 7 were calf thymus dsDNA or poly (rG) free, respectively. The concentrations of calf thymus dsDNA and poly (rG) were 1 μg/ml (lanes 2 and 8), 3 μg/ml (lanes 3 and 9), 10 μg/ml (lanes 4 and 10), 30 μg/ml (lanes 5 and 11) and 100 μg/ml (lanes 6 and 12), respectively.

identical with the corresponding sequences of p34 and 28RNP. Recently, Chen and Stern [12] purified and characterized a 33 kDa RNA-binding protein, which specifically binds to the stem-loop structure on the mRNA 3′ end, from the chloroplast fraction of spinach. Although the complete primary sequences of the 33 kDa RNA-binding protein and p34 have not yet been determined, there are biochemical similarities between these two chloroplast proteins in their RNA-binding ability and molecular size.

Recent reports concerning biochemical characterizations of 28–33 kDa RNPs purified from the chloroplast fractions of spinach [10,12] and tobacco [11,15,16] have revealed that they: (i) are nuclear encoded proteins

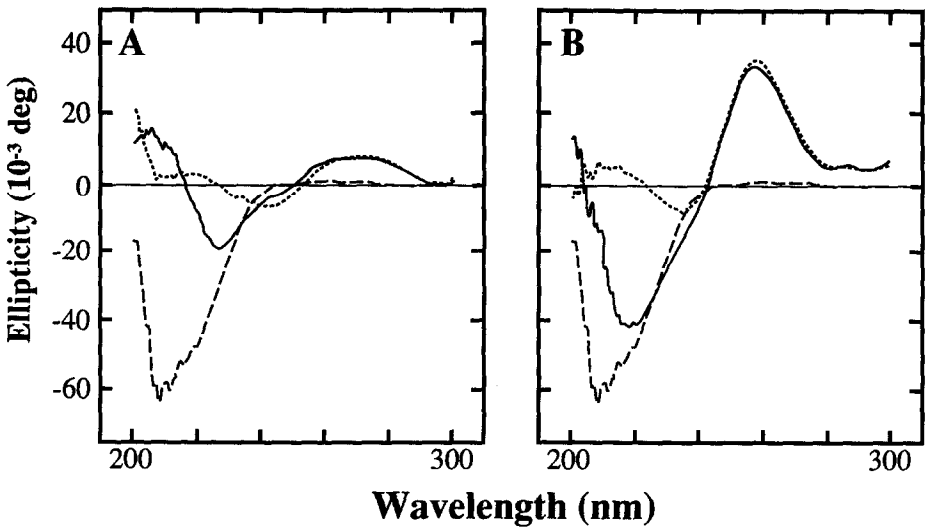

Fig. 4. Conformational changes of p34 induced by calf thymus dsDNA and poly (rG). The CD spectra of p34 (0.2 mg), calf thymus dsDNA (40 μg/ml) and poly (rG) (40 μg/ml) alone, and p34 in the complex with 40 μg/ml of calf thymus dsDNA (A) or poly (rG) (B) were determined. – – – –, p34; ·····, calf thymus dsDNA (A) or poly (rG) (B); and ———, p34 in the complex with calf thymus dsDNA (A) or poly (rG) (B).

[10,11]; (ii) specifically bind to plastid mRNA at the 3′ end, which contains an inverted repeat sequence that can fold into a stem-loop structure [10,12]; and (iii) have characteristic domains in common (two highly conserved RNA-binding domains and an acidic N-terminal domain) [10,11]. From these biochemical characteristics, it has been postulated that these RNPs may play an important role in plastid mRNA 3′ end processing and stability. Moreover, earlier studies have described: (i) RNase activity detected in spinach chloroplast RNP fraction purified by 28RNP-IgGs-affinity chromatography [10]; and (ii) an acidic N-terminal domain containing phosphorylating sites (S-E-X-E, S-D-X-E and S-D-E-X) for CK-II [2] in 28RNP which may be engaged in the protein-protein interaction with specific splicing RNases [10]. These indirect observations suggest that the N-terminal domain of these RNPs may be responsible for the molecular interaction with splicing RNases, which specifically cleave at the inverted repeat regions of mRNA at the 3′ end, after its specific phosphorylation by CK-II in the chloroplast.

The experimental results that the binding of p34 with DNAs results in significant stimulation of its CK-II catalyzed phosphorylation (Fig. 3) and induction of its conformational change (Fig. 4A), suggest that specific phosphorylation of p34-bound DNA by CK-II may play important roles in the regulation of transcription and/or DNA replication in chloroplasts. This possibility is supported by further preliminary experimental results: (i) unphosphorylated p34 has a higher binding affinity with DNAs than that of the phosphorylated one; and (ii) p34 is released from the p34-DNA complex when the protein in the complex is fully phosphorylated by CK-II. Moreover, Klimczak et al. have demonstrated that the specific binding of GBF-1 (G box binding factor 1) with the *cis*-element (G-box) on the promoter region of a plant light-regulated gene [17] is controlled by its specific phosphorylation by CK-II [8]. Although p34 has no base-specificity, both proteins (p34 and GBF-1) function as effective phosphate acceptors for CK-II in plant cells. This leads us to suggest that CK-II may be a key enzyme regulating light-induced signal transduction and functional mediator to specifically activate the regulatory proteins p34 and GBF-1 through their specific phosphorylation. However, the biological significance of p34 phosphorylation by CK-II and the stimulatory effect of DNAs (dsDNA and ssDNA) in the chloroplast are presently unclear.

To understand clearly the biological significance of specific phosphorylation of p34 by CK-II in post-transcriptional regulation, further analytical studies are required (i) to determine the molecular interaction between p34, mRNAs (or chloroplast DNA) and splicing RNases; and (ii) to investigate the biological role of the CK-II mediated control mechanisms involved in post-transcription and DNA replication in chloroplasts.

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