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Phosphorylation by protein kinase CKII modulates the DNA-binding activity of a chloroplast nucleoid-associated protein

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Abstract Plastid DNA, like bacterial and mitochondrial DNA, is organized into protein–DNA complexes called nucleoids. Plastid nucleoids are believed to be associated with the inner envelope in developing plastids and the thylakoid membranes in mature chloroplasts, but the mechanism for this localization is unknown. MFP1 is a DNA-binding, coiled-coil protein associated with the thylakoid membranes of mature chloroplasts. It is also a component of nucleoids, suggesting a function at the interface of the chloroplast genome and the photosynthetic membranes. Several thylakoid proteins are phosphorylated by a protein kinase CKII-like activity and the alpha subunit of a chloroplast-located CKII has recently been identified as a component of the chloroplast transcription complex. Here, we show evidence for the phosphorylation of MFP1 in purified chloroplasts from tobacco (*Nicotiana tabacum* L.). We demonstrate that the DNA-binding domain of MFP1 is a substrate for CKII and that phosphorylation by CKII inhibits DNA binding. Using site-directed mutagenesis, we identify a conserved twin CKII site in the DNA-binding domain that is required for the inhibition of DNA binding. Phosphorylation of MFP1 by chloroplast CKII as a possible means to modulate its DNA-binding activity is discussed.

Keywords Chloroplast · DNA-binding protein · *Nicotiana* · Protein kinase CKII

Abbreviations CKII: Casein kinase II · MFP1: MAR-binding filament-like protein 1 · PTK: Plastid transcription kinase

Introduction

Plastid DNA, like bacterial and mitochondrial DNA, is organized into protein–DNA complexes called nucleoids. Plastid nucleoids are believed to be associated with the inner envelope in developing plastids and the thylakoid membranes in mature chloroplasts, but the mechanism for this relocalization is unknown (Miyamura et al. 1986; Liu and Rose 1992; Sato et al. 1993). The nuclear-encoded protein MFP1 (MAR-binding filament-like protein 1) from tomato (LeMFP1) was identified by its ability to bind to matrix attachment region DNA (Meier et al. 1996). MFP1 is an 80-kDa, predominantly coiled-coil protein with a C-terminal DNA-binding domain. It was subsequently shown to be localized in plastids and associated with thylakoid membranes, with the DNA-binding domain oriented towards the stroma. In mature *Arabidopsis* chloroplasts, MFP1 is a major DNA-binding activity which binds to all tested chloroplast DNA fragments without detectable sequence specificity (Jeong et al. 2003). The expression of MFP1 is tightly correlated with the accumulation of thylakoid membranes. Importantly, MFP1 is associated in vivo with nucleoids, suggesting a function at the interface between chloroplast nucleoids and the thylakoid membrane system (Jeong et al. 2003).

Phosphorylation has been shown to play a major role in modulating the function and DNA-binding activity of many nuclear proteins, including transcription factors and proteins involved in chromatin organization (e.g., Dang et al. 1994; Armstrong et al. 1997; Hoffmann et al. 1998). Recently, a chloroplast protein (cpCK2 α) with high similarity to the alpha subunit of casein kinase II (CKII) has been reported as a component of the chloroplast transcription apparatus (Ogrzewalla et al. 2002). cpCK2 α has the functional characteristics of a

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biochemically identified serine-specific protein kinase associated with the plastid encoded PEP-A RNA polymerase (plastid transcription kinase, PTK; Baginsky et al. 1999). Because nuclear CKII has been shown to phosphorylate a number of transcription factors and chromatin-associated proteins, it is intriguing to find a member of this kinase family associated with the transcriptionally active plastid genome. It is presently not clear what the *in vivo* substrates of plastid CKII are or what function CKII phosphorylation has in the context of the chloroplast nucleoid.

Here, we show that chloroplast-localized MFP1 is phosphorylated *in vivo* and that *in vitro* phosphorylation by CKII inhibits its DNA-binding activity. A tandem CKII site in the DNA-binding domain was identified which is involved in the phosphorylation-dependent loss of DNA-binding activity. These features of MFP1 make CKII-dependent phosphorylation a possible mode of regulating the DNA-binding activity of the protein *in vivo*.

Materials and methods

Chloroplast protein isolation and two-dimensional electrophoresis

Nicotiana tabacum L. plants were grown in soil under 16 h light, 8 h dark at 22°C for 2 months. Chloroplast isolation was according to Maliga et al. (1995). Two-dimensional (2-D) electrophoresis was performed as described previously (Calikowski et al. 2003), except that ampholytes 4.7–5.9 (BioRad, Hercules, CA, USA) were used. The gel was transferred to nitrocellulose, stained with SyproRuby, and de-stained for immunoblotting.

MFP1 C-terminal mutations and protein purification

Constructs for protein expression were cloned into pRSET (Invitrogen) as described by Meier et al. (1996). Mutagenesis was performed by incorporation of a phosphorylated oligonucleotide during PCR amplification (Michael 1994). Positive clones were identified by restriction digestion and confirmed by sequencing. Mutants were subcloned into pRSET/MFP1 Δ Hinc as a *SpeI/KpnI* fragment for expression. Constructs were transformed into *Escherichia coli* BL21 (DE3) pLysS (Novagen), and protein expression and purification were performed as described by Meier et al. (1996).

Phosphorylation with CKII, immunoblots, and “South-western” DNA-binding blots

For phosphorylation of MFP1 Δ Hinc (wild type and alanine mutants), 0.6–2 μ g of protein was combined with 1 \times CKII buffer [10 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM MgCl₂], 3–4 nmol of ATP (with or without 74 kBq [³²P] γ -ATP), and 250–800 units of recombinant human CKII (NEB P6010) in a total volume of 15–20 μ l, and incubated at 30°C for 30–60 min. One unit of CKII is defined as the amount required to catalyze the transfer of 1 pmol of phosphate to RRREEETEEE (400 μ M) in 1 min at 30°C in a total volume of 25 μ l. Reactions were terminated by an equal volume of SDS gel loading buffer (BioRad) and boiled for 3 min. For immunoblots, proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with 1:5,000-diluted anti-express antibody (Invitrogen) (Fig. 2b) or with 1:3,000-diluted anti-MFP1 R50 antiserum (Harder et al. 2000; Fig. 2d) and 1:5,000-diluted horseradish

peroxidase-conjugated anti-rabbit secondary antibody. Antibody incubations and enhanced chemiluminescence detection were as described in the ECL manual (Amersham). “South-western” blot analysis was essentially performed as described by Meier et al. (1996). Binding reactions contained 25–40 ng ml⁻¹ DNA fragment (1.0 \times 10⁶–1.7 \times 10⁶ cpm ml⁻¹) and 10–25 μ g ml⁻¹ sheared *E. coli* DNA as non-specific competitor. The DNA probe was a 0.66-kb *Drosophila* histone H1/H3 spacer MAR DNA (von Kries et al. 1991).

Results

A search for putative phosphorylation sites for CKII in the LeMFP1 sequence identified 17 sites that match the consensus recognition motif T/SXXD/E (Cardenas et al. 1993). To investigate whether MFP1 is phosphorylated in tobacco chloroplasts, a total chloroplast protein extract was resolved by 2-D gel electrophoresis over a pH range expected to resolve MFP1 isoforms (Fig. 1a). A 2-D immunoblot showed a series of up to 12 spots at the predicted molecular weight of 80 kDa, covering a pI range of ca. 5.0 to 5.4, consistent with the predicted pI of 5.4 (Fig. 1b). The multiple, more negatively charged isoforms are likely due to multiple phosphorylation

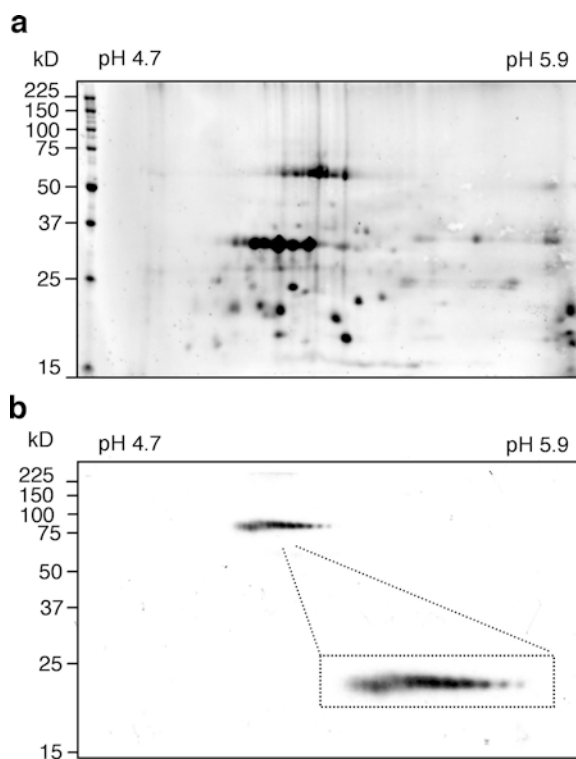


Fig. 1a, b Identification of multiple isoforms of tobacco (*Nicotiana tabacum*) MFP1 in a chloroplast extract. **a** CyproRuby staining of tobacco chloroplast proteins separated by isoelectric focusing between pH 4.7 and pH 5.9 in the first dimension and by SDS-PAGE in the second dimension. Molecular weight markers are indicated on the left. **b** Two-dimensional immunoblot of a replica gel of **a** that was probed with the anti-MFP1 288 antibody (Harder et al. 2000). The immunoblot reveals up to 12 separate spots at a size of 80 kDa and a pI range of ca. 5.0–5.4. The inset shows a magnification of the signals

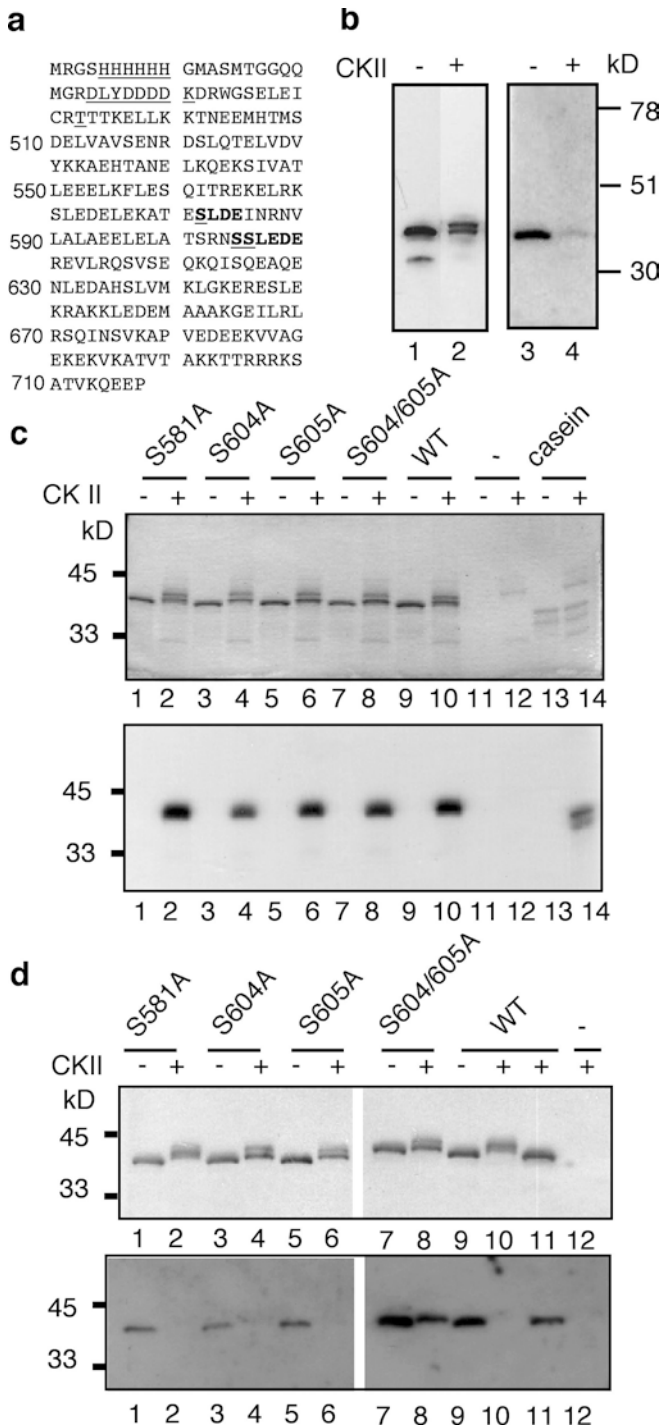


Fig. 2 a Sequence of MFP1 Δ Hinc protein with the conserved CKII sites in *bold* and the serines exchanged to alanines *bold and underlined*. The 6 \times His tag, express tag, and first amino acid of the DNA-binding domain of MFP1 (T493) are *underlined*. Numbers on the *left* indicate the amino acid position in full-length MFP1. **b** Inhibition of DNA binding by phosphorylation of the MFP1 DNA-binding domain. MFP1 Δ Hinc was incubated with (+) or without (-) CKII. *Lanes: 1, 2* immunoblot with anti-express antibody; *3, 4* "South-western" DNA-binding experiment. **c** Phosphorylation of MFP1 Δ Hinc alanine mutants with CKII. Purified proteins were incubated in the presence of [32 P] γ -ATP with (+) or without (-) CKII. *Lanes: 11, 12* no protein; *13, 14* purified casein (Sigma) as positive control. *Top panel* Coomassie stain; *bottom panel* [32 P] γ -ATP incorporation. **d** DNA binding of unphosphorylated and phosphorylated MFP1 Δ Hinc alanine mutants. Purified proteins were incubated with (+) or without (-) CKII. *Lanes: 11* wild-type MFP1 Δ Hinc without ATP; *12* no protein. *Top panel* immunoblot with anti-MFP1 antibody; *bottom panel* "South-western" DNA-binding experiment. The blot used for the "South-western" was re-probed with anti-MFP1 antibody and gave results identical to the top panel (data not shown)

served in all MFP1 sequences known, indicating a functional significance (Harder et al. 2000; *bold* in Fig. 2a). The second site (SSLEDE) consists of two overlapping sites in the tomato and tobacco sequences, while it is a single site in the *Arabidopsis*, maize, and soybean sequences.

We tested the C-terminal 258 amino acids of LeMFP1 fused to an N-terminal histidine tag (MFP1 Δ Hinc, Fig. 2a) for phosphorylation by CKII and examined its ability to bind DNA in the presence and absence of phosphorylation. Phosphorylation of MFP1 Δ Hinc was demonstrated by a change in mobility in the presence of ATP and CKII (Fig. 2b, left panel). No shift in mobility was observed in the absence of kinase but presence of ATP, in the presence of kinase but absence of ATP, and when heat-inactivated kinase was used (Fig. 2c, and data not shown). In a "South-western" blot experiment (Fig. 2b, right panel), the unphosphorylated MFP1 Δ Hinc protein bound to DNA as shown previously (Meier et al. 1996). In contrast, phosphorylated MFP1 Δ Hinc protein did not bind DNA, indicating that phosphorylation of the MFP1 carboxy-terminal DNA-binding domain inhibits its DNA-binding activity.

To investigate whether DNA binding of MFP1 Δ Hinc is affected by CKII phosphorylation at particular sites, the three conserved CKII sites at positions 581, 604, and 605 were mutated individually and the 604/605 site in tandem, by replacing the phosphate-acceptor serine residue with an alanine. Figure 2c shows that all mutant proteins remained a substrate for CKII phosphorylation, consistent with the presence of more than one acceptor site. To determine whether phosphorylation at serines 581, 604, or 605 is involved in blocking the DNA-binding activity, we examined the ability of each of the alanine mutants to bind to DNA in both their unphosphorylated and phosphorylated forms. Immunoblot analysis (Fig. 2d, upper panel) demonstrated that equivalent amounts of each protein were loaded on the gels, and showed the change in migration of the phosphorylated forms relative to the unphosphorylated form.

events; however, other types of modification of MFP1 can presently not be excluded.

Because MFP1 is a non-specific DNA-binding protein that is associated with chloroplast nucleoids, we investigated whether phosphorylation of its DNA-binding domain modulates its DNA-binding affinity. The DNA-binding domain was previously mapped to amino acids 492–717 at the C-terminus of MFP1 (Fig. 2a; Meier et al. 1996). Nine predicted CKII sites are present in this domain. Of those, two sites are con-

When probed with DNA, MFP1 Δ Hinc wild type, S581A, S604A, and S605A exhibited DNA binding only in the unphosphorylated state (Fig. 2d, lower panel, lanes 1, 3, 5, and 9). In contrast, MFP1 Δ Hinc S604/605A showed DNA-binding activity in both the unphosphorylated and phosphorylated state (lanes 7 and 8). This indicates that mutating the twin CKII sites at positions 604 and 605 strongly reduces the inhibitory effect of phosphorylation on DNA binding, and suggests therefore that a phosphate at either serine 604 or serine 605 is required for the inhibition of DNA binding.

Discussion

The data presented here suggest that chloroplast-located MFP1 is an *in vivo* target for phosphorylation and show that *in vitro* phosphorylation of the C-terminal domain by CKII inhibits DNA binding. No effect on phosphorylation-dependent inhibition of DNA binding was observed in the S581A mutant, indicating that this site is either not a target for phosphorylation, or that phosphorylation of serine 581 is not required for inhibition of DNA binding. In contrast, the difference in DNA-binding activity of wild type versus mutant S605/604A suggests that this double CKII site can modulate MFP1 DNA-binding activity. The MFP1 DNA-binding domain is clearly phosphorylated by CKII at least at one site in addition to serine 604 and 605, demonstrated by both the shift in mobility and incorporation of ³²P-phosphate by the S604/605A mutant protein. However, the S604/605A mutant protein is equally capable of binding to DNA in the presence or absence of phosphorylation. In contrast, mutating only the serine at position 604 or at position 605 renders the mutant protein susceptible to phosphorylation-dependent inhibition of DNA binding. These data indicate that a phosphate at either serine 604 or serine 605 disrupts DNA binding, while other phosphorylation events have a minor effect on the DNA-binding activity.

Until recently, protein kinase CKII has been considered a multifunctional kinase with substrates limited to the nucleus and the cytoplasm. The recent finding that a member of the CKII family is also present in the chloroplast, where it is associated with the bacterial-type RNA-polymerase complex (PEP) opens up the question of whether DNA-binding or chromatin-organizing proteins in the chloroplast are also substrates for this kinase. The dual interactions of MFP1 with the thylakoid membranes and the nucleoid would position the protein closely to the nucleoid-associated PTK and its previously proposed substrates, which are either integral membrane proteins of the thylakoid or connected to the transcriptional machinery (Kanekatsu et al. 1995, 1998; Testi et al. 1996). It is therefore conceivable that MFP1 is a substrate for cpCK2 α and that the degree of MFP1 phosphorylation could modulate its DNA-binding activity.

Three other DNA-binding, chloroplast nucleoid proteins have been previously described, PEND, CND41, and DCP68 (Sato et al. 1993; Nakano et al. 1997;

Chi-Ham et al. 2002). While currently nothing is known about the effects of phosphorylation on PEND and CND41, dephosphorylation by alkaline phosphatase affects the binding of purified DCP68 to DNA *in vitro* (Chi-Ham et al. 2002). It is therefore conceivable that modulation of DNA-binding activity by phosphorylation is a mechanism that affects several nucleoid-associated proteins. Further experiments will be required to probe into the *in vivo* interactions between DCP68, MFP1, chloroplast protein kinases, and DNA. Having mapped a phosphorylation site in the DNA-binding domain that is required for inhibition of DNA-binding, it is now feasible to construct an MFP1 mutant protein that cannot be inhibited by phosphorylation and to test the effect of such a mutation *in vivo*.

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References

- Armstrong SA, Barry DA, Leggett RW, Mueller CR (1997) Casein kinase II-mediated phosphorylation of the C terminus of Sp1 decreases its DNA binding activity. *J Biol Chem* 272:13489–13495
- Baginsky S, Tiller K, Pfannschmidt T, Link G (1999) PTK, the chloroplast RNA polymerase-associated protein kinase from mustard (*Sinapis alba*), mediates redox control of plastid *in vitro* transcription. *Plant Mol Biol* 39:1013–1023
- Calikowski TT, Meulia T, Meier I (2003) A proteomic study of the *Arabidopsis* nuclear matrix. *J Cell Biochem* 90:361–378
- Cardenas ME, Walter R, Hanna D, Gasser SM (1993) Casein kinase II copurifies with yeast DNA topoisomerase II and reactivates the dephosphorylated enzyme. *J Cell Sci* 104:533–543
- Chi-Ham CL, Keaton MA, Cannon GC, Heinhorst S (2002) The DNA-compacting protein DCP68 from soybean chloroplasts is ferredoxin:sulfite reductase and co-localizes with the organellar nucleoid. *Plant Mol Biol* 49:621–631
- Dang Q, Alghisi GC, Gasser S (1994) Phosphorylation of the C-terminal domain of yeast topoisomerase II by casein kinase II affects DNA-protein interaction. *J Mol Biol* 243:10–24
- Harder PA, Silverstein RA, Meier I (2000) Conservation of matrix attachment region-binding filament-like protein I among higher plants. *Plant Physiol* 122:225–234
- Hoffmann R, Craik DJ, Pierens G, Bolger RE, Otvos L Jr (1998) Phosphorylation of the C-terminal sites of human p53 reduces non-sequence-specific DNA binding as modeled with synthetic peptides. *Biochemistry* 37:13755–13764
- Jeong SY, Rose A, Meier I (2003) MFP1 is a thylakoid-associated, nucleoid-binding protein with a coiled-coil structure. *Nucleic Acids Res* 31:5175–5185
- Kanekatsu M, Ezumi A, Nakamura T, Ohtsuki K (1995) Chloroplast ribonucleoproteins (RNPs) as phosphate acceptors for casein kinase II: purification by ssDNA-cellulose column chromatography. *Plant Cell Physiol* 36:1649–1656
- Kanekatsu M, Saito H, Motohashi K, Hisabori T (1998) The beta subunit of chloroplast ATP synthase (CF0CF1-ATPase) is phosphorylated by casein kinase II. *Biochem Mol Biol Int* 46:99–105
- Liu JW, Rose RJ (1992) The spinach chloroplast chromosome is bound to the thylakoid membrane in the region of the inverted repeat. *Biochem Biophys Res Commun* 184:993–1000
- Maliga P, Klessig D, Cashmore A, Gruissem W, Varner J (1995) *Methods in plant molecular biology: a laboratory course manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Meier I, Phelan T, Gruissem W, Spiker S, Schneider D (1996) MFP1, a novel plant filament-like protein with affinity for matrix attachment region DNA. *Plant Cell* 8:2105–2115

- Michael S (1994) Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. *Biotechniques* 16:410–412
- Miyamura S, Nagata T, Kuroiwa T (1986) Quantitative fluorescence microscopy on dynamic changes of plastid nucleoids during wheat development. *Protoplasma* 133:66–72
- Nakano T, Murakami S, Shoji T, Yoshida S, Yamada Y, Sato F (1997) A novel protein with DNA-binding activity from tobacco chloroplast nucleoids. *Plant Cell* 9:1673–1682
- Ogrzewalla K, Piotrowski M, Reinbothe S, Link G (2002) The plastid transcription kinase from mustard (*Sinapis alba* L.). A nuclear-encoded CK2-type chloroplast enzyme with redox-sensitive function. *Eur J Biochem* 269:3329–3337
- Sato N, Albrieux C, Joyard J, Douce R, Kuroiwa T (1993) Detection and characterization of a plastid envelope DNA-binding protein which may anchor plastid nucleoids. *EMBO J* 12:555–561
- Testi MG, Croce R, Polverino-De Laureto P, Bassi R (1996) A CK2 site is reversibly phosphorylated in the photosystem II subunit CP29. *FEBS Lett* 399:245–250
- von Kries JP, Buhrmester H, Stratling WH (1991) A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding. *Cell* 64:123–135