Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells

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Abstract CND41 is a 41 kDa DNA-binding protein isolated from chloroplast nucleoids of cultured tobacco cells. The presence of the active domain of aspartic protease in the deduced amino acid sequence of CND41 suggests that it has proteolytic activity. To confirm this, CND41 was highly purified from cultured tobacco cells and its proteolytic activity was characterized with fluorescein isothiocyanate-labeled hemoglobin as the substrate. The purified CND41 had strong proteolytic activity at an acidic pH (pH 2–4). This activity was inhibited by various chemicals, including the nucleoside triphosphates, NADPH, Fe^{3+} and sodium dodecyl sulfate.

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1. Introduction

A few DNA-binding proteins are known to have protease activity: the Lon protease of *Escherichia coli* ([1] and references therein) is the most investigated DNA-binding protease. Lon contributes to the regulation of several important cellular functions, including radiation resistance, cell division and proteolytic degradation of certain regulatory and abnormal proteins. AEBP1, a mouse carboxypeptidase, is reported to have site-specific DNA-binding activity and is responsible for transcriptional repression [2]. The yeast homologue of mammalian bleomycin hydrolase, GAL6, is a cysteine protease that has a high binding affinity for RNA and single-strand DNA but a much lower affinity for double-strand DNA [3,4]. To our knowledge, however, very few DNA-binding proteins with aspartic protease activity have been reported [5]. DNA-binding protein with protease activity would be an important regulatory factor in both prokaryotic and eukaryotic genetic systems. Although Lon homologues are present in several widely divergent bacteria, as well as in the mitochondria of yeasts, humans and higher plants [6–8], no Lon homologue has been found in chloroplasts/plastids.

We previously isolated a 41 kDa DNA-binding protein, from the plastid nucleoids (DNA–protein complex) of cultured tobacco cells [9]. Characterization of antisense transgenic tobacco cells indicated that decreases in the CND41 protein increased the chloroplastic gene transcripts [10]. These results suggest that this novel DNA-binding protein is involved in the negative regulation of gene expression in chloroplasts, although the actual regulation mechanism is not clear.

In contrast, the deduced amino acid sequence indicates that CND41 has active domains conserved for aspartic protease [10]. Therefore, we became interested in the bifunctional nature of this novel protein which may degrade transcriptional factors such as sigma factors or RNA polymerase, etc., and decrease the level of chloroplast transcripts. To examine this possibility, we purified the CND41 from cultured tobacco cells and characterized its protease activity, with fluorescein isothiocyanate-labeled hemoglobin (FTC-Hb) as the substrate.

2. Materials and methods

2.1. Plant material

Photomixotrophic cultured cells of *Nicotiana tabacum* cv Samsun NN were maintained, as described previously, under continuous light (about 60 μmol/m^2/s) in modified Linsmaier and Skoog liquid medium containing 3% (w/v) sucrose [9].

2.2. Purification of CND41

All procedures were done below 4°C. Cells from an 8 day culture (about 1 kg fresh weight) were homogenized in a Polytron (Kinematica, Luzern, Switzerland) in the same volume of buffer A (50 mM Na phosphate, pH 7.2, and 10 mM 2-mercaptoethanol) containing 10 mM EDTA. The homogenate was filtered through two layers each of gauze and Miracloth (Calbiochem, La Jolla, CA, USA) and then centrifuged at 7000×*g* for 30 min. The precipitate was resuspended for 1 h in 1/10 volume of buffer A containing 0.5 M NaCl and then centrifuged at 15 000×*g* for 20 min. The supernatant diluted with the same volume of buffer A was applied to an SP-Sepharose (Amersham Pharmacia, Bucks) column equilibrated with buffer A containing 0.25 M NaCl. Proteins were eluted with a 0.25–1.2 M NaCl gradient. The CND41 fractions were applied to an Octyl-Sepharose CL-4B (Amersham Pharmacia) column, which had been equilibrated with buffer A containing 0.25 M NaCl, then proteins eluted in a gradient of 0–50% ethylene glycol. The eluted CND41 fractions were dialyzed in 50 mM HEPES buffer (pH 7.2), 10 mM 2-mercaptoethanol and 0.25 M NaCl, and then layered on a HiTrap heparin column (Amersham Pharmacia). Proteins were eluted with a gradient of 0.25–1.5 M NaCl. The CND41 fractions were concentrated using Centriplus-10 (Millipore, Bedford) and stored at −70°C in 25% (v/v) glycerol until used. The filtrate was similarly stored and used as the mock control in the protease assay. The elution of CND41 was monitored by immunoblotting or proteolytic activity with FTC-Hb as the substrate. The concentration of the purified CND41 was measured by the absorbance at 280 nm [11]. Purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining, immunoblotting and Southwestern blotting as described by Nakano et al. [10]. N-terminal amino acid sequences of the purified

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Abbreviations: FTC-Hb, fluorescein isothiocyanate-labeled hemoglobin

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proteins were determined with an ABI 476A peptide sequencer (Perkin-Elmer, Norwalk).

2.3. Protease activity assay

FTC-Hb was used as substrate because of its digestibility and purity. The preparation of FTC-Hb and determination of protease activity were done by the method described by Twining [12] with some modification. FTC-Hb (final concentration 0.5 mg/ml) and purified CND41 (20 nM) were mixed on ice in Na citrate buffer at the indicated pH (final concentration 50 mM, final volume 50 μl), and the proteolytic reaction was started by an immediate shift of temperature to 30°C. After 40 min of incubation, the reaction was stopped by the addition of 120 μl of 5% TCA. After the precipitation of TCA-insoluble protein by centrifugation at 10 000 × *g* for 10 min at 4°C, a portion of the supernatant was diluted with 500 mM Tris buffer (pH 8.5), and its fluorescence was measured in a fluorescence spectrophotometer (excitation: 490 nm, emission: 525 nm, Model F-2000, Hitachi, Tokyo, Japan).

3. Results

3.1. Purification of CND41

CND41 was first identified as a DNA-binding protein, but no proteolytic activity was found for it at the beginning of purification. We therefore purified CND41 using immunological detection with rabbit antiserum prepared against truncated CND41 produced in *E. coli* [10]. This specific antiserum detected two proteins extracted from cultured tobacco cells. Because only the lower band of these proteins (named CND41-II) showed DNA-binding activity on Southwestern blotting analysis, we further purified it. The major protein detected by the anti-CND41 antiserum that showed no DNA-binding activity was soluble in the extraction buffer. Because this protein (named CND41-I) also was decreased in antisense transgenic tobacco plants with reduced CND41 transcripts, we speculate that it is an isoform of CND41 or a precursor form of CND41, but further characterization is needed.

CND41-II was insoluble in extraction buffer with a low salt concentration. It was made soluble from precipitates of cell homogenates by the use of high salt buffer containing 0.5 M NaCl, then purified through successive SP-Sepharose, Octyl-Sepharose and heparin column chromatographies (Fig. 1). After the heparin column chromatography, three bands of about 41, 26 and 19 kDa were detected in SDS–PAGE analysis with silver staining. All three bands were detected by the anti-CND41 antiserum. Determination of the N-terminal amino acid sequences indicated that both the 41 and 26 kDa bands had the N-terminal amino acid sequence of mature CND41 and that the 19 kDa band had the internal sequence of CND41 (data not shown). We concluded that this fraction contained only CND41 and its degradation products. Because no other contaminant was detected in this fraction, after heparin column chromatography, we designated the fraction highly purified CND41 in order to characterize its biochemical activity.

3.2. Protease activity of CND41

As stated in Section 1, CND41 has all the residues of the active sites conserved in aspartic proteases [10]. Because aspartic protease is an acidic protease, we first examined the effect of pH with FTC-Hb as the model substrate. Purified CND41 had a very high activity at an acidic pH (pH 2–4); the highest being at pH 2.5 and half maximum was pH 4.0. Although about 10% activity of the maximum activity was found with FTC-Hb at pH 7.0, SDS–PAGE analysis with ribulose 1,5-bis-phosphate carboxylase/oxygenase showed no degradation of protein at this pH (data not shown). The maximum activity of CND41 with FTC-Hb at pH 2.5 was about 1/10 of pepsin purified from porcine stomach mucosa (Sigma) of 45 000 U/mg protein on the molecule basis.

Next, we investigated conditions which might affect the proteolytic activity of CND41. First, we examined the effect of known protease inhibitors. None of those examined had a strong effect on activity (Table 1). Even pepstatin, an aspartic protease inhibitor, only weakly inhibited the proteolytic activity of CND41, whereas the same concentration of pepstatin completely inhibited pepsin activity (data not shown). Of the cations examined, Fe^{3+} had a marked inhibitory effect on the CND41 protease, whereas monovalent and divalent cations, including Fe^{2+}, had very little effect.

Because metabolic activities in chloroplasts are often regulated by the redox potential as well as nucleotides [13], the effects of redox reagents and nucleotides were examined. As Table 1 shows, nucleoside triphosphates and their non-hydrolyzable analogs, e.g. ATP/GTP-γ-S, as well as NADPH strongly inhibited the protease activity of CND41 at an acidic pH. Because NADPH is very unstable at an acidic pH, the actual inhibitory mechanism of NADPH on protease activity is not clear. SDS, which is known to stimulate protease activity [14,15], also strongly inhibited the activity of CND41.

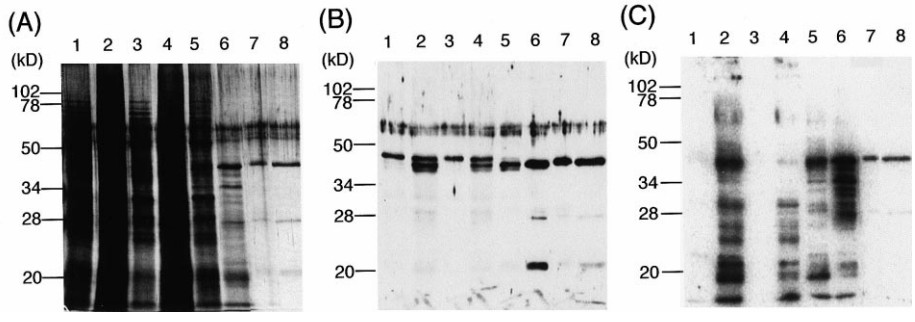

Fig. 1. Purification of CND41 from cultured tobacco cells. 10 μl fractions were subjected to SDS–PAGE. Proteins were detected by silver staining (A), immunoblot analysis with anti-CND41 antiserum (B) or Southwestern analysis with ^{32}P-labeled chloroplast DNAs (C). Lanes: 1, crude homogenate of cultured tobacco cells; 2 and 3, precipitate and supernatant after 7000 × *g* centrifugation of the crude homogenate; 4 and 5, insoluble and soluble fractions after 500 mM NaCl extraction of the 7000 × *g* precipitate; 6, 7 and 8, CND41 fractions respectively after SP-Sepharose, Octyl-Sepharose and heparin column chromatography.

Table 1
Effect of various compounds on proteolytic activity of CND41 at pH 2.5

Compounds	Concentration	Activity (%)	S.D.
Protease inhibitors			
PMSF	1 mM	103	4.1
Antipain	3 μg/ml	98.4	7.8
Chymostatin	3 μg/ml	106	9.6
Leupeptin	3 μg/ml	101	6.2
Pepstatin	3 μg/ml	81.6	1.1
Phosphoramidon	3 μg/ml	85.0	5.2
Aprotinin	5 μg/ml	100	1.8
EDTA	1 mM	122	7.2
Ions			
KCl	20 mM	92.6	4.3
NaCl	20 mM	97.2	6.9
$CaCl_2$	2 mM	78.5	4.6
$MgCl_2$	2 mM	95.0	1.8
$MnCl_2$	2 mM	89.8	5.7
$ZnCl_2$	2 mM	93.7	2.7
$FeCl_2$	2 mM	99.8	5.4
$FeCl_3$	2 mM	14.5	2.3
Redox reagents			
2-Mercaptoethanol	5 mM	147	9.2
Dithiothreitol	1 mM	129	10
GSH	10 mM	156	2.9
GSSG	5 mM	130	20
NADH	3 mM	82.3	23
NAD	3 mM	139	4.4
NADPH	3 mM	32.1	3.5
NADP	3 mM	105	4.0
Na ascorbate	5 mM	92.1	6.6
Nucleotides and related compounds			
Na phosphate	1 mM	109	5.2
Na pyrophosphate	1 mM	79.4	1.8
AMP	1 mM	99.3	10
ADP	1 mM	102	11
ATP	1 mM	33.8	3.2
GTP	1 mM	17.9	6.1
CTP	1 mM	41.1	5.1
UTP	1 mM	31.6	6.6
dATP	1 mM	10.2	1.0
dGTP	1 mM	15.9	2.7
dCTP	1 mM	55.5	9.1
dTTP	1 mM	28.9	5.3
ATP-γ-S	1 mM	25.8	3.1
GTP-γ-S	1 mM	14.6	0.77
DNA[a]	0.02 mg/ml	119	9.0
RNA[b]	0.02 mg/ml	103	5.0
Detergents			
SDS	0.05%	0.00	1.6

Mean values and S.D.s of three independent experiments are shown. The mean value for CND41 alone was taken as 100%.
Abbreviations: PMSF, phenylmethylsulfonyl fluoride; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate).
[a]Salmon sperm DNA (Sigma, St. Louis, MO, USA).
[b]Calf liver RNA (Sigma).

Although CND41 has DNA-binding activity, the protease activity was not effected when salmon sperm DNA or calf liver RNA were added at an acidic pH.

4. Discussion

The chloroplast nucleoid DNA-binding protein CND41 was highly purified, and its proteolytic activity was confirmed. Like the other aspartic proteases, CND41 has a high proteolytic activity under an acidic condition, indicative that a component of the nucleoid CND41 has both DNA-binding and protease activities. Although only a few proteases are known to have DNA-binding activity, these DNA-binding proteases would be important in the regulation of both prokaryotic and eukaryotic gene expression: the Lon protease of *E. coli* [1], AEBP1 (a mouse carboxypeptidase [2]), GAL6 (the yeast homologue of mammalian bleomycin hydrolase [3,4]). Our results suggest that CND41 may be a chloroplastic protease with a function similar to that of the Lon protease of *E. coli*.

CND41, the first aspartic protease to be found in chloroplasts, would have the broad substrate specificity of the Clp protease (data not shown). CND41 might be involved not only in the regulation of gene expression, but also in the biogenesis of the functional apparatus of chloroplasts and in the degradation of denatured proteins. Only a few proteolytic enzymes that are involved in processing for the generation of functional components have been well characterized in chloroplasts [16,17]. Although CND41 has an unusually low optimal pH and such a low pH would not occur in chloroplasts under normal physiological conditions, certain environmental stresses, e.g. freeze-induced dehydration stress, are known to lower the cytosolic pH due to disturbance of the tonoplast membranes [18]. CND41 may function under such stress conditions.

CND41 does not need ATP or other nucleoside triphosphates for proteolysis, whereas some proteolytic enzymes such as Clp protease, Lon protease and proteasome do. Indeed, ATP and other nucleoside triphosphates (ribonucleosides or deoxyribonucleosides as well as such non-hydrolyzable nucleosides as ATP/GTP-γ-S) strongly inhibit the protease activity of CND41. Because CND41 degrades proteins non-specifically at a low pH, the photosynthetic products may regulate protease activity under normal conditions.

A mechanism that may control the proteolytic activity of CND41 is modification of the substrate, or of CND41 itself, to enhance its activity at a physiological pH. Our preliminary results suggest that some modification of the substrate is important for degradation by CND41 at a physiological pH. A more detailed investigation is now underway.

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