Volume 316, number 1, 93–98 FEBS 12023 January 1993

Chloroplast envelope protein encoded by chloroplast genome

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Received 5 October 1992; revised version received 24 November 1992

The gene product of an open reading frame of chloroplast genome, ORF 231 in pea, was immunochemically detected in chloroplast and etioplast envelopes. This is the first protein of a Chloroplast Envelope Membrane encoded by a chloroplast genome. It was named CEM A and the gene, *cem* A. CEM A is an acidic protein having an apparent molecular mass of 34 kDa on SDS-PAGE, and a minor component detected in the fractionated inner envelope.

Chloroplast envelope protein; Chloroplast genome; Open reading frame; Pea; Spinach

1. INTRODUCTION

Complete DNA sequences of plastid genome were reported for liverwort [1], tobacco [2] and rice [3], and about 30 open reading frames (ORF) were found in each genome. Identification of the gene product of ORF is needed to understand chloroplast biogenesis. An ORF 231 in the pea plastid genome, is located in the *zfp*A-*pet*A operon and the homologue is conserved in other plant species [1–3]. The deduced amino acid sequence of the ORF 231 protein [4] seems to contain four membrane spanning domains [5]. Transcripts of ORF 231 were detected by Northern blot analysis and by primer extension analysis [4,6], so in this report we set out to detect immunochemically its gene product in the membrane fraction of the pea chloroplasts. We found that the gene product was present in the envelope fraction.

2. MATERIALS AND METHODS

2.1. *Construction of the chimeric bgal-231 plasmid and the chimeric MAL-73 plasmid*

Chimeric β-galactosidase-ORF231 (βgal-231) plasmid: The *Xma*I–*Bam*HI fragment of the 17.3-kb *Pst*I DNA fragment from pea chloroplasts [6] that contains ORF 231-*pet* A was inserted into the *Sma*I–*Bam*HI site of pUC119. The *Eco*RI fragment of this plasmid, which had lost 16 nucleotides (6 amino acid residues from the N-terminal) of the ORF231 coding region, was ligated into the *Eco*RI site of pUEX2 containing *cro-lacZ* [7]. Chimeric maltose-binding protein-73 amino acid residues (MAL-73) plasmid: the DNA fragment encoding 73 amino acid residues from 30 to 102 of the ORF 231 was amplified by PCR, and ligated into pIH 889 containing *malE* [8].

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2.2. *Expression and purification of βgal-231 fused protein and of MAL-73 fused protein*

The chimeric βgal-231 plasmid was introduced into *E. coli* JM109. The fused protein was induced for 2 h at 42°C and formed inclusion bodies. The inclusion bodies were isolated [9], made soluble in 5% SDS and electrophoresed on SDS-PAGE (5% gel) [10]. Protein with a molecular mass of 140 kDa (estimated from the fused protein) was cut out from the gel and electroeluted. SDS in the protein solution was partially removed by dialyzing against 20 mM Tricine-KOH (pH 8) containing 0.1% SDS. The chimeric MAL-73 plasmid was introduced into *E. coli*XLI-Blue. The fused protein was purified by affinity chromatography as described in the manufacturer's protocol (New England BioLabs).

2.3. *Antibody preparation*

Antibodies against βgal-231 fused protein were obtained from rabbits as described [11]. Preimmune sera were obtained from non-immunized rabbits. An oligopeptide (KKSLESWITHWYNTKESE) containing 18 amino acid residues from 28 to 45 of ORF 231 was synthesized, and conjugated to bovine serum albumin [12]. Antibodies against this conjugated protein (18mer/231 protein) were similarly obtained from rabbits. These antisera were subjected to $(NH_4)_2SO_4$precipitation and the IgG fractions were purified on a DEAE-cellulose column. Antibodies against βgal-231 were further purified by affinity chromatography using Affigel 10 (Bio-Rad) conjugated with MAL-73 protein as described in manufacturer's protocol. The IgG against β-galactosidase was purchased (5Prime-3Prime).

2.4. *Fractionation of chloroplast proteins*

Pea plants (*Pisum sativum*, cv. Alaska) were grown under a cycle of 12 h light and 12 h dark. Intact chloroplasts were isolated with a Percoll gradient from 10- to 12-day-old pea leaves, and ruptured by three cycles of freezing and thawing in hypertonic buffer [13]. Thylakoid and stromal fractions were obtained by the procedure of Soll and Bennett [14], and the envelope fraction was prepared as described elsewhere [15]. The thylakoid pellet was treated with 80% acetone to remove chlorophyll, dried, and made soluble in 5% SDS. The envelope fraction was separated into inner and outer envelope fractions by centrifugation on a discontinuous sucrose density gradient [13]. Fractions were pelleted and washed with 10 mM Tricine-KOH (pH 7.5) and 2 mM EDTA by being centrifuged at 40 000 × *g* for 30 min at 4°C, and were then dissolved directly in the loading buffer [10]. Protein concentration was measured by an assay kit (Bio-Rad). Pea etioplasts and spinach chloroplasts were isolated and their proteins were similarly fractionated.

2.5. *Immunoblotting*

Proteins were separated by SDS-PAGE and electrophoretically blotted onto a nitrocellulose membrane [16]. The blots were probed with IgG (10 μg/ml) followed by a 1:3000 dilution of goat anti-rabbit IgG conjugated to peroxidase (Bio-Rad), and developed using an immunostaining system (Konica) or an ECL-kit (Amersham). In competition experiments, blots were incubated with anti-βgal-231 IgG (10 μg/ml) in the presence of β-galactosidase or fused βgal-231 protein, and analyzed with an ECL-kit (Amersham).

2.6. *Enzyme assays*

Acyl-CoA synthetase activity was measured as described in [17]. ATPase activity was measured as described in [18]

2.7. *Two-dimensional gel electrophoresis*

Proteins from the crude envelope fraction were made soluble in 2.2% SDS and treated as described elsewhere [19]. The sample was separated as described elsewhere [20,21].

3. RESULTS

3.1. *Preparation of fused protein*

We fused the ORF 231 gene into the heat-inducible *cro-lac Z* sequence in an expression vector, pUEX2 [7]. The fused protein, βgal-231 protein, was produced in *Escherichia coli* as inclusion bodies. The inclusion bodies were isolated and electrophoresed on SDS-PAGE. A protein with a molecular mass of about 140 kDa was a major component of the inclusion bodies (Fig. 1, lane 1), and the 140-kDa protein was purified (Fig.1, lane 2). The estimated molecular mass of the fused protein is 144,782 and the 140-kDa protein seems to be the βgal-231 protein. To confirm that the fused protein has the amino acid sequence of ORF 231, we prepared antibodies against an oligopeptide of 18 amino acid residues that is present in a hydrophilic region of ORF 231. The antibodies reacted with the purified 140 kDa-fused protein but not with the β-galactosidase (Fig. 1, lanes 4 and 3), indicating that the fused protein contained the amino acid sequence of ORF 231. This fused βgal-231 protein was used as an antigen to prepare antibodies in rabbits.

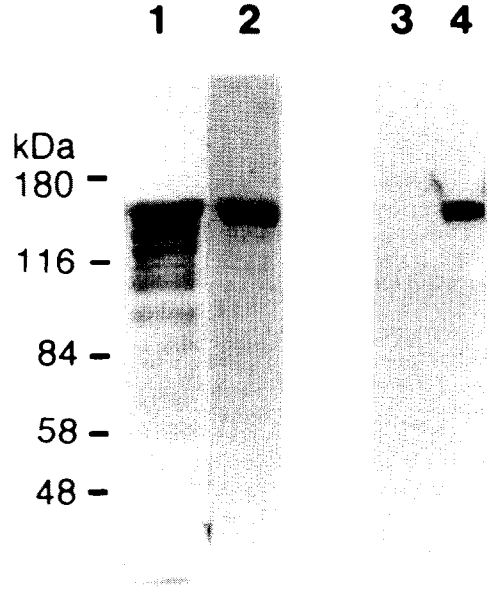

Fig. 1. Immunochemical identification of βgal-231 fused protein. Inclusion bodies (lane 1) and purified βgal-231 protein (lane 2), each 20 μg protein, were separated on SDS-PAGE and stained. 100 ng of β-galactosidase (lane 3) and βgal-231 protein (lane 4) were separated on SDS-PAGE and probed with anti-18mer/231 IgG.

The fused βgal-231 protein was a hydrophobic protein and insoluble in water, and we prepared another soluble fused protein containing the ORF 231 protein sequence. The DNA fragment encoding 73 amino acid residues of a hydrophillic region of ORF 231 protein was fused into an expression vector containing the maltose-binding protein. The fused protein, MAL-73 protein, was purified, conjugated to Affigel 10, and used for purification of anti-βgal-231 IgG by affinity chromatography.

3.2. *Antibodies against the fused protein reacted with envelope protein*

Intact chloroplasts were isolated from pea leaves, and stroma, thylakoid and envelope were fractionated. Proteins from stromal, thylakoid and envelope fractions were electrophoresed, blotted, and probed with specific antibodies (Fig. 2). IgG against βgal-231 fused protein (anti-βgal-231 IgG) reacted with a protein in the envelope with the apparent molecular weight of 34,000, but preimmune IgG and anti-βgal IgG did not (Fig. 2A). The 34-kDa protein was found only in envelope but not in stromal and thylakoid fractions. The affinity-purified anti-βgal-231 IgG reacted with the 34-kDa protein about 5–10 times stronger than the IgG before purification, suggesting that the MAL-73 protein and the 34-kDa protein are related. The fused βgal-231 protein competed with the 34-kDa protein (Fig. 2B) for the reaction with anti-βgal-231 IgG, but βgal protein did not. This indicated that ORF 231 protein and the 34-kDa protein are closely related. A protein with the apparent molecular weight of 16000 in the stroma also reacted with anti-βgal-231 IgG, but the fused βgal-231 protein did not compete with this protein (data not shown), suggesting that the 16-kDa protein is not a gene product of ORF 231. The molecular weight of the ORF 231 protein is 27409 [4]. The 34-kDa protein had less mobility during electrophoresis than expected, probably because the protein is extremely hydrophobic or has

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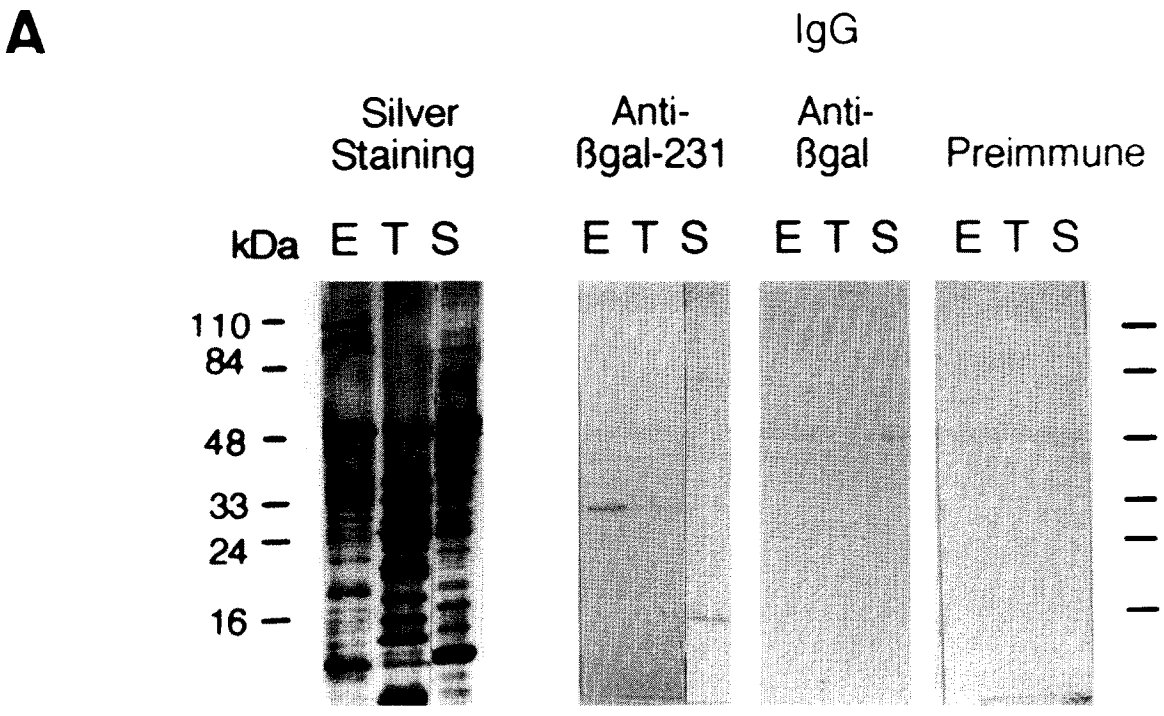
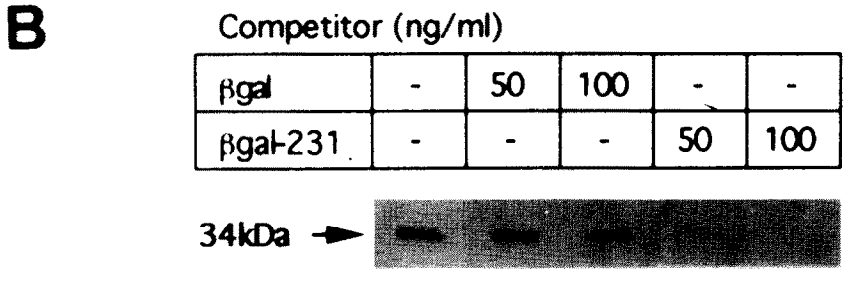
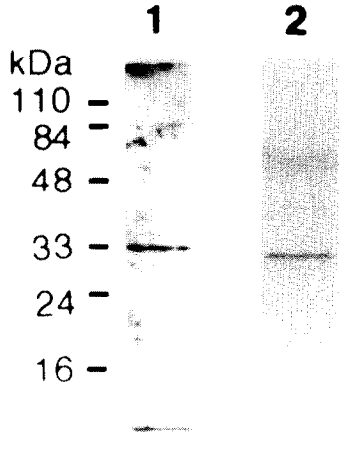
Fig. 2. Immunoblotting of chloroplast proteins. (A) Proteins (30 μg) from fractions of the envelope (E), thylakoid (T) and stroma (S) were probed with 10 μg/ml anti-βgal-231 IgG, anti-βgal IgG or preimmune IgG. For silver staining, 5 μg of protein was used. (B) Envelope proteins (15 μg) were separated by SDS-PAGE and probed with anti-βgal-231 IgG in the presence of the indicated concentrations of a competitor. (C) Proteins (30 μg) from the envelope fraction of pea etioplasts (lane 1) and spinach chloroplasts (lane 2) were probed with anti-βgal-231 IgG.


A
IgG
Silver Staining
Anti-ßgal-231
Anti-ßgal
Preimmune
kDa E T S E T S E T S E T S
110
84
48
33
24
16


B
Competitor (ng/ml)
βgal - 50 100 - -
βgal-231 - - - 50 100
34kDa


1
2
kDa
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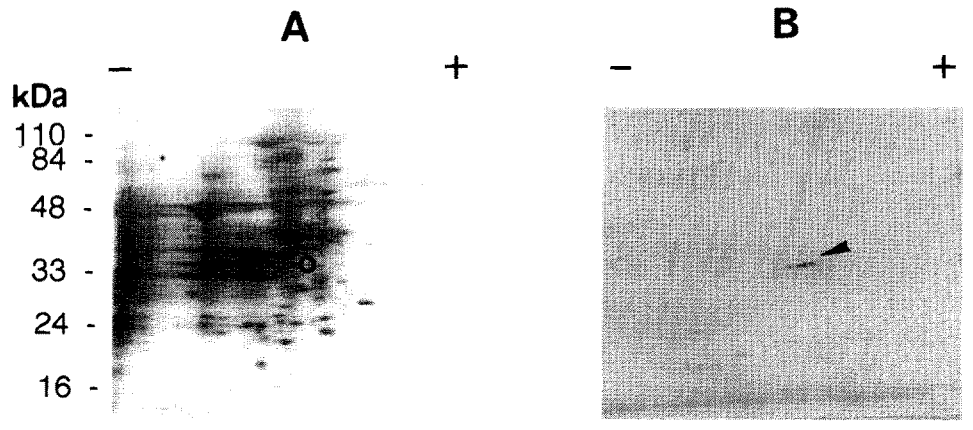

Fig. 3. Immunoblotting of proteins in crude envelope fraction separated by a two-dimensional gel. Proteins (50 μg) from crude envelope fraction were first analyzed with a pH range of 3.5–10 in the isoelectric focussing dimension and then with 12.5% SDS-PAGE gel. (A) Proteins were silver-stained. The position corresponding to CEM A detected by immunoblotting is circled. (B) Proteins were probed with anti-βgal-231 IgG. The arrow indicates the CEM A band.

undergone modification. These results suggest that the 34-kDa protein in envelope is the gene product of ORF 231. This protein may not be processed during translocation into the envelope, and the targeting signal is unclear. We named the ORF 231 gene *cem* A (Chloroplast Envelope Membrane protein A) and the protein, CEM A.

The 34-kDa protein was found in the pea etioplast envelope as well as in the spinach chloroplast envelope (Fig. 2C) and is probably a general protein in a plastid envelope.

When 50 μg of crude envelope proteins were separated on a two-dimensional gel by electrofocussing and SDS-PAGE (Fig. 3), the 34-kDa protein was found in the acidic region but was not specified as a remarkable spot. In stained SDS-PAGE gel, the 34-kDa band was undetectable, and CEM A was a minor component of the envelope.

When the envelope proteins were made soluble with a detergent buffer containing 0.9% cholate, CEM A was found in fractions with the apparent molecular mass of 100–150 kDa by Sephacryl A 300 chromatography (data not shown). This size was also confirmed by glycerol gradient centrifugation (data not shown). CEM A may be present as a complex in the envelope.

3.3. *CEM A was found in the fractionated inner envelope*

We fractionated the envelope into inner and outer envelopes by sucrose gradient centrifugation. The activities of the marker enzymes, ATPase for the inner envelope and acyl-CoA synthetase for the outer envelope, showed that the inner-envelope marker-protein was present in the fractionated inner envelope only but the outer-envelope marker-protein was present in both fractions, which indicated that outer envelope fraction was not contaminated but the inner envelope fraction was contaminated with the outer envelope (Fig. 4B). The 34-kDa band was not found in the outer envelope fraction, indicating that CEM A was present in the inner envelope when fractionated (Fig. 4A).

When isolated chloroplasts were treated with trypsin (200 μg/mg of chlorophyll), no change in the mobility of 34-kDa protein during electrophoresis was observed (data not shown). This result suggests that the exogenous trypsin is not accessible to CEM A in the intact chloroplast under the conditions tested and that CEM A is not exposed on the chloroplast surface.

4. DISCUSSION

The chloroplast genome has limited coding capacity. A number of genes encoding proteins of known functions in the stroma and thylakoid have been identified from their complete DNA sequences [1–3]. However, no gene that codes for an envelope protein has been identified, and this is the first report of an envelope protein encoded by the chloroplast genome. We immunochemically detected the gene product of ORF 231 using one of the antibodies, and the finding was supported by the accompanying several experiments.

In the progress of our experiment, the ORF 231 was reported to probably encode a haem-binding polypeptide, possibly a *b*-type cytochrome, from the partial sequence similar to cytochrome *b* from chromaffin granules and neutrophil membranes, and was suggested to be a component of the thylakoid membrane [4]. But such a gene product has not yet been found in thylakoid membrane. Two membrane-spanning regions (amino acid residues 106–125 and 151–170) are depicted in the predicted topology [4]. The KKD method [5] is the best among the currently available programs for delineating membrane-spanning regions according to the comparative investigation of Fasman and Gilbert [22]. The four membrane-spanning domains predicted by the KKD

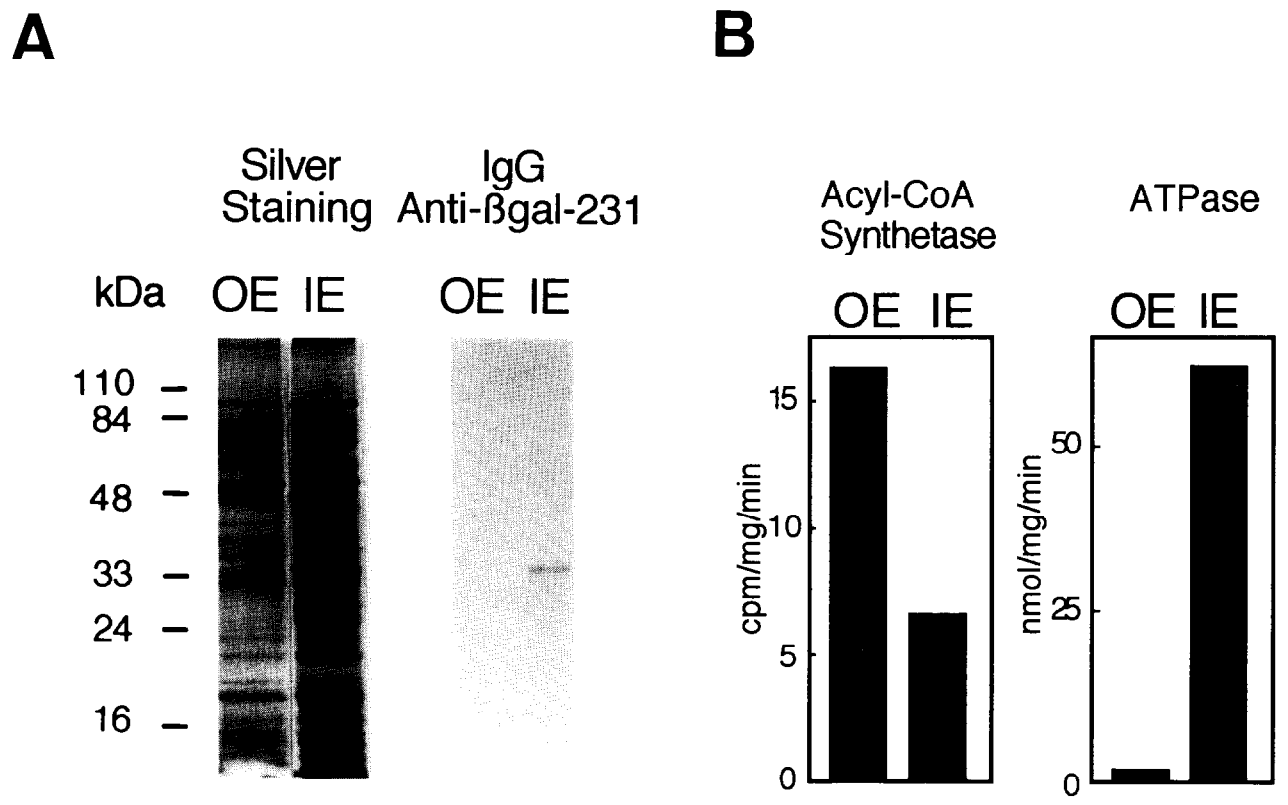

Fig. 4. Immunoblotting of proteins in fractionated inner and outer envelope. (A) Proteins (5 μg) from isolated outer envelope (OE) and inner envelope (IE) were silver-stained, and the proteins (30 μg) were probed with anti-βgal-231 IgG. (B) The activities of marker enzymes (acyl-CoA synthetase for the outer envelope and ATPase for the inner envelope) in isolated outer and inner envelope fraction were measured.

method are 17 amino acid residues from 7 to 23, from 106 to 122, from 148 to 164 and from 189 to 205, respectively. Our results do not agree with the proposed localization and topology.

Despite the importance of chloroplast envelope proteins, little is known about these proteins because of their scarcity. Recently a few genes encoding envelope proteins have been isolated: two outer membrane proteins, a 14-kDa protein from pea [23] and a 6.7-kDa protein from spinach [24], and two inner membrane proteins, a 37-kDa protein from spinach [25] and a phosphate translocator [26]. The function of these genes, other than phosphate translocator, are unknown. The sequence homology of CEM A is 48–72% among different species and is not as high a value as those in photosynthesis-related components, such as *rbc*L and *psb*A. Taken together, CEM A may play an important role in plastid maintenance and intracellular communication. Further experiments are needed to characterize chloroplast envelope proteins to understand chloroplast biogenesis.

Acknowledgements: We thank Akira Watanabe and Tomokazu Konishi for their valuable comments and suggestions. This work was supported by grants from the Japanese Ministry of Education, Science and Culture, and from the Inamori Foundation.

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