Anabaena flos-aquae and other cyanobacteria possess diadenosine 5',5'''-P¹,P⁴-tetrphosphate (Ap₄A) phosphorylase activity

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Diadenosine 5',5'''-P¹,P⁴-tetrphosphate (Ap₄A) phosphorylase, previously only known in lower eukaryotes, has been detected in extracts of the cyanobacteria *Anabaena flos-aquae*, *Anabaena variabilis* and *Synechococcus* sp. The 32 kDa enzyme was partially purified from *A. flos-aquae* and separated from a 23 kDa hydrolytic activity. It had a pH optimum of 9.5 and required a bivalent cation for activity: Mg²⁺, Mn²⁺, Ca²⁺, Co²⁺ or Zn²⁺. Diadenosine tri-, tetra- and penta-phosphates were all effective substrates (relative rates 0.85, 1.00 and 0.27 respectively), while the hexaphosphate was a poor substrate and the diphosphate

was inactive. ADP was always one of the products of phosphorolysis. Arsenate and vanadate could substitute for phosphate (relative rates 1.80, 2.25 and 1.00 respectively), but tungstate and sulphate could not. Chromate and molybdate were poor substrates. A search of the GenBank non-redundant database revealed a putative Ap₄A phosphorylase gene in the cyanobacterium *Synechocystis* sp. The gene showed significant blocks of identity/similarity with yeast Ap₄A phosphorylases I and II, particularly the latter.

INTRODUCTION

The nucleotide diadenosine 5',5'''-P¹,P⁴-tetrphosphate (Ap₄A) is found in the cytosol of all prokaryotes and eukaryotes in concentrations ranging from 0.05 μM to approx. 10 μM [1,2]. It, and related dinucleoside polyphosphates such as adenosine 5'-guanosine 5'-P¹,P⁴-tetrphosphate and diadenosine 5',5'''-P¹,P³-triphosphate (Ap₃A), are synthesized primarily by aminoacyl-tRNA synthetases by the addition of the AMP moiety of an aminoacyl-AMP to an acceptor nucleotide [3,4]. The secretory granules of certain specialized cell types, such as platelets, chromaffin cells and neurons, also contain appreciable quantities of diadenosine nucleotides which are released extracellularly upon degranulation [5–7]. While there is now clear evidence for various extracellular signalling functions for these compounds, their possible intracellular roles remain unclear. In many cases, particularly in prokaryotes, their concentrations increase after exposure of cells to various forms of molecular stress (heat, oxidative, nutritional, DNA damage) and they have, therefore, been implicated in the regulation of the cellular response to such stresses [8–11]. Other evidence has supported a role for Ap₄A in DNA replication in eukaryotes [1,12]. Alternatively, these dinucleotides may be inevitable by-products of protein synthesis which must be degraded before they attain levels which could prove toxic to the cell through their inhibition of essential enzymes such as adenylate kinase [13,14] and protein kinases [15–17].

One approach to understanding the intracellular role(s) of these dinucleotides is to study the enzymes responsible for their degradation [18]. Higher eukaryotes (vertebrates, invertebrates and plants) have a small (17–21 kDa) Ap₄A hydrolase (EC 3.6.1.17) which cleaves Ap₄A asymmetrically to give AMP plus

ATP. The human [19] and pig [20] enzymes have recently been cloned and sequenced, and shown to possess the MutT nucleotide pyrophosphohydrolase sequence motif. Among the lower eukaryotes, the phylogenetic picture is somewhat confusing. The acellular slime mould *Physarum polycephalum* has a symmetrically cleaving Ap₄A hydrolase (EC 3.6.1.41) which generates two molecules of ADP [21], while the fission yeast *Schizosaccharomyces pombe* has a dimeric (49 kDa) asymmetrically cleaving Ap₄A hydrolase whose properties and sequence are quite unlike those of the enzyme from higher eukaryotes [22]. Indeed, this enzyme may be related to the 'specific' Ap₃A hydrolase detected in *Saccharomyces cerevisiae* [23]. Recently, the gene for the probable human homologue of this enzyme, the FHIT protein, has been shown to be defective in a wide variety of common renal, digestive tract and lung cancers [24,25], suggesting that increased levels of Ap₃A and/or Ap₄A may indeed be highly detrimental to the cell. Most other lower eukaryotes examined have a quite distinct Ap₄A-metabolizing enzyme: a reversible Ap₄A phosphorylase (ADP:ATP adenylyl-transferase; EC 2.7.7.53) which catalyses the reaction Ap₄A + P_i ↔ ATP + ADP [26–29]. *S. cerevisiae* has, in fact, two phosphorylases (I and II; 36–38 kDa), both of which have been cloned and shown to be distinct gene products sharing 60% sequence identity [30], while some organisms, e.g. the green alga *Scenedesmus obliquus*, appear to have both specific hydrolytic and phosphorolytic activities capable of degrading Ap₄A and related nucleotides, in addition to an Ap₃A hydrolase [28].

Among the bacteria, the picture has so far appeared more straightforward. *Escherichia coli* and related enterobacteria have a symmetrically cleaving 33 kDa Ap₄A hydrolase, the product of the *apaH* gene [31,32], which is related to the eukaryotic protein serine-threonine phosphatases [33]. A similar activity is present

Abbreviations used: Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetrphosphate; Ap₂A, diadenosine 5',5'''-P¹,P²-diphosphate; Ap₃A, diadenosine 5',5'''-P¹,P³-triphosphate; Ap₅A, diadenosine 5',5'''-P¹,P⁵-pentaphosphate; AMPS, adenosine 5'-phosphosulphate; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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in the Gram-negative *Acidaminococcus fermentans* and the archaeon *Pyrodictum occultum* [31]. *E. coli* 5'-nucleotidase [34] and NADH pyrophosphatase [35] are also able to hydrolyse diadenosine nucleotides; however, no phosphorolytic activity has so far been detected in a prokaryote. Here we demonstrate the presence in cyanobacteria of an Ap_4A phosphorylase with properties similar to those of the eukaryotic enzymes.

This work has been reported in a preliminary form [36].

EXPERIMENTAL

Materials

PMSF, benzamidine, E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] and all nucleotides were from Sigma, and Ultrogel AcA44 was from Réactifs IBF. Buffer A was 30 mM Hepes/KOH, pH 8.0, 5 mM EDTA, 1 mM PMSF, 1 mM benzamidine and 5 μM E-64. Buffer B was 30 mM Hepes/KOH, pH 8.0, 50 mM KCl, 1 mM 2-mercaptoethanol and 0.1 mM EDTA. Buffer C comprised 20 mM Tris/HCl, pH 8.0, and 10% (v/v) glycerol.

Cells and culture conditions

Small-scale cultures of *Anabaena flos-aquae* CCAP1403/13A, *Anabaena variabilis* PCC 7118 and *Synechococcus* sp. PCC 7942 (*Anacystis nidulans*) were grown at 30 °C in BG11 medium [37] in an illuminated orbital incubator gassed with 1–2% (v/v) CO_2 in air, and harvested by centrifugation at 7000 *g* for 30 min at 20 °C. Bulk cultures of *A. flos-aquae* were grown in BG11 medium in a 10 litre fermenter at 30 °C, stirred at 190 rev./min. Light was provided by 61 cm (24 inch) warm white fluorescent tubes surrounding the vessel, 6 cm from its surface. The number of tubes illuminating the vessel was increased gradually from two, when the cultures were first inoculated, to a maximum of 16 when the cultures had reached a high cell density. Cultures were gassed initially with air at approx. 100 ml/min and later with 1% (v/v) CO_2 in air at 100 ml/min, increasing to 800 ml/min at high cell densities. Cells were harvested as above and frozen at –70 °C until use.

Analysis of cyanobacterial cell extracts

Samples of frozen cells (2–3 g) were resuspended in 3 vol. of buffer A and broken by two passages through a French pressure cell at 120 MPa. Homogenates were centrifuged at 150 000 *g* for 1 h and the supernatants were concentrated 10–15-fold by dialysis against solid sucrose at 4 °C. Samples (0.5 ml) were applied to a HiLoad 16/60 Superdex 75 column in buffer B, and fractions were analysed for Ap_4A hydrolase and Ap_4A phosphorylase activities.

Partial purification of Ap_4A phosphorylase from *Anabaena flos-aquae*

Frozen cells (48.7 g wet wt.) were thawed, and a crude homogenate and cell-free extract were prepared as described above. The high-speed supernatant was concentrated by dialysis against solid sucrose and applied at 80 ml/h to a 95 cm $\times$ 5 cm column of Ultrogel AcA44 in buffer B. The hydrolase and phosphorylase activities eluted close together. Active fractions were pooled, precipitated with $(\text{NH}_4)_2\text{SO}_4$ (70% saturation), redissolved in 10 ml of buffer C and dialysed overnight against buffer C. The material was then applied in three separate portions to a Mono Q HR10/10 column (Pharmacia), and each was eluted with a 50 ml linear gradient of 0–0.4 M NaCl in buffer C. Phosphorylase

Table 1 Purification of Ap_4A phosphorylase from *A. flos-aquae*

Ap_4A phosphorylase was purified from 48.7 g of cells as described in the Experimental section and assayed using the luminescence assay [28]. One unit of enzyme activity is the amount that degrades Ap_4A at the rate of 1 $\mu\text{mol/min}$. The data are from a single purification which was representative of three individual purifications.

Fraction	Protein (mg)	Specific activity (m-units/mg)	Purification (fold)	Activity yield (%)
Crude extract	1125	0.18	1	100
Ultrogel AcA44	164	1.0	5.6	81
Mono Q	15.2	10.2	57	77
Superdex 75	3.2	37.0	206	59

and hydrolase activities were completely separated, eluting at 0.26 M and 0.35 M NaCl respectively. Fractions containing phosphorylase activity from each run were pooled, concentrated to 2 ml by dialysis against solid sucrose and then applied to a HiLoad 16/60 Superdex 75 column in buffer B at 1 ml/min. Active fractions were pooled, concentrated and stored at –20 °C in 50% (v/v) glycerol. The final fraction was free of Ap_4A hydrolase and nucleotide phosphatase activities, but did contain adenylate kinase as a contaminant. Attempts to purify the phosphorylase further resulted in substantial losses of activity. The purification is summarized in Table 1.

Other methods

Ap_4A hydrolase and phosphorylase activities were assayed by luminometry and by HPLC as previously described [28,38]. Diadenosine 5',5'''- P^1, P^5 -pentaphosphate (Ap_5A ; 0.1 μM) was included in all assays to inhibit contaminating adenylate kinase activity. Control experiments showed that this concentration was completely effective and did not interfere with the interpretation of the results. Protein concentrations were measured by the Coomassie Blue binding method [39]. Molecular masses of the native enzymes were determined by gel filtration on a HiLoad 16/60 Superdex 75 column (16 mm $\times$ 600 mm; Pharmacia) calibrated with molecular mass standards [28].

RESULTS

Demonstration of Ap_4A phosphorylase activity in cyanobacteria

When crude protein extracts of *A. flos-aquae*, *A. variabilis* and *Synechococcus* sp. were analysed by high-performance gel filtration on a calibrated Superdex 75 column, both hydrolytic (phosphate-independent) and phosphorolytic (phosphate-dependent) activities that could degrade Ap_4A were detected (Figure 1). No Ap_4A phosphorylase could be measured when a similar extract of *E. coli* was analysed (results not shown). The calculated native molecular masses for the hydrolases and phosphorylases were 23 and 32 kDa respectively for *A. flos-aquae* and *A. variabilis*, while *Synechococcus* sp. had a 39 kDa phosphorylase and two hydrolytic activities at 48 kDa and 70 kDa.

In order to determine whether the observed phosphorolytic activity was due to an enzyme similar to the well characterized yeast Ap_4A phosphorylases, the enzyme from *A. flos-aquae* was purified further (Table 1). Although not homogeneous, the phosphorylase was freed from contaminating hydrolase activity (which was not characterized further), while residual adenylate kinase (which might interconvert the primary products) was

Figure 1 Demonstration of Ap₄A phosphorylase and Ap₄A hydrolase activities in cyanobacterial extracts by gel filtration

Crude extracts (0.5 ml) were applied to a HiLoad 16/60 Superdex 75 column and the column was run at 1 ml/min in buffer B. Fractions (1 ml) were collected and 12.5 μ l samples assayed for Ap₄A phosphorylase (●) and Ap₄A hydrolase (○) activities using the luminescence assay (50 μ l total). Phosphorylase assays contained 5 mM potassium phosphate. For fractions containing both hydrolase and phosphorylase activities, phosphorylase activity was calculated from the difference in rates before and after the addition of phosphate to the assay. The column was calibrated with the following molecular mass standards as previously described [28]: conalbumin (77 kDa), BSA (67 kDa), ovalbumin (45 kDa), β -lactoglobulin (36.8 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen A (25 kDa), soybean trypsin inhibitor (20.1 kDa), myoglobin (17 kDa) and cytochrome *c* (12.4 kDa).

Table 2 Nucleotide substrate specificity of Ap₄A phosphorylase from *A. flos-aquae*

Reaction rates (nmol of nucleotide degraded/min per mg of protein) were determined in triplicate by HPLC with 100 μ M nucleotide, 5 mM potassium phosphate and 0.1 μ M Ap₂A as previously described [28]. Products were identified from the retention times of authentic standards, except for p₅A (adenosine 5'-pentaphosphate), whose identity was assumed from its increased retention time relative to p₄A (adenosine 5'-tetraphosphate). Rates are also expressed relative to that obtained with Ap₄A. Abbreviation: Ap₆A, diadenosine 5',5'''-P¹,P⁶-hexaphosphate.

Substrate	Reaction rate		Products
	Actual (nmol/min per mg)	Relative (%)	
Ap ₂ A	0	0	—
Ap ₃ A	25.8 $\pm$ 1.5	85	ADP
Ap ₄ A	30.4 $\pm$ 1.8	100	ADP + ATP
Ap ₅ A	8.2 $\pm$ 2.0	27	ADP + p ₄ A
Ap ₆ A	0.9 $\pm$ 0.3	3	ADP + p ₅ A
AMP	0	0	—
ADP	0	0	—
ATP	0	0	—

completely inhibited by the inclusion of 0.1 μ M Ap₅A in all assays.

Properties of the enzyme

The *A. flos-aquae* Ap₄A phosphorylase degraded adenine dinucleotides with three or more bridging phosphates in a phosphate-dependent reaction (Table 2). ADP was always one of the products, and the *K_m* for Ap₄A was 0.6 μ M. Mononucleotides and diadenosine 5',5'''-P¹,P²-diphosphate (Ap₂A) were resistant, while activity directed against NAD⁺ was not phosphate-dependent and probably represented a contaminant (results not shown). These results are consistent with the proposed mechanism for the *S. cerevisiae* Ap₄A phosphorylases, in which P_i displaces the AMP from a covalent enzyme-AMP complex with the formation of ADP in each case [40].

The enzyme had an alkaline pH optimum of around 9.5, and was completely dependent on a bivalent cation for activity:

Table 3 Anion substrate specificity of Ap₄A phosphorylase from *A. flos-aquae*

Reaction rates (nmol of Ap₄A degraded/min per mg of protein) were determined by the luminescence assay in the presence of 5 mM anion and 20 μ M Ap₄A [28]. Rates are also expressed relative to that obtained with phosphate. Control assays revealed no significant inhibition of luciferase under these conditions. Values are means $\pm$ S.E.M. from three determinations.

Anion	Reaction rate	
	Actual (nmol/min per mg)	Relative (%)
None	0	0
Phosphate	29.6 $\pm$ 2.5	100
Arsenate	53.9 $\pm$ 1.6	182
Vanadate	66.6 $\pm$ 6.2	225
Chromate	2.4 $\pm$ 0.7	8
Molybdate	0.6 $\pm$ 0.2	0
Tungstate	0	20
Sulphate	0	0

Mg²⁺, Mn²⁺, Ca²⁺, Co²⁺ and Zn²⁺ all promoted degradation of Ap₄A. It was also totally dependent on a bivalent anion for anionolysis (Table 3). As expected, in the cases of arsenate and vanadate, the resulting NMP-anion intermediates were unstable and were broken down to give AMP and ATP as the final products of Ap₄A anionolysis [26,41].

Sequence of a putative Ap₄A phosphorylase gene from *Synechocystis* sp.

While the present work was in progress, a search of the GenBank non-redundant database using the NCBI BLAST server revealed the presence of a hypothetical 33.9 kDa protein product of the sl10509 gene contained within the SYCSLLH locus of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803 [42] which shows highly significant sequence identity to Ap₄A phosphorylases I (Apa1) and II (Apa2) from *S. cerevisiae* [26,27,29] and Ap₄A phosphorylase II from *Kluyveromyces lactis* [43] (Figure 2). Three blocks of identity/similarity are apparent:

Figure 2 Sequence alignments between the putative *Synechocystis* sp. *Ap*₄*A* phosphorylase, *S. cerevisiae* *Ap*₄*A* phosphorylases I and II (*Apa1* and *Apa2*) and *K. lactis* *Ap*₄*A* phosphorylase II

Alignments were performed using the program CLUSTAL W 1.6 [44]. The three 'homology blocks' (shaded) were considered significant using the BLOSUM62 comparison matrix and the SIM alignment tool on the ExPASy World Wide Web server [45]. Numbers along the top of each row refer to the alignment positions, and numbers in the right-hand margin refer to the actual positions in each sequence. Circles indicate amino acid identities and vertical lines indicate amino acid similarities. *K. lac.*, *K. lactis* *Ap*₄*A* phosphorylase II; *Syn.*, putative *Synechocystis* sp. *Ap*₄*A* phosphorylase.

Table 4 Quantitative sequence comparisons between the putative *Synechocystis* sp. *Ap*₄*A* phosphorylase and the *S. cerevisiae* *Apa1* and *Apa2* and *K. lactis* *Apa2* gene products

'Homology blocks' 1 (38 amino acids), 2 (25 amino acids) and 3 (51 amino acids) were scored for identical amino acids and conservative substitutions (similarities).

	<i>S. cerevisiae</i> <i>Apa1</i>		<i>S. cerevisiae</i> <i>Apa2</i>		<i>K. lactis</i> <i>Apa2</i>	
	Identities	Similarities	Identities	Similarities	Identities	Similarities
Block 1	17/38 (45%)	28/38 (74%)	22/38 (58%)	29/38 (76%)	14/38 (37%)	27/38 (71%)
Block 2	14/25 (56%)	21/25 (84%)	15/25 (60%)	23/25 (92%)	15/25 (60%)	22/25 (88%)
Block 3	14/51 (27%)	31/51 (61%)	17/51 (33%)	34/51 (67%)	20/51 (39%)	36/51 (71%)

1, positions 115–152; 2, positions 169–193; 3, positions 280–330 (Figure 2; Table 4). Block 2 shows the greatest similarity and contains sequences believed to be involved in substrate binding and catalysis, including the pair of glycine residues Gly-175 and Gly-179, and the sequence motif H[*RK*]HLQ, which may be related to the histidine triad (HIT) motif found in the distantly related *S. pombe* *Ap*₄*A* hydrolase [22].

DISCUSSION

Although we have not directly correlated the putative *Synechocystis* protein, whose existence is inferred from the *slr0509* gene sequence, with the enzyme activity reported here, it can be concluded from both lines of evidence that certain cyanobacteria express a prokaryotic homologue of the *Ap*₄*A* phosphorylases

previously only detected in lower eukaryotes. In its sequence this enzyme is more closely related to *Apa2* from *S. cerevisiae* and *K. lactis* than to *S. cerevisiae* *Apa1*. In terms of its substrate specificity, it is similar to the enzyme from *Euglena gracilis*, which can also use *Ap*₃*A* [29], but not those from *S. cerevisiae* and *Sc. obliquus*, which cannot. The *Euglena* enzyme is also the only other phosphorylase unable to use tungstate for *Ap*₄*A* anionolysis [18].

It would be premature to conclude that this *Ap*₄*A* phosphorylase is ubiquitous among the cyanobacteria, as so few have been examined. However, it is likely to be widespread, as there are great morphological and biochemical differences between the unicellular *Synechococcus* and the heterocystous, filamentous, *Anabaena* spp. For the same reason, it would also be premature to conclude that the enzyme is unique among prokaryotes to the

cyanobacteria. However, a potential gene is clearly absent from *Haemophilus influenzae*, which does have an *apaH* homologue [46], and *Mycoplasma genitalium* [47], and so far neither the gene nor the activity has been found in *E. coli*. It is unlikely that the presence of the Ap₄A phosphorylase is simply related to the photosynthetic ability of cyanobacteria, as no such activity has yet been found in whole cells or chloroplasts of higher plants [18,28], while it is present in a range of non-photosynthetic fungi and protozoa [28]. Hence, what might its function be? The ability of the yeast Ap₄A phosphorylases to synthesize Ap₄A *in vitro* has highlighted the possibility that these enzymes might act as synthetases *in vivo*. Although measurements of *K_{eq}* at physiological pH and the fact that disruption of both yeast Ap₄A phosphorylase genes leads to an increase in the concentration of intracellular Ap₄A suggest that *in vivo* they normally act as catabolic enzymes [30,48,49], overexpression of *Apal* in *S. cerevisiae* leads to a surprising increase in Ap₄A of up to 20-fold [50]. Thus a synthetic role is not excluded under certain circumstances. In cyanobacteria, such circumstances may include metabolic stress. When *Synechococcus* is subjected to heat or heavy-metal stress, the levels of a number of dinucleoside tri- and tetra-phosphates increase [11]. The relative levels of the tri- and tetra-phosphates are the same in the light and dark when heat stress is applied but, when induced by heavy metals, tetra-phosphates predominate in the dark and triphosphates in the light, something which cannot be simply explained by changes in precursor pool sizes. Although we were unable to demonstrate synthesis of Ap₄A by the *A. flos-aquae* Ap₄A phosphorylase *in vitro* (results not shown), it is possible that this enzyme may contribute to the levels of dinucleotides in stressed cells, through either differential synthesis or differential degradation of dinucleotides synthesized in the light and dark by aminoacyl-tRNA synthetases.

An alternative role that has been suggested for Ap₄A phosphorylase is in sulphur metabolism. Yeast *Apal* can catalyse the phosphorolysis of adenosine 5'-phosphosulphate (AMPS) according to the scheme $\text{AMPS} + \text{P}_i \leftrightarrow \text{ADP} + \text{SO}_4^{2-}$, and the irreversible conversion of ATP and AMPS into Ap₄A and sulphate [49], and it has been suggested that this enzyme may be identical to the previously described ADP sulphurylase [51]. However, in higher plants, ADP sulphurylase appears to be located in the chloroplasts [52], and all such enzymes studied so far have molecular masses in excess of 120 kDa [53]. These facts are inconsistent with any relationship between Ap₄A phosphorylase and ADP sulphurylase.

Analysis of the genomic context of the *sll0509* gene does not provide any significant clues as to the function of the product. The gene is the second of two open reading frames within the SYCSLLH locus that are transcribed in the same direction. The first open reading frame, *sll0508*, encodes a potential product of unknown function, while the DNA immediately upstream of *sll0509* contains potential promoter and ribosome binding sites, suggesting that this gene is transcribed and translated on its own.

The Ap₄A hydrolase activity that was separated from the phosphorylase was not characterized further. Thus we have no details of its specificity, whether it cleaves Ap₄A asymmetrically or symmetrically, and whether it or the phosphorylase is more significant *in vivo* in terms of diadenosine nucleotide metabolism. Not all prokaryotic enzymes with the ability to hydrolyse Ap₄A *in vitro* are specific Ap₄A hydrolases, e.g. 5'-nucleotidase [34] and NADH pyrophosphatase [35]. Indeed, even in those eukaryotes where a duality of Ap₄A-degrading enzymes has been observed, e.g. *Sc. obliquus* [28], the hydrolase may be another member of the MutT family of nucleotide pyrophosphohydrolases rather than a true Ap₄A hydrolase. The relationships among these

various enzymes will only be understood when further sequence information becomes available, while clarification of the function of the cyanobacterial Ap₄A phosphorylase will probably require a genetic analysis in an appropriately amenable organism such as *Anabaena* sp. PCC 7120. Nevertheless, the demonstration of Ap₄A phosphorylase in cyanobacteria should prove to be an important step towards understanding the function of this enzyme and of Ap₄A itself.

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