PiUS (Pi uptake stimulator) is an inositol hexakisphosphate kinase

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Abstract A cDNA cloned from its ability to stimulate inorganic phosphate uptake in *Xenopus* oocytes (phosphate uptake stimulator (PiUS)) shows significant similarity with inositol 1,4,5-trisphosphate 3-kinase. However, the expressed PiUS protein showed no detectable activity against inositol 1,4,5-trisphosphate, nor the 1,3,4,5- or 3,4,5,6-isomers of inositol tetrakisphosphate, whereas it was very active in converting inositol hexakisphosphate ($InsP_6$) to inositol heptakisphosphate ($InsP_7$). PiUS is a member of a family of enzymes found in many eukaryotes and we discuss the implications of this for the functions of $InsP_7$ and for the evolution of inositol phosphate kinases.

Key words: Inositol; Inositol trisphosphate kinase; Inositol hexakisphosphate kinase; Phosphate uptake

1. Introduction

Following the original discovery of the phosphorylation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) by a 3-kinase [1], there has been a huge proliferation of inositol phosphates in animal cells, many of which have at present no defined function (see [2] for a recent review). This proliferation demands the existence of a wide range of inositol phosphate kinases, but apart from the original $Ins(1,4,5)P_3$ 3-kinase and its immediate family (see e.g. [3] for review), only $Ins(1,3,4)P_3$ 5/6-kinase from animals and *Arabidopsis* [4,5] and $Ins(1,3,4,5,6)P_5$ 2-kinase [6] from yeast have been cloned and these show no obvious homology with $Ins(1,4,5)P_3$ 3-kinase. Thus, there remains an unknown number of inositol phosphate kinases to be cloned.

Particularly intriguing amongst the inositol phosphates are the pyrophosphate-containing species, known to exist probably in all eukaryotic cells (see [2] for review). Their metabolism is much more rapid than that of their probable precursor, inositol hexakisphosphate ($InsP_6$), and their intracellular levels can be modulated by, for example, the emptying of Ca^{2+} stores with thapsigargin [7] or by elevation of cAMP [8]. Their cellular function, however, remains enigmatic. One of the phosphatases catalysing their removal has been cloned [9] and the inositol phosphate kinases probably responsible for the synthesis of PP-$InsP_5$ and $(PP)_2$-$InsP_4$ (also known more loosely as inositol heptakisphosphate ($InsP_7$) and $InsP_8$, respectively) have been purified in Snyder's laboratory [10,11]. These are able to transfer inorganic phosphate back to ADP (i.e. the phosphorylation of $InsP_6$ and $InsP_7$ is reversible) and $InsP_7$ and $InsP_8$ may therefore be a form of energy currency similar to ATP, but perhaps with a more specialised role. For example, because they bind with a high affinity to a number of proteins involved in secretion, they may act as a localised energy source to drive this process [10,12].

A few years ago, we cloned a cDNA from a rabbit enterocyte library that caused the stimulated uptake of inorganic phosphate when injected into *Xenopus* oocytes and called it phosphate uptake stimulator (PiUS), but its mode of action was unknown [13]. As we describe below, we have found a significant similarity between part of this protein and the catalytic domain of $Ins(1,4,5)P_3$ 3-kinase and have therefore sought an inositol phosphate substrate for the bacterially expressed PiUS enzyme.

2. Materials and methods

2.1. Materials

[^{3}H]$Ins(1,4,5)P_3$ (33 Ci/mmol) was from Amersham, [^{3}H]$InsP_6$ (21.4 Ci/mmol) was from NEN and [^{3}H]$Ins(3,4,5,6)P_4$ of unknown specific activity was prepared as in [14]. PQE-30 expression vector was from Qiagen, UK. Talon metal affinity resin was from Clontech.

2.2. Bacterial expression and purification of PiUS

A cDNA encoding rabbit PiUS in the pSPORT vector [13] was used as a PCR template with the following primer set: 5′-CGGGATCC-ATGAGCCCAGCCTTCAGG-3′/5′-ACGCGTCGACTCACTCCC-CACTGTCCTCAC-3′. Underlined regions correspond to *Bam*HI and *Sal*I restriction sites, respectively. Digested, gel-purified DNA was cloned into the pQE-30 vector, which incorporates a (His)6 expression tag upstream from PiUS. A 200 ml culture of bacteria (*Escherichia coli*) harbouring this vector was induced overnight with 100 μg/ml IPTG at 18°C. Cells were pelleted at 4°C and resuspended in 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, before sonication and extraction with 0.1% Triton X-100. The extract was centrifuged for 15 min at $13\,000 \times g$. Following this step, almost all of the recombinant protein detectable by Coomassie-stained gels was recovered in the pellet fraction. Bacterial membranes were then extracted in a denaturing buffer containing 8 M urea, 300 mM NaCl and 50 mM sodium phosphate, pH 7.5, before affinity purification of the recombinant protein using the Talon metal affinity resin, according to the manufacturer's instructions. Protein was eluted with denaturing buffer containing 150 mM imidazole and peak fractions were immediately pooled and dialyzed at 4°C overnight against a renaturing buffer (25% sucrose, 0.1% Tween-40, 84 mM HEPES pH 7.5, [15]). The next day, the renatured protein was centrifuged at 13 000 rpm for 30 min, 4°C, and the supernatant of this spin was collected, aliquotted and stored at −20°C. This fraction contained one major protein band of about 57 kDa (Fig. 2) and was used for all inositol phosphate kinase assays.

2.3. Inositol phosphate kinase assays

In our initial experiments, $Ins(1,4,5)P_3$ 3-kinase assays were performed as described [16], using 20 000 dpm [^{3}H]$Ins(1,4,5)P_3$. Initial $Ins(3,4,5,6)P_4$ kinase assays were performed as for $Ins(1,4,5)P_3$ 3-ki-

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nase, using 6000 dpm [^{3}H]Ins(3,4,5,6)P_4. All later assays, including all the assays for InsP_6 kinase, were performed according to [10], in a buffer containing 20 mM HEPES, pH 6.8, 1 mM dithiothreitol, 6 mM $MgCl_2$, 5 mM ATP and 10 000 dpm substrate. Incubations were carried out at room temperature or 37°C, stopped by precipitation of protein with 10% trichloroacetic acid and after centrifugation, the supernatants were extracted three times with diethyl-ether before high performance liquid chromatography (HPLC) analysis.

2.4. HPLC analysis

This was based on the method of Stephens et al. [17], but instead of NaH_2PO_4, we used $(NH_4)_2HPO_4$ buffered to pH 3.8 with H_3PO_4. Stephens [18] has shown that higher inositol polyphosphates elute from Partisil SAX columns with this eluent, with Ins(1,3,4,5,6)P_5 eluting at a concentration lower than 1.0 M. The inositol phosphates were eluted from a 250×4.6 mm Partisil 10-SAX column (Phenomenex), running at 1.0 ml/min, with the following profile (A is H_2O, B is 1.2 M $(NH_4)_2HPO_4$ buffered to pH 3.8 with H_3PO_4): 100% A, 5 min; linear gradient of 0% B to 100% B over 25 min; 100% B, 20 min; 100% A, 10 min. Nucleotide markers (AMP, ADP, ATP) were included and monitored at 254 nm [19] and all samples were spiked with 300 dpm ^{32}P-labelled Ins(1,3,4,5)P_4 prepared as in [20] as an internal marker. Ins(1,3,4,5)P_4 eluted at about 28 min, with InsP_6 eluting at around 40 min. Fractions were collected at 30 s intervals and counted for ^{3}H and ^{32}P by scintillation counting using Packard Ultima-Flo-AP (Groningen, The Netherlands) as scintillant.

2.5. Sequence analysis and phylogeny

Inositide kinase protein sequences were aligned using ClustalW set to the following parameters: pairgap, 0.1; matrix, blosum; gap open and end, 10; gap extension and distances, 0.05. Output was highlighted using MacBoxshade v2.11 for phylogenetic analysis, the probable inositide binding region was located from the initial alignment and other parts of the sequences were trimmed off. This set was then re-aligned in ClustalW before analysis using a distance-based method from the PHYLIP v3.573c software package. Distance matrices were computed in PROTDIST and a least squares method (KITCH) was used to construct the phylogenetic tree. Tree bootstrap confidence values were generated by 100 replications using SEQBOOT and the consensus best tree was obtained in CONSENSE, with the tomato PiUS sequence as the outgroup.

3. Results and discussion

A routine BLAST v2.0 database search for homologues of Ins(1,4,5)P_3 3-kinase revealed that the rabbit PiUS gene showed close similarity with the conserved, catalytic domain (see [3]) of all the known Ins(1,4,5)P_3 3-kinases. This is shown in Fig. 1. Critical residues involved with binding of the Ins(1,4,5)P_3 substrate and ATP [3] also appear to be conserved (see legend to Fig. 1). However, PiUS contains no obvious calmodulin binding site and also does not contain the consensus CaM kinase II site found in the Ins(1,4,5)P_3 3-kinases [21]. PiUS is also sufficiently different from these Ins(1,4,5)P_3 3-kinases that we thought it might phosphorylate an inositol phosphate other than Ins(1,4,5)P_3.

We expressed the enzyme in a bacterial system and purified it as described in Section 2 to give a single band of molecular weight 57 kDa (Fig. 2), similar to the apparent molecular weight of about 60 kDa reported for PiUS [13]. This enzyme preparation was tested for kinase activity against some of its possible substrates. With Ins(1,4,5)P_3 as a substrate, we could detect no activity at 37°C and even when we incubated the enzyme overnight at room temperature, only [^{3}H]Ins(1,4,5)P_3 was recovered, with no InsP_4 or Ins(1,3,4,5,6)P_5 detected when the products were analysed by HPLC (not shown). This showed that PiUS is not an Ins(1,4,5)P_3 3-kinase and it also rules out the most likely alternative, that it might have been one of the Ins(1,4,5)P_3 3/6-kinases that can also apparently phosphorylate their Ins(1,4,5,6)P_4 or Ins(1,3,4,5)P_4 products further into Ins(1,3,4,5,6)P_5. These enzymes have

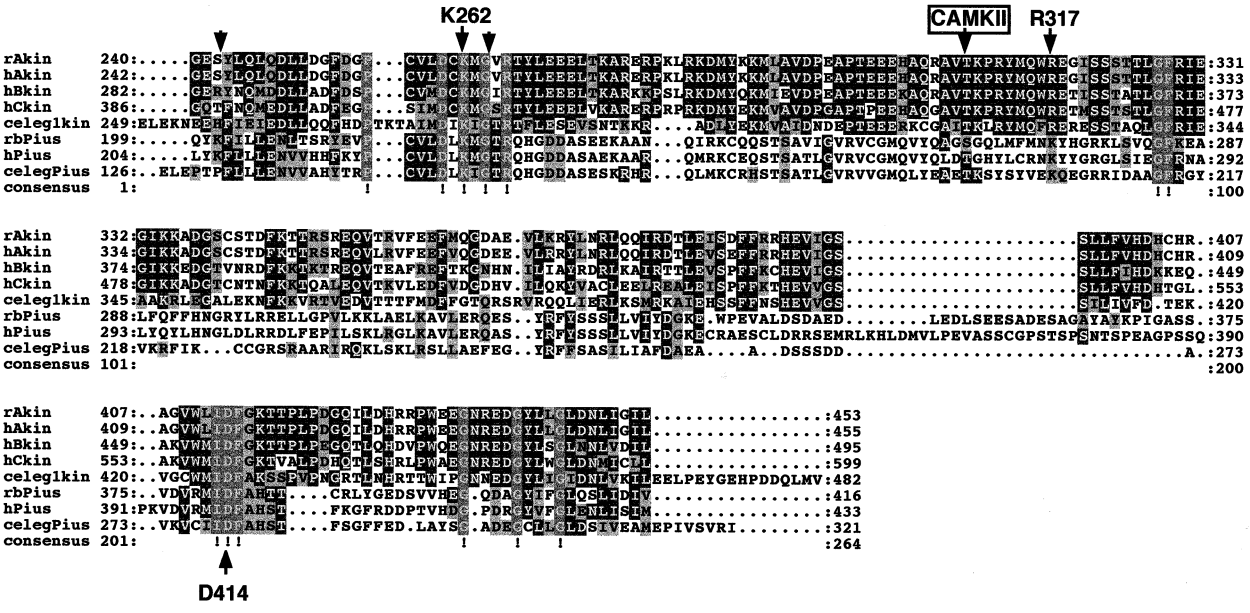

Fig. 1. Interrelationships between Ins(1,4,5)P_3 3-kinases and PiUS homologues. The regions corresponding to the catalytic regions of Ins(1,4,5)P_3 3-kinases were aligned with PiUS family members. Residues conserved among all sequences are indicated by an exclamation point. The arrow at K262 indicates a reside crucial for binding Ins(1,4,5)P_3, while the arrows at R317 and D414 indicated residues crucial for ATP binding (see [3]). The unlabelled arrowheads bracket the sequence corresponding to a conserved exon in the gene structure [27]. Also marked is the threonine that is the substrate for CaM kinase II [21]. Abbreviations (and accession numbers): rAkin, rat Ins(1,4,5)P_3 3-kinase A form (AAA41457); hAkin, human A form (CAA38700); hBkin, human B form (S17682); hCkin, human C form (D38169); celeg1kin, *Caenorhabditis elegans* Ins(1,4,5)P_3 3-kinase spliced form 1 (AAC38960); rbPiUS, rabbit PiUS (AAB49289); hPiUS, human PiUS homolog (BAA13393); celegPius, *C. elegans* PiUS homolog (CAB03023).

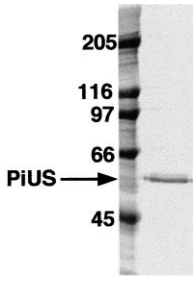

Fig. 2. Expression of PiUS. Recombinant purified PiUS prepared as described in Section 2 was run on an 8% acrylamide/sodium dodecyl sulfate gel and stained with Coomassie blue. Molecular weight markers are indicated.

been reported from yeast [6,22,23], higher plants [24] and probably also *Chlamydomonas* [25], though none of them has yet been cloned. We could detect no activity against [^{32}P]Ins(1,3,4,5)P_4 (not shown) and we should note also that PiUS shows no obvious sequence similarity with the mammalian or *Arabidopsis* Ins(1,3,4)P_3 5/6-kinases [4,5] nor with the recently identified Ins(1,3,4,5,6)P_5 2-kinase from yeast [6].

Another intriguing possibility is that PiUS is an Ins(3,4,5,6)P_4 1-kinase. This enzyme plays an important role in regulating chloride efflux in some tissues by regulating levels of Ins(3,4,5,6)P_4 (see [2] for references), but it has not yet been cloned. It is not inconceivable that Ins(3,4,5,6)P_4 could also have a direct or indirect connection with phosphate uptake, so we investigated this possibility. However, incubation of our PiUS preparations with [^{3}H]Ins(3,4,5,6)P_4 (see Section 2) produced no detectable Ins(1,3,4,5,6)P_5 formation, either at 37°C or overnight at room temperature (not shown).

Another group of possible inositol phosphate kinase(s) remaining to be explored were those involved in synthesis of the pyrophosphate-containing inositol phosphates, InsP_7 and InsP_8 (see Section 1), and when we tested PiUS in this context, we found that it had a robust and very active InsP_6 kinase activity at 37°C. Indeed, incubation overnight at room temperature with InsP_6 under these first order conditions caused a 95% conversion of InsP_6 to InsP_7 (Fig. 3). We show this particular experiment in Fig. 3 because it was carried out in parallel with examples of those overnight incubations described above, in which Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(3,4,5,6)P_4 were completely inactive as substrates, and this illustrates the high specificity of the enzyme for InsP_6. The K_m of the enzyme for InsP_6 was 6.1 ± 0.8 μM and the V_{max} 143 ± 30 pmol/μg/h (both means of three experiments ± S.E.M., data not shown) and an equivalent preparation from vector-transfected *E. coli* showed no InsP_6 kinase activity. The affinity of the enzyme for InsP_6 is lower than that of the enzyme purified from brain by Voglmaier et al. [10] (their reported K_m was 0.7 ± 0.192 μM). However, as with the Ins(1,4,5)P_3 3-kinases [3], it may be that a brain-specific isoform exists and given that the source of PiUS is an intestinal epethlial library [13], we are most likely studying an enzyme from peripheral tissue.

We could not detect any InsP_8 formation at 37°C although we extended the HPLC elution time with 1.2 M $(NH_4)_2HPO_4$. As we have no standard for InsP_8, we cannot be sure of its elution time. However, in overnight incubations as shown in Fig. 3, we were able to recover approximately 95% of the added InsP_6 as InsP_7, so we conclude that PiUS probably does not phosphorylate InsP_7. Note that Huang et al. [11] have purified an InsP_7 kinase as a different enzyme from InsP_6 kinase [10]. Thus, we conclude that PiUS is an InsP_6 kinase and given that its molecular weight is very close to that of the InsP_6 kinase purified by Voglmaier et al. from rat brain [10], we think it is very likely that it is closely related to that activity.

There are two particular implications that stem from the identification of PiUS as an InsP_6 kinase. Firstly, the connection between an InsP_6 kinase activity and the stimulation of inorganic phosphate uptake [13] remains unclear and to be explored. The most likely possibilities are either (a) that InsP_7 (or InsP_8) regulates this process, which must await further exploration by injecting pure InsP_7 made by PiUS into *Xenopus* oocytes, or (b) that the effect on Pi uptake is an indirect consequence of the increased phosphorylation of InsP_6. InsP_6 is found at significant levels in most tissues, estimates varying from a few hundred μM to a few mM (see [2] for review), and if InsP_7 and InsP_8 do function as some form of high energy currency [2,10], a large increase in InsP_6 phosphorylation could well affect cellular phosphate metabolism sufficiently to increase the uptake of radiolabelled inorganic phosphate.

The second interesting conclusion stems from the light it sheds on the evolution of inositol phosphate synthesis and of Ins(1,4,5)P_3 3-kinases in particular. PiUS shows significant homology with the known mammalian Ins(1,4,5)P_3 3-kinases, as well as with a number of other genes found in the databases (see Fig. 1). A dendrogram of the conserved (presumed inositol phosphate binding) regions of these (Fig. 4) suggests that the PiUS enzymes and Ins(1,4,5)P_3 3-kinases form a distinct family, separate from distantly related higher plant enzymes. We could find no sequence homology of PiUS or Ins(1,4,5)P_3 3-kinase in the sequences of the rat Ins(1,3,4)P_3 5/6-kinase [5] or the yeast Ins(1,3,4,5,6)P_5 2-kinase [6] (not shown in Fig. 1). This is in fact an unexpected result, because Ins(1,3,4)P_3 5/6-kinase and Ins(1,3,4,5,6)P_5 2-kinase share with Ins(1,4,5)P_3 3-kinase the property that they phosphorylate hydroxyl moieties on an already partly phosphorylated *myo*-

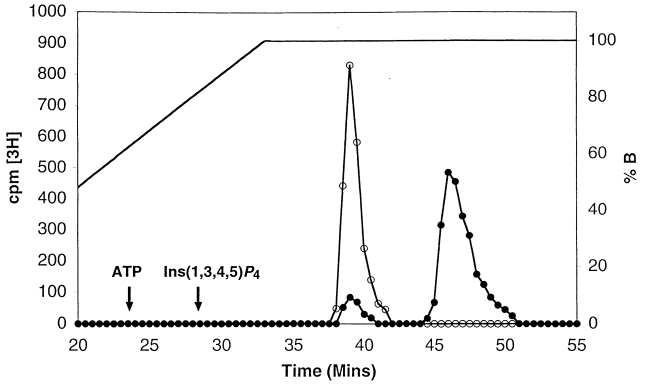

Fig. 3. InsP_6 kinase activity of expressed PiUS. Samples of [^{3}H]InsP_6 were incubated overnight at room temperature in the presence or absence of PiUS protein and aliquots of these incubations were analysed by HPLC. For details of incubation and HPLC analysis, see text and Section 2. Two HPLC runs are shown. Filled circles, presence of PiUS; open circles, absence of PiUS. The gradient profile (A is H_2O; B is 1.2 M $(NH_4)_2HPO_4$, buffered to pH 3.8 with H_3PO_4) is superimposed on the figure and the elution positions of two internal markers, ATP and [^{32}P]Ins(1,3,4,5)P_4, are indicated.

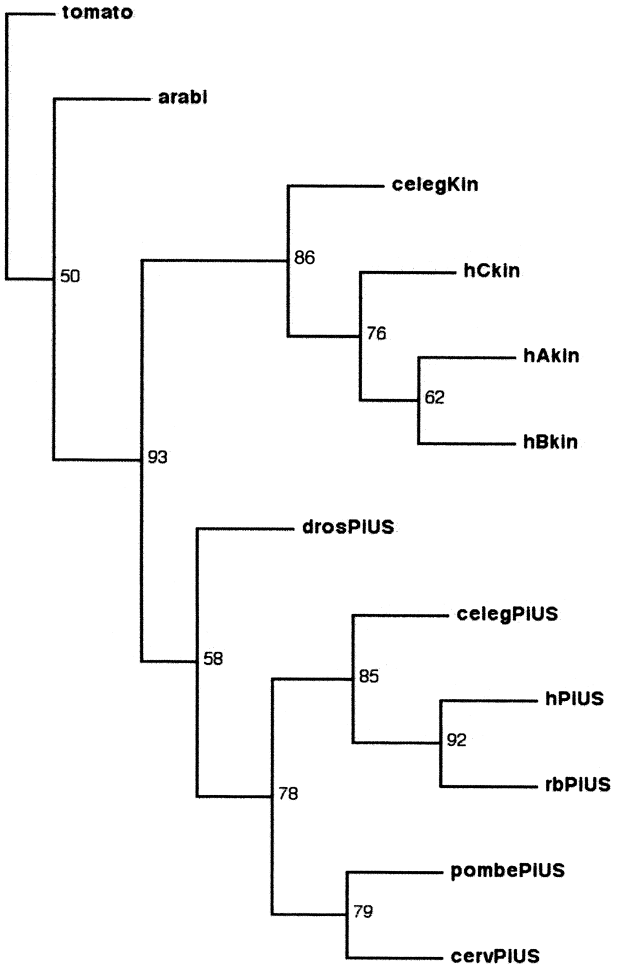

Fig. 4. Phylogenetic relationship between the PiUS family of inositol phosphate kinases and Ins(1,4,5)P_3 3-kinases. The first 100 amino acids of the line-up depicted in Fig. 1 were expanded to include more distantly related genes and then, a dendrogram was constructed and tested as described in Section 2. The numbers located at each node indicate bootstrap support. Abbreviations and accession numbers are the same as in the legend for Fig. 1. Other abbreviations (and accession numbers): tomato, possible homologue from tomato ovary (translated EST247403; AI489064); arabi, probable homologue from *Arabidopsis thaliana* (H36803); pombePiUS, *Schizosaccharomyces pombe* homologue (CAA20701); cervPiUS, *Saccharomyces cervevisiae* homologue (S54640).

inositol ring. Thus, one might have expected them to be very similar, whereas InsP_6 kinase, which is actually phosphorylating a phosphate moiety on a fully phosphorylated *myo*-inositol ring to form a pyrophosphate, might have been expected to be the 'outlier'. However, certainly from the sequences that are currently available, it seems (Fig. 4) that the Ins(1,4,5)P_3 3-kinases evolved from InsP_6 kinases.

InsP_7 and InsP_8 probably appeared early in eukaryotic evolution, they were first discovered in *Dictyostelium* [17], and this would be consistent with an early origin of the PiUS family. Originally, one of us suggested that the appearance of Ins(1,4,5)P_3 3-kinase was also early and preceded the split between plants and animals [26]. However, the *Chlamydomonas* Ins(1,4,5)P_3 kinase [25], whose existence led to this suggestion, can now be seen probably to be akin to the Ins(1,4,5)P_3 3/6-kinases discussed above [6,22,23] in so far as it has a low affinity for Ins(1,4,5)P_3, no detectable regulation by Ca^{2+} and (assuming it is only one enzyme) it converts its InsP_4 product(s) directly into Ins(1,3,4,5,6)P_5. Thus, the 'classic' Ca^{2+}-regulated Ins(1,4,5)P_3 3-kinases [1,3] may now be viewed as not so much a central part of inositol phosphate metabolism, but rather as separate, and late, addition to the repertoire of Ins(1,4,5)P_3 metabolism following the evolution of this molecule as a second messenger.

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