Recombinant ArtinM activates mast cells

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Abstract

Background: Mast cells are hematopoietically derived cells that play a role in inflammatory processes such as allergy, as well as in the immune response against pathogens by the selective and rapid release of preformed and lipid mediators, and the delayed release of cytokines. The native homotetrameric lectin ArtinM, a D-mannose binding lectin purified from Artocarpus heterophyllus seeds, is one of several lectins that are able to activate mast cells. Besides activating mast cells, ArtinM has been shown to affect several biological responses, including immunomodulation and acceleration of wound healing. Because of the potential pharmacological application of ArtinM, a recombinant ArtinM (rArtinM) was produced in Escherichia coli. The current study evaluated the ability of rArtinM to induce mast cell degranulation and activation.

Results: The glycan binding specificity of rArtinM was similar to that of jArtinM. rArtinM, via its CRD, was able to degranulate, releasing β-hexosaminidase and TNF-α, and to promote morphological changes on the mast cell surface. Moreover, ArtinM induced the release of the newly-synthesized mediator, IL-4. rArtinM does not have a co-stimulatory effect on the FcεRI degranulation via. The IgE-dependent mast cell activation triggered by rArtinM seems to be dependent on NFκB activation.

Conclusions: The lectin rArtinM has the ability to activate and degranulate mast cells via their CRDs. The present study indicates that rArtinM is a suitable substitute for the native form, jArtinM, and that rArtinM may serve as an important and reliable pharmacological agent.

Keywords: Mast cells, rArtinM, ArtinM, Degranulation, Lectin

Background

Mast cells are hematopoietically derived cells that reside in the connective tissue [1–5] and play a major role in the immune response in both physiological and pathological processes such as allergy, inflammation, cardiac disease, cancer, autoimmune diseases, and wound healing [6–9]. The activation of mast cells in these processes results in degranulation, release of lipid mediators and cytokines [10]. The mast cell mediators then recruit other cell types including T lymphocytes, neutrophils, and dendritic cells to inflammatory sites [11–13].

Lectins are among the diverse molecules that have been shown to activate mast cells [14–16]. It has previously been shown that the native tetrameric ArtinM (jArtinM), a D-mannose-binding lectin from Artocarpus heterophyllus (jackfruit) seeds, induces the recruitment of rat mast cells from bone marrow to the peritoneal cavity [17], as well as inducing degranulation of rat peritoneal mast cells [11]. In the rat mast cell line RBL-2H3, jArtinM stimulates NFAT (nuclear factor of activated T-cells) and NFKB (nuclear factor kappa-light-chain-enhancer of activated B cells) in an IgE independent manner [18]. In addition to its action on mast cells, jArtinM also recruits neutrophils [19] by binding to glycans of CXCR2 that stimulate signal transduction via G protein [20], thus activating the cells and increasing their phagocytic activity against pathogens [21]. jArtinM also has immunomodulatory activity. Systemic administration of jArtinM confers protection against intracellular parasites such as Leishmania major and Paracoccidioides brasiliensis, by inducing IL-12 production through interaction with TLR2 N-glycans, resulting in a Th1-type immune response [22, 23].

A recombinant form of jArtinM, rArtinM, has been heterologously expressed in Escherichia coli [24, 25].
rArtinM is produced as soluble monomers with its CRDs preserved and active [25]. Furthermore, the binding affinity of rArtinM to the trimannoside Manα1-3 [Manα1-6] Man from HRP, a N-glycosylated protein, is similar to the native form [26]. Additionally, rArtinM showed both prophylactic and therapeutic effects during the course of *P. brasiliensis* infection in mice [27]. The present investigation was undertaken to evaluate if rArtinM, as a monomeric molecule, has the same ability as jArtinM to activate mast cells. In the current study, rArtinM was shown to have the same binding affinity to N-glycans as the native form, jArtinM, and was also able to activate and degranulate mast cells through its CRDs.

**Results**

**Analysis of rArtinM**

The objective of the present study was to characterize the effect of monomeric rArtinM on mast cells. Therefore, it was essential to confirm that rArtinM was indeed monomeric. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare the homogeneity of native and recombinant ArtinM preparations. Under nondenaturing conditions or after thermal dissociation rArtinM exhibited a single protein band of approximately 13 kDa that corresponds to rArtinM monomers (Fig. 1a, lanes 1 and 2). jArtinM, the native tetrameric form, was used as a control. When undenatured jArtinM was loaded onto the gel, a protein band of approximately 60–80 kDa was observed. This band corresponds to jArtinM tetramers (Fig. 1a, lane 3). When jArtinM was submitted to thermal dissociation, a single protein band of approximately 13 kDa, corresponding to the dissociated tetramers (Fig. 1a, lane 4), was observed. These results indicate that *E. coli* expresses a monomeric form of ArtinM. It is also plausible that *E. coli* expresses oligomeric forms of ArtinM but these forms cannot be detected by electrophoresis, since their bonds could be dissociated by exposure to SDS.

jArtinM and rArtinM were also submitted to size exclusion chromatography on a Superdex 75 column, which was calibrated by using protein molecular weight standards. jArtinM presented two distinct peaks, the first with the apparent molecular mass of 42 kDa and the second peak with the apparent molecular mass of 22 kDa, together these two peaks had a molecular mass of 64 kDa (Table 1). This estimate is compatible with previous data from mass spectrometry analysis [28]. rArtinM had the lowest molecular mass, 13 kDa, thus reinforcing the hypothesis that rArtinM is expressed in a monomeric form (Table 1).

Because both of these analytical techniques could result in the dissociation of weakly associated monomers,
Table 1 Estimate of the molecular weight by size exclusion gel filtration chromatography

| Sample       | Kav  | MW    |
|--------------|------|-------|
| jArtinM (peak 1) | 0.1077 | 41.6584 |
| jArtinM (peak 2) | 0.2184 | 22.8336 |
| rArtinM     | 0.3214 | 13.0524 |

jArtinM and ArtinM were submitted to size exclusion chromatography on a Superdex 75 column. Eluted peaks were analyzed by retention volume and molecular weight was calculated by its partition coefficient (Kav). 41.6 kDa (jArtinM peak 1), 22.8 kDa (jArtinM peak 2), and 13 kDa (rArtinM).

an analytical ultracentrifugation assay (AUC) was performed to determine if rArtinM monomers are able to self-associate in solution. The AUC analysis, based on the sedimentation velocity and also on the sedimentation equilibrium, showed that in solution, rArtinM appeared to be trimers, and exhibited a molecular weight of approximately 50 kDa (Fig. 1b). Minor peaks were absent from rArtinM. A previous AUC analysis of jArtinM demonstrated that it has an S value of 4.24 and is a 64 kDa tetramer. However, jArtinM is capable of forming higher order oligomeric species since there were minor peaks at S values of approximately 6, 7, 9, 10 [29]. This is in agreement with previous results using molecular modeling [30]. To understand these differences, crystal trials are being conducted on the two ArtinM forms using the crystallization conditions published for native artocarpin [30].

The molecular weights obtained from the sedimentation equilibrium were confirmed by sedimentation equilibrium, which makes no assumption regarding the hydrodynamic shape of the protein. This analysis shows that, in solution, rArtinM monomers are able to self-oligomerize, mostly into trimers.

jArtinM and rArtinM share carbohydrate binding specificity

It has been previously shown that rArtinM exhibits the same binding affinity for the trimannoside Manα1-3[Manα1-6] Man as does jArtinM. [25, 26] To assess the carbohydrate-binding properties of jArtinM and rArtinM at a higher resolution, glycan microarray analyses was carried out using a panel of 255 lipid-linked oligosaccharide probes representing diverse mammalian glycan sequences and their analogs. Fifty N-glycan related probes were included in the array. jArtinM (Fig. 2a) and rArtinM (Fig. 2b) bound exclusively to the N-glycan sequences, in agreement with published data on the specificity of the native form [31]. There was no significant difference in the specificities of the recombinant and native lectins, suggesting that the features that allow for the modified oligomerization of rArtinM do not affect the three-dimensional structure of its CRD, which is responsible for the glycan binding specificity.

rArtinM induces mast cell degranulation and activation through its carbohydrate recognition domains (CRDs)

The next step was to investigate if the recombinant form of ArtinM would be able to induce mast cell degranulation. A dose–response curve for the release of β-hexosaminidase and TNF-α was performed (Fig. 3). In the absence of IgE, only the higher concentrations of rArtinM were able to release β-hexosaminidase (20 and 40 µg/ml) and TNF-α (40 µg/ml) (Fig. 3a and b). On the other hand, in the presence of IgE, all concentrations of rArtinM induced β-hexosaminidase release (Fig. 3a), and for TNF-α there was a greater release at 10 and 40 µg/ml of rArtinM (Fig. 3b). The ability of rArtinM to induce the production of the newly formed lipid mediator, LTC4, was also examined. The production of LTC4 was observed only in sensitized cells and occurred in a dose-dependent manner (Fig. 3c). The synthesis of the newly-synthesized mediator, IL-4, was also investigated. Only the higher concentrations of rArtinM (20 and 40 µg/ml) induced the synthesis and release of IL-4 either in the presence or absence of IgE (Fig. 3d).

It was then of interest to determine if the degranulation and activation induced by rArtinM was dependent on its CRDs. Therefore, rArtinM (40 µg/ml) was pre-incubated with its specific sugar, D-mannose, and β-hexosaminidase release assay was performed. Pre-incubation of rArtinM with D-mannose abolished the release of β-hexosaminidase, in the absence or presence of IgE (Fig. 4), indicating that mast cell degranulation mediated by rArtinM is dependent on its CRDs. The effect of rArtinM on mast cell activation was also assessed by scanning electron microscopy (SEM). By SEM unstimulated RBL-2H3 cells are spindle shaped and their surface is covered with fine microvilli (Fig. 5). After stimulation via FcεRI the cells have deep ruffles on their surface and are spread over the substrate (Fig. 5). Incubation with rArtinM (40 µg/ml) produced similar changes, which are consistent with mast cell activation (Fig. 5). When rArtinM (40 µg/ml) was pre-incubated with D-mannose, the cells had the same morphological characteristics as the unstimulated cells (Fig. 5). Taken together, these results show that mast cell activation and degranulation induced by rArtinM is mediated by its CRDs.

rArtinM does not have a co-stimulatory role in mast cell degranulation via FcεRI

Since the recombinant form of ArtinM is produced as monomers, it was of interest to investigate if rArtinM could act as a co-stimulatory molecule in the FcεRI signaling pathway. Co-stimulatory molecules act in parallel with a major signaling pathway, either by amplifying the intensity of the stimuli or by extending/prolonging its time-response [32]. For this, rArtinM was incubated for 30 min along with the IgE-TNP specific antigen, DNPα-
HSA, and β-hexosaminidase release assay was performed. Interestingly, when rArtinM was jointly incubated with DNP$_{48}$-HSA, the levels of β-hexosaminidase released were diminished by 37 % (Fig. 6) compared to cells stimulated with DNP$_{48}$-HSA alone, suggesting that rArtinM disrupts the IgE-DNP$_{48}$-HSA binding, probably blocking the interaction between DNP$_{48}$-HSA and IgE.

**rArtinM activates the transcription factor NFkB**

Since rArtinM was able to activate and degranulate mast cells, it was of interest to investigate if rArtinM is activating transcription factors such as NFkB and NFAT. For this, transfected RBL-2H3 cell lines with a GFP-gene reporter for NFkB and NFAT expression were used. rArtinM was able to activate NFkB in an IgE-dependent manner, but rArtinM did not activate NFAT (Fig. 7).

**Discussion**

The present study shows that the recombinant monomeric form of ArtinM, rArtinM, expressed in *Escherichia coli*, has the ability to induce mast cell activation and degranulation. Activation by rArtinM resulted in the release of HSA and β-hexosaminidase.
of preformed, newly formed, and newly-synthesized mediators. Also, mast cell activation triggered by rArtinM results in morphological changes that are characteristic of activated mast cells. Furthermore, mast cell activation induced by rArtinM is dependent on its CRDs. Additionally, the IgE-dependent activation of mast cells triggered by rArtinM is dependent on NFkB activation.

The ubiquitous distribution of mast cells places them in a privileged position to act as sentinel cells, responding rapidly to external signals by releasing their stored preformed mediators and secreting newly-synthesized lipid mediators [10]. One of the principal preformed mediators released during mast cell degranulation is TNF-α [6, 33]. TNF-α is an important chemoattractant for neutrophils and T cells during inflammatory processes [11, 34–36]. Besides TNF-α, leukotrienes also play a role in recruiting neutrophils and T cells to sites of inflammation [7, 37]. The release of TNF-α and LTC4 induced by rArtinM may help explain some of the biological activities attributed to this lectin such as accelerated tissue regeneration [38, 39] and amplified recruitment of neutrophils [11].

It has been shown previously that monomers of rArtinM share their primary structure with the native form of ArtinM (jArtinM), which contributes to its correct folding and exposure of its CRDs, leading to its proper lectin-like activity [25]. This data supports our current findings showing that the ability of rArtinM to activate and degranulate mast cells is dependent on their CRDs.

The fact that rArtinM is expressed as monomers and is able to oligomerize in solution, most likely because of a high monomer concentration, supports our findings, since mast cell activation and degranulation occurred at high concentrations of rArtinM. A similar dose-dependent effect was observed when rArtinM was assayed for its effect on spleen cells. In spleen cells, only high concentrations of rArtinM induced cell proliferation and IL-2 production [40]. Although it shares the sugar-binding specificity of jArtinM, rArtinM differs in its avidity for glycotargets due to its unique quaternary structure. The requirement for high concentrations of rArtinM in order to trigger cellular responses may be associated with its oligomerization upon binding to glycoligands on the cell surface, as has been well established for Galectin 3 [41]. Both IgE [42] and FcεRI [43] are highly glycosylated. IgE contains several mannose residues that could be targets for ArtinM [44, 45]. FcεRI, also presents structural characteristics that could favor its recognition by ArtinM such as several N- glycosylation sites on the FcεRI α subunit [46]. Therefore, rArtinM may be activating mast cells by cross-linking IgE or FcεRI.
However, previous studies have demonstrated that jArtinM is able to degranulate the rat mast cell line, RBL-2H3, as well as peritoneal rat mast cells in an IgE-independent manner [11, 18]. The possibility that rArtinM interacts with other receptors on the mast cell surface, such as TLR4 [6, 47], TLR2 [48, 49], the chemokine receptor CXCR2 [50–52] and complement receptors [7], should not be completely discounted, since degranulation, IL-4 release and the morphological changes on the mast cell surface triggered by rArtinM all occurred in an IgE-independent manner.

It is well established that mast cells can respond in different manners to the same stimulus. For example, FcεRI cross-linking results in NFkB activation leading to mast cell degranulation [53]. However, exposure of IgE sensitized mast cells to low concentrations of specific antigen induces NFAT activation in the absence of degranulation [54]. The same appears to be true for ArtinM. At 10 μg/ml, jArtinM induces mast cell degranulation as well as NFkB and NFAT activation [18], while rArtinM at the same concentration does not induce degranulation. The fact that rArtinM can activate mast cells in a pro-inflammatory manner, without inducing degranulation, makes it an attractive candidate for pharmacological use.

rArtinM was also able to induce IL-4 release. It is known that IL-4 and another cytokines such as, IL-6, VEGF, IL-13 and, TNF-α play a role in allergic inflammatory processes, leading to IgE production by B cells [55, 56]. The ability of rArtinM to induce IL-4 release agrees with our previous data showing that ArtinM can trigger an allergic pro-inflammatory response [11, 18]. However, higher concentrations of rArtinM (20 and 40 μg/ml) were required to trigger responses similar to those observed for jArtinM [18].

Conclusions

In the current investigation, monomeric rArtinM was able to activate and stimulate mediator release by mast cells through its CRDs. The mechanisms by which rArtinM leads to mast cell activation is dependent on the transcription factor NFkB, but not NFAT. These results demonstrate that the mast cell response depends on the nature and concentration of the stimuli. The present study indicates that rArtinM is a suitable substitute for the native form, since it shares some of the biological activities already described for jArtinM. Therefore, rArtinM may serve as an important and reliable pharmacological agent.

Methods

rArtinM preparations

rArtinM was expressed in Escherichia coli BL21- Codon-Plus(DE3)-RP and purified as previously reported [25]. rArtinM preparations containing less than 0.05 ng/ml of bacterial endotoxin, as determined by the Limulus amoeobaocyte lysate assay, were used in this study (Sigma-Aldrich, St. Louis, MO).

Size exclusion chromatography

Native and recombinant forms of ArtinM were submitted to size exclusion chromatography for molecular
weight determination, on a Superdex 75 column (Sigma Aldrich) coupled to an AKTA protein purification system (GE Healthcare, Uppsala, Sweden), which was calibrated by using protein molecular weight standards (Protein Mixture, GE Healthcare). The molecular weight of proteins was determined by partition coefficient (Kav) using this formula: $Kav = \frac{Ve - Vo}{Vt - Vo}$, where $Ve$ is the elution volume of the samples, $Vt$ is the total volume and $Vo$ is the void volume of the gel bed. High molecular weight blue dextran was used to determine the void volume.

**Analytical ultracentrifugation**

Sedimentation velocity measurements were performed using a Beckman XL-A analytical centrifuge equipped with both absorbance and interference optics. All data were acquired at a rotor-speed of 50,000 rpm at 20 °C using a Beckman An60Ti rotor. For each sample, 100 scans were acquired at 120 s intervals. Buffer density and viscosity as well as the partial specific volume of the protein were calculated using SEDNTERP (Alliance Protein Laboratories, Thousand Oaks, CA.).
Glycan array analysis
The native and recombinant ArtinM forms were biotinylated as previously described [38] and quantified by determining their absorbance at 280 nm (OD280). Microarrays were composed of lipid-linked oligosaccharide probes robotically printed in duplicate on nitrocellulose-coated glass slides at 2 and 7 fmol per spot (in-house designation sets 18–21bis) using a non-contact instrument, as previously described [57]. The microarray binding assays of biotinylated ArtinM proteins were performed at 19 °C–20 °C, as previously described [58]. In brief, the slide arrays were blocked with 1 % w/v bovine serum albumin (Sigma Aldrich) in casein blocking solution (Pierce Chemical Co, Thermo Fisher, Waltham, MA) for 1 h. The biotinylated ArtinM (50 μg/mL) was overlaid, and binding was detected with streptavidin conjugated to Alexa Fluor 647 (Molecular Probes, Thermo Fisher, Waltham, MA) at 1 μg/mL in blocking solution. Glycoarray data analysis was performed with dedicated software [59]. The binding signals were probe-dose dependent.

Cells
RBL-2H3 cells, a rat mast cell line, were used in this study [60]. The stable transgenic RBL-2H3 derived cell lines, VB9 and NFκB 2, were also used. The VB9 cell line is a GFP-reporter cell line for NFAT activation [54]. The NFκB 2 cell line is a GFP-reporter cell line for NFκB activation [61], which presents a genome transduction with a reporter vector that possess 4 copies of the binding site for NFκB that regulate GFP expression. All cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Thermo Fisher, Waltham, MA) supplemented with 15 % fetal calf serum (Sigma-Aldrich) as previously described [60]. Transfected cells were

Fig. 7 rArtinM activates NFκB, but not NFAT. rArtinM (40 μg/ml) induced NFκB activation only in the presence of IgE (a), but did not activate the transcription factor NFAT either in the absence or in the presence of IgE (b). Data are expressed as mean ± SEM and are representative of three separate experiments. *** p < 0.001 between samples and the controls
selected with Geneticin (0.4 mg/ml) (Sigma-Aldrich). All cell lines were generously provided by Dr. Reuben P. Siraganian, NIDCR, NIH, Bethesda, MD.

Cell sensitization and stimulation
As a positive control for FcεRI-stimulated cells, the cells were cultured in the presence of a 1:5000 dilution of mouse IgE anti-TNP ascites fluid (kindly provided by Dr. Reuben P. Siraganian) for 16 h, and then stimulated with the multivalent specific antigen DNP4κ-HSA (Sigma-Aldrich) at 50 ng/ml. As positive control for FcεRI-independent stimulation, the cells were incubated with calcium ionophore-A23187 (Sigma-Aldrich) at 0.1 μM for degranulation assays and, at 0.7 μM for NFκB and NFAT activation assays. In experimental conditions, the cells were sensitized or not with IgE anti-TNP ascites fluid (1:5000) for 16 h and then stimulated with rArtinM. In some experiments, rArtinM was preincubated with D-mannose 100 mM (Sigma-Aldrich) for 1 h at 4 °C. To evaluate release of preformed mediators (β-hexosaminidase and TNF-α) and the lipid mediator LTC4, cells were stimulated for 45 min. For scanning electron microscopy, the cells were stimulated for 20 min. For newly-synthesized IL-4, the cells were stimulated for 12 h. For NFκB and NFAT activation, the cells were stimulated for 5 and 17 h respectively.

β-Hexosaminidase activity
3.0 × 10⁴ cells/well were plated in 96 well tissue culture plates (Corning Life Sciences, Lowell, MA) in the absence or presence of IgE and cultured overnight. The cells were washed 2 times with Tyrode's buffer (137 mM NaCl; 2.7 mM KCl; 12 mM NaHCO₃; 0.37 mM NaH₂PO₄; 0.1 mM MgCl₂; 1.3 mM CaCl₂; 10 mM Heps; pH 7.3) supplemented with 0.1 % BSA (Sigma-Aldrich) and 0.01 % gelatin (Sigma-Aldrich) and then incubated with the stimulus diluted in Tyrode's buffer for 45 min as described above in Cell sensitization and stimulation. After stimulation, the supernatants were transferred to clean wells and β-hexosaminidase activity measured as previously described [1]. All assays were run in triplicate.

Leukotriene C₄ and cytokine detection assays
IgE-sensitized or unsensitized cells were incubated with rArtinM for 45 min or 12 h. The concentrations of LTC4, TNF-α and IL-4 in the cell culture supernatants were measured by ELISA (Leukotriene C₄ EIA kit, Cayman Chemical Company, MI, USA; OPTIEA™ Rat TNF ELISA kit II; OPTIEA™ Rat IL-4 ELISA kit II, BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Scanning electron microscopy (SEM)
Cells (3x10⁵) were cultured on 13 mm diameter glass coverslips in 24 well plates (Corning Life Sciences). The cells were cultured in the presence or absence of IgE for 16 h. The cells were then stimulated as described in Cell sensitization and stimulation. The samples were prepared as previously described [62], and were examined with a JEOL JSM-6610 LV scanning electron microscope (JEOL, Ltd.; Tokyo, Japan).

Flow cytometric measurements of NFκB and NFAT activation
NFκB2 and VB9 cells (1x10⁵) were sensitized or not with IgE, and then stimulated. Fluorescence levels were measured using a Guava Personal Cell Analysis-96 System and data were processed by Guava InCyte Software (Millipore Co., Billerica, MA).

Statistics
Data was analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Results were expressed as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Tukey's Multiple Comparison Test. *p < 0.05; **p < 0.01; ***p < 0.001.

Abbreviations
CRD, carbohydrate recognition domain; TNF-α, tumor necrosis factor alpha; IgE, immunoglobulin E; NFκB, nuclear factor kappa B; NFAT, nuclear factor of activated T-cells; RBL-2H3, rat basophilic leukemia cell line; CXCR2, chemokine receptor 2; HRP, horseradish peroxidase; TLR2, toll-like receptor 2; AUC, analytical ultracentrifugation; LTC4, leukotriene C 4; SEM, scanning electron microscopy; FcεRI, high affinity receptor for IgE; VEGF, vascular endothelial growth factor; kD, kilodaltons; MW, molecular weight

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Availability of data and material
The data sets supporting the conclusions of this article are included within the article.

Authors' contributions
VCBL carried out all the experimental assays and analyzed the data. NTC performed the size-exclusion chromatography, AUC and glycoarray analysis. PAAB participated in the design of the study and performed the statistical analysis. MCP and MHSG carried out the expression and purification of rArtinM. GPS and MCRB contributed intellectually with this study and also provided reagents for this study. VCBL, MCI and CO designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.
Consent for publication
Not Applicable.

Ethics approval and consent to participate
Not applicable.

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