Inhibition of Protein Phosphatase-1 by Clavosines A and B

NOVEL MEMBERS OF THE CALYCU LIN FAMILY OF TOXINS*

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Site-directed mutagenesis was used to investigate the mechanism of interaction between the catalytic subunit of human protein phosphatase-1 (PP-1c) and members of the calyculin family of toxins. Clavosines A and B are related to calyculins but are glycosylated with a trimethoxy rhamnose group. We provide experimental evidence implicating Tyr-134 as an important residue in PP-1c that mediates interactions with the calyculins. Mutation of Tyr-134 to Phe, to prevent hydrogen bond formation, resulted in a slight increase in sensitivity of PP-1c to clavosines A and B and calyculin A. In contrast, a Y134A mutant was 10-fold less sensitive to inhibition by all three inhibitors. The greatest effect on inhibition was found by substituting an Asp for Tyr-134 in PP-1c. Inhibition was found by substituting an Asp for Tyr-134 in PP-1c and PP-2Ac (12). This suggests that the phosphate group of the phosphorylated substrate or inhibitor. However, a dephosphorylated analog of calyculin A has been found to strongly inhibit PP-1c and PP-2Ac (12). This suggests that the phosphate group may not play a major role in the interaction of calyculin A with these protein phosphatases. Recently, the NMR solution structures of calyculin A and dephosphonocalyculin A were determined (21). The structure of dephosphonocalyculin A was found to be quite similar to that of calyculin A in both methanol and nodularins from cyanobacteria, and the spiroketal calyculins isolated from marine sponges (3).

The first member of the calyculin family of phosphatase inhibitors was identified in 1986 (4). Calyculin A was originally purified from a hydrophobic extract of the marine sponge Discoeodermia calyx. It was found to be cytotoxic toward P388 and L1210 leukemia cells and to be a strong inhibitor of starfish egg development (4, 5). It was subsequently characterized as a powerful inhibitor of the catalytic subunits of type 1 and 2A protein phosphatases, PP-1c (1) and PP-2Ac, two of the four major eukaryotic serine/threonine protein phosphatases (6). Calyculin A was also shown to have tumor-promoting activity as potent as that of okadaic acid (7). Many additional calyculins (8–13), and the structurally related calyculinamides (11, 14), have since been identified.

Recently we reported the isolation of two novel glycosylated members of the calyculin family, clavosines A and B from the marine sponge Myriastra clavosa (15). Clavosines A and B are potent inhibitors of mammalian PP-1c and PP-2Ac and were found to be more cytotoxic overall than the calyculins and the calyculinamides (14, 15) in the National Cancer Institute (NCI) screening panel of tumor cell lines (16). The polyfunctional structure of clavosines A and B and calyculin A is depicted in linear diagrams (Fig. 1a) and space filling models (Fig. 1b). The clavosines (Fig. 1) have many structural features in common with the calyculins but differ markedly from them in having a methyl group at C-32 and an unusual trimethoxy rhamnose group at position C-21. Like the calyculinamides, and unlike calyculin A, the clavosines possess an amide at the end of their tetraene tail, as found in the calyculinamides (14). Clavosine A differs from clavosine B in having Z (cis) rather than E (trans) geometry at the C-2=C-3 double bond. Despite the large number of calyculins identified and characterized, we lack a detailed understanding of the molecular mechanisms underlying their interactions with the protein phosphatases.

The mode of interaction of calyculin family members with protein phosphatases is controversial (17–20). Several models found in the literature are based on the assumption that the phosphate-containing calyculins bind in the same fashion as a phosphorylated substrate or inhibitor. However, a dephosphorylated analog of calyculin A has been found to strongly inhibit PP-1c and PP-2Ac (12). This suggests that the phosphate group may not play a major role in the interaction of calyculin A with these protein phosphatases. Recently, the NMR solution structures of calyculin A and dephosphonocalyculin A were determined (21). The structure of dephosphonocalyculin A was found to be quite similar to that of calyculin A in both methanol and

Serine/threonine protein phosphatases are highly conserved enzymes, which have been isolated from numerous eukaryotic and prokaryotic organisms (1, 2). Eukaryotic protein phosphatases are inhibited by a structurally diverse array of natural product toxins. These include the polyether okadaic acid from marine dinoflagellates, cyclic peptide microcystins and prokaryotic organisms (1, 2). Eukaryotic protein phosphatases are inhibited by a structurally diverse array of natural product toxins. These include the polyether okadaic acid from marine dinoflagellates, cyclic peptide microcystins and the spiroketal calyculins isolated from marine sponges (3).

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chloroform, implying that the phosphate group is not solely responsible for determining or stabilizing the calyculin structure.

To evaluate the roles played by specific amino acid residues in clavosine binding to PP-1c, we have prepared a series of PP-1c mutants. In this study we have characterized the effects of these mutations on the ability of clavosines A and B and calyculin A to inhibit PP-1c. We have exploited this data to develop structural models that describe features important in mediating interactions between the clavosines and PP-1c.

**EXPERIMENTAL PROCEDURES**

**Materials**—Clavosines were purified as described previously (15). Calyculin A was obtained from Calbiochem. Other reagents were obtained from Sigma unless otherwise stated.

**Expression and Purification of PP-1c Mutants**—The cDNA encoding PP-1c was cloned from a human teratocarcinoma NT2 cDNA library (Stratagene) and subcloned into pBluescriptSK (Stratagene). The PP-1c insert was sequenced in its entirety and compared with the published sequence of the γ-isoform (22). PP-1c mutants were generated using the QuikChange Mutagenesis kit and native *Pfu* polymerase (Stratagene). All mutant constructs were validated by DNA sequencing. Fragments comprising the wild-type and mutant PP-1c cDNA sequences were cloned into the expression vector pCW (generously provided by Dr. Dahlquist, University of Oregon) 17 bases downstream of the promoter and transformed into the *E. coli* strain DH5α. The recombinant PP-1c enzymes (Y134F, Y134A, Y134D, V223A, V223C, I133Y, and C291A) were expressed and purified to homogeneity as described previously (23, 24). Preparations of PP-1c mutants had specific activities comparable to wild-type recombinant PP-1c.

**Protein Phosphatase Inhibition Assays**—Protein phosphatase-1 inhibition was assayed using 10 μM [32P]phosphorylase a substrate as described previously (25). Control phosphatase activity was standardized to 15% release of total phosphate from the substrate for each enzyme. Reactions were initiated by the addition of substrate, and assays were performed in duplicate. Reactions involving the clavosines were performed under low light conditions to prevent photochemical isomerization of the terminal tetraene unit.

**Molecular Modeling Studies**—Structures and binding models were generated on a Silicon Graphics Indigo® Impact 10000 workstation using Insight II (Biosym/MSI Technologies Inc.) on a Silicon Graphics Indigo 2 Impact 10,000 workstation.

The structure of Calyculin A (4) was obtained from the Cambridge Crystallographic Data Center (code DUPSUW). In contrast to previous work published in collaboration with our laboratory (26), this calyculin A structure was inverted to obtain the correct, natural enantiomer (27). Although the absolute configurations of the clavosines have not been determined, they are assumed to be the same as those established for calyculins (15). Charges were adjusted to –2 for the phosphate group, but the dimethylamine was maintained in the free base form (27). The x-ray crystallographic coordinates of PP-1c bound to microcystin-LR (28) were obtained from the Protein Data Bank (PDB code 1FJM). Chain B of the structure was deleted, as was microcystin-LR and all water molecules. To complete the structure, amino acid side chains only partially detected by crystallographic methods were added, as were hydrogens appropriate for pH 7.

Docking was carried out with half of the amino acid residues of PP-1c (those comprising the active site “face” of the protein) designated as the ligand-binding subset. We employed Metropolis docking at a temperature of 300 K with 200 minimization steps between structure evaluations. Several starting positions were investigated for each inhibitor. The lowest energy structures generated were examined and subjected to more rigorous energy minimization using Discover 3. Promising structures were used as the starting position for a second round of
docking. During minimization of the docked models, subsets of the protein were defined so that amino acid residues within 10 Å of the inhibitor were allowed to be flexible, while the rest of the protein was held fixed. The PP-1c-inhibitor complex was soaked with water molecules at a thickness of 5 Å and minimized to a convergence of 0.05 kcal/mol/Å using the steepest descent and conjugate gradient algorithms.

RESULTS

Inhibition of Protein Phosphatases 1c and 2Ac by the Clavosines and Calyculin A—Clavosine A inhibited PP-1c and PP-2Ac was obtained from bovine cardiac muscle; both were purified to homogeneity. PP-1c and PP-2Ac were assayed for activity using 10 μM [32P]phosphorylase a substrate. Dose-response curves for the inhibition of PP-1c and PP-2Ac activity by calyculin A (●), clavosine A (○), and clavosine B (■) are shown. All assays were carried out in duplicate, and each point displayed represents the average of 3–5 independent experiments.

Inhibition of tyrosine 134 PP-1c mutants. Site-directed mutagenesis was employed to substitute Phe, Ala, and Asp residues in place of Tyr-134 in PP-1c, as described under “Results.” Mutant enzymes were expressed in E. coli and purified to homogeneity. Inhibition of protein phosphatase activity was assayed using 10 μM [32P]phosphorylase a substrate. Dose-response curves for the inhibitors (a) clavosine A, (b) clavosine B, and (c) calyculin A are shown for each of the following enzymes: Y134F (●), Y134A (○), Y134D (■), and wild-type PP-1c (x). The x axis is graphed on a logarithmic scale to accommodate the large range of effective concentrations determined. All assays were carried out in duplicate, and each point displayed represents the average of 3–5 independent experiments.

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FIG. 3. Inhibition of tyrosine 134 PP-1c mutants. Site-directed mutagenesis was employed to substitute Phe, Ala, and Asp residues in place of Tyr-134 in PP-1c, as described under “Results.” Mutant enzymes were expressed in E. coli and purified to homogeneity. Inhibition of protein phosphatase activity was assayed using 10 μM [32P]phosphorylase a substrate. Dose-response curves for the inhibitors (a) clavosine A, (b) clavosine B, and (c) calyculin A are shown for each of the following enzymes: Y134F (●), Y134A (○), Y134D (■), and wild-type PP-1c (x). The x axis is graphed on a logarithmic scale to accommodate the large range of effective concentrations determined. All assays were carried out in duplicate, and each point displayed represents the average of 3–5 independent experiments.
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PP-1c mutants were generated as described under "Experimental Procedures." PP-1cγ activity was measured using 10 μM [32P]phosphorylase a as a substrate. Each value (± S.D.) represents the average of 3–5 independent experiments with a full range of inhibitor concentrations.

| PP-1cγ | Calyculin A | Clavosine A | Clavosine B |
|--------|------------|------------|------------|
| Wild-type | 0.7 (± 0.28) | 0.5 (± 0.1) | 13 (± 0.58) |
| Y134F | 0.3 (± 0.06) | 0.2 (± 0.06) | 9.5 (± 3.5) |
| Y134A | 7.0 (± 2.1) | 5.5 (± 0.5) | 145 (± 9.4) |
| Y134D | 125 (± 11) | 93 (± 6.7) | 4050 (± 44) |
| V223A | 0.1 (± 0.05) | <0.1 | 1.0 (± 0.4) |
| V223C | 0.9 (± 0.26) | 0.6 (± 0.10) | 22 (± 3.6) |
| H133Y | 1.2 (± 0.40) | 0.6 (± 0.15) | 15 (± 3.9) |
| C291A | 1.5 (± 0.56) | 0.8 (± 0.25) | 17 (± 2.5) |

13, and 0.7 nM, respectively). In contrast, Y134A was inhibited less potently by clavosine A, clavosine B, and calyculin A (IC50 = 5.5, 145, and 2 nM, respectively). Y134D was significantly resistant to inhibition by clavosine A, clavosine B, and calyculin A (IC50 = 93, 4050, and 125 nM, respectively).

Calcineurin (protein phosphatase-2B) is a protein phosphatase homologous to PP-1 but strongly resistant to inhibition by calyculin A (3). In calcineurin the residue corresponding to Val-223 in PP-1cγ is a cysteine (Cys-256). It was therefore of interest to mutate PP-1cγ Val-223 to Ala and Cys in order to determine whether this residue may be involved in clavosine inhibition of PP-1cγ (Fig. 4).

PP-1cγ V223C was inhibited by clavosine A, clavosine B, and calyculin A with similar potency (IC50 = 0.6, 22, and 0.9 nM, respectively) to wild-type PP-1cγ (IC50 = 0.5, 13, and 0.7 nM, respectively). Surprisingly, V223A was more strongly inhibited by clavosine A, clavosine B, and calyculin A (IC50 = <0.1, 1, and 0.1 nM, respectively) than the wild-type enzyme. These experiments suggest that replacing the more bulky side chain of valine with alanine may allow the calyculins, especially clavosine B, to interact more closely with PP-1c in this region, thus reducing the IC50 values for each inhibitor.

Clavosine Inhibition of Other PP-1cγ Mutants—The clavosines and calyculin A were additionally tested for inhibitory activity against other PP-1cγ mutant enzymes. The results from these studies are depicted in Table I. Despite its proximity in sequence number to Tyr-134, mutation of Ile-133 to Tyr resulted in no significant change in response to the clavosines and calyculin A. We also examined the effect of replacing Cys-291 with Ala on toxin inhibition. This residue was chosen for our mutagenesis studies because it has been proposed (29) to be part of an essential binding motif that is involved in the interaction of PP-1c with inhibitor-1, a phosphoprotein inhibitor specific to PP-1c. PP-1cγ C291A was inhibited by the clavosines and calyculin A with almost identical potency to wild-type recombinant PP-1cγ.

Computer Modeling Studies—We carried out modeling studies on the interaction between the clavosines and PP-1c to address three aspects of calyculin and clavosine structures: the position of the phosphate group with respect to the phosphatase, correlation of IC50 data with the orientation of the amide/nitrile portion of the tetraene tail, and accommodation of the rhamnose moiety in the PP-1c-clavosine complex. Using the experimental data generated in our mutagenesis studies and exploiting PP-1c structure-function information found in the literature (9, 11, 12, 15, 30, 31), we generated hypothetical models for clavosine binding to PP-1c. Our models of clavosine A, clavosine B, and calyculin A binding are depicted in Fig. 5.

Use of the extensible subset forcefield allowed us to perform all docking and minimization steps with manganese ions present at the active site of PP-1c. We were therefore able to initiate docking without fixing any portion of the clavosines in the active site. In this way, our modeling studies were performed without initial bias. It was necessary to use coordinates of the α-isoform of PP-1c (28) for the modeling studies as coordinates on the interaction between the clavosines and PP-1c to address three aspects of calyculin and clavosine structures: the position of the phosphate group with respect to the phosphatase, correlation of IC50 data with the orientation of the amide/nitrile portion of the tetraene tail, and accommodation of the rhamnose moiety in the PP-1c-clavosine complex. Using the experimental data generated in our mutagenesis studies and exploiting PP-1c structure-function information found in the literature (9, 11, 12, 15, 30, 31), we generated hypothetical models for clavosine binding to PP-1c. Our models of clavosine A, clavosine B, and calyculin A binding are depicted in Fig. 5.

The inhibitor/phosphatase structures generated in our modeling studies were similar for each inhibitor. Tyrosine 134, one of the PP-1c residues mutated in this study, is predicted to lie located almost exclusively in the first 5 residues of the amino terminus and the last 29 residues of the carboxyl terminus.

The inhibitor/phosphatase structures generated in our modeling studies were similar for each inhibitor. Tyrosine 134, one of the PP-1c residues mutated in this study, is predicted to lie within 3 Å of the dimethylamine group in all of the inhibitor models, in close proximity with the amine (Fig. 1a) in the clavosine A and calyculin A structures and the C-35 hydroxyl in clavosine B. Val-223 is within 3.5 Å of each inhibitor, residing closer to calyculin B (<3 Å) than the other two compounds.

From the model, the phosphate group in clavosine A and calyculin A is predicted to form a hydrogen bond with Arg-96 in

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PP-1c. All three models suggest a contact between the C-13 hydroxyl of the calyculin or clavosine and Arg-221 in PP-1c, with the clavosine B predicted to lie the closest to this amino acid. In both clavosines models, the C-1 amide carbonyl group is predicted to form a contact with Lys-211, whereas the amide NH group in clavosine B may also interact with Asp-210. Asn-124 appears to reside within 4 Å of the C-33 carbonyl of each inhibitor. Additional residues in PP-1c predicted to reside within 4 Å of the inhibitor in every model include: His-66, Asp-92, His-125, Ile-130, Arg-132, Trp-206, Asp-208, Pro-209, Asp-210, Asn-219, Asp-220, Thr-226, His-248, Gln-249, Val-250, and Tyr-272. In addition, the manganese ions in the active site of the enzyme are within 4 Å of the inhibitors in all of the models. Fig. 6 displays residues in PP-1c mutated in this study, relative to the active site of the phosphatase. In addition, some of the amino acids shown to be involved in calyculin A inhibition of PP-1c by other researchers (30, 31) are highlighted. Asp-210 and Lys-211, two residues proposed to be involved in clavosine and calyculin binding by computer modeling studies contained in this paper, are also indicated.

**DISCUSSION**

**Site-directed Mutagenesis of Tyr-134**—In this study clavosines A and B have been identified as powerful protein phosphatase inhibitors, comparable to the other members of the calyculin family of natural product toxins. We have also shown that the Tyr-134 residue of PP-1c is an important mediator of inhibition by these compounds. Mutation of Tyr-134 to Phe resulted in a slight decrease in IC_{50} for all three inhibitors, possibly indicative of an increased hydrophobic interaction with the Phe side chain in the absence of a hydroxyl group. Substitution of the aromatic side chain in this position for alanine significantly increased the IC_{50} values of clavosines A, B, and calyculin A. The most dramatic effect on clavosine inhibition of PP-1c was found by substituting Asp in place of Tyr-134. Clavosine B inhibited PP-1c Y134D with an approximately 310-fold decrease in potency compared with its inhibition of wild-type enzyme. Clavosine A and calyculin A were also markedly poorer inhibitors of the Y134D mutant (200- and 175-fold worse, respectively). These results clearly show that the presence of a negative charge at residue 134 in PP-1c is unfavorable for calyculin or clavosine inhibition.

**Examination of Existing Calyculin A Binding Models**—A recent computer modeling paper (18) has suggested that Tyr-134 may be involved in calyculin binding to PP-1c. Our mutagenesis data is the first experimental evidence to confirm the importance of this residue for inhibitor binding. The only known three-dimensional structure of an inhibitor bound to PP-1c is the x-ray structure of a microcystin-LR-PP-1c complex determined by Goldberg et al. (28). One of the three areas of interaction between microcystin-LR and the phosphatase involved a possible hydrogen bond between the D-erythro-β-methyl aspartic acid carboxylate group and Tyr-134 of PP-1c. Therefore, mutagenesis of Tyr-134 serves as an important first starting point to test proposed models for binding of other toxins to PP-1c. In fact, the PP-1c Y134F mutant is actually more susceptible to inhibition by clavosine A/B and calyculin A than wild-type enzyme, thus arguing against the importance of hydrogen bonding between this residue and the calyculins.

In addition to the present work, there are three recent docking/modeling papers that address calyculin A binding to PP-1c: Gauss et al. (17), Gupta et al. (18), and Lindvall et al. (19). The Gupta et al. (18) publication proposes a direct role for Tyr-134 in calyculin binding to PP-1c. Their modeling studies propose hydrogen bonding between 1) the C-17 phosphate and C-35 hydroxyl of calyculin A with Arg-96 and 2) the C-34 hydroxyl and C-37 methoxy group of calyculin A with Tyr-134. We chose to test whether Tyr-134 was likely to make any hydrogen bond contact with the calyculins by mutating this residue to Phe. In view of the data presented here, it is possible that this model is incorrect with respect to hydrogen bond formation between calyculin A and PP-1c. The calyculin binding models of Gauss et al. (17) and Lindvall et al. (19) make no direct mention of the involvement of Tyr-134 in calyculin A binding. In contrast, our mutagenesis experiments suggest a role for the bulky aromatic ring of the Tyr-134 sidechain in phosphatase inhibition by the calyculins.

**Modeling of the Interaction between PP-1c and the Clavosines**—The models of calyculin A and clavosine binding presented in Fig. 5 allow for close interaction with Tyr-134 but not Ile-133 (see Fig. 6). This is an important point, because in this study we showed that mutation of Ile-133 to Tyr did not affect the potency of PP-1c inhibition by the clavosines (Table I).
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26-fold greater than that observed for clavosine A. This increase in IC$_{50}$ is in agreement with the model shown in Fig. 5b. Our model suggests that the C-2/C-3 geometry of clavosine B forces the amide portion of the inhibitor toward the phosphatase structure, which results in a slight “downward” shift away from the active site of PP-1c. In both clavosine models, the C-1 amide carbonyl appears to form a contact with Lys-211 of PP-1c. However, the orientation of clavosine B may allow formation of an additional contact between Asp-210 and the amide NH$_2$ group of the inhibitor, perhaps explaining the shift in the modeled complex. This movement away from the active site of the phosphatase may account for the reduction in effectiveness of clavosine B as an inhibitor of the recombinant enzyme.

Our models of clavosine binding to PP-1c orient the phosphate group of each of the clavosines and calyculin A such that it interacts closely with Arg-96 of the phosphatase. However, given the strong inhibitory activity of dephospho-calyculin A toward PP-1c (12), it is unlikely that this interaction forms the sole driving force for inhibition of the phosphatase. We found that calyculin A does not appear to depend solely on the phosphate group for maintaining its internal structure or its interaction with PP-1c, as dephospho-calyculin A docked similarly to the models in Fig. 5, despite loss of contact with Arg-96 (data not shown). During docking and energy minimization, the dephosphoanalog exhibited internal structural changes consistent with previously determined experimental and structural observations (12). It will be interesting to compare our model to the recently published NMR structure of the dephosphoanalog when the coordinates become available (21). The calyculin phosphate group is an important part of an “extended” model of calyculin binding to PP-1c that has been presented in the literature, which proposes that the phosphate-containing calyculin is a mimic of the phosphorylated PP-1c inhibitor protein inhibitor-1 (or the related protein DARPP-32). Several models of calyculin binding (17, 19) appear to be based, at least in part, upon models of phospho-DARPP-32 or inhibitor-1 interactions with PP-1c. Our computer simulations of clavosine binding suggest that the calyculin family of inhibitors do not bind as linear, phospho-peptidomimetics as has been previously proposed (19).

In our models the trimethoxy rhamnose group found in the clavosine structures is positioned so that it cannot interfere with toxin/protein binding. This is in accordance with data from our PP-1c inhibition assays (Fig. 2), which indicate that calyculin A and clavosine A inhibit PP-1c with similar potencies. In both clavosine models the rhamnose resides close to Glu-275 and Phe-276 in PP-1c but is clearly oriented away from the enzyme.

**Cytotoxicity of the Clavosines**—The average cytotoxicity of the clavosines was higher than that of calyculin A in the NCI cell culture screens (15). This implies a targeting role for the rhamnose group. Lectins are a group of proteins that recognize specific carbohydrates and are involved in host-pathogen interactions, targeting of proteins within cells, and cell-cell interactions (32–34). It is possible that a rhamnose-specific lectin could be involved in clavosine binding and uptake by a particular cell type or in an intracellular targeting mechanism. Rhamnose-specific lectins have been predominantly identified in fish eggs (35–37). Perhaps the clavosines discourage predation of marine sponges and/or associated microorganism(s) that produce the compounds; species of fish with rhamnose-specific lectins could be harmed if the clavosines were ingested. Interestingly, some cancer cell types may have receptors that recognize a rhamnose or rhamnose-like residue. Binding of salsodine glycosides (rhamnose-containing sugars) to tumor cells...
is thought to occur via a rhamnose moiety. These sugars selectively destroy tumor cells in vivo (38).

In conclusion, there is a great need for further NMR or x-ray crystallographic work in this area. Determination of the structure of the clavosines or calyculin A in complex with PP-1c would address many of the controversial questions regarding the specific mode of interaction between the calyculins and type 1 protein phosphatase. Investigations along these lines are currently underway in our laboratory. Further characterization of the anti-tumor activity of the calyculin family members is also needed if we are to take full advantage of this cell permeable class of phosphatase inhibitors.

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