Identification of heterochromatin protein 1 (HP1) as a phosphorylation target by Pim-1 kinase and the effect of phosphorylation on the transcriptional repression function of HP1[1]

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Abstract Pim-1, a protooncogene product, is a serine/threonine kinase and is thought to play a role in signal transduction in blood cells. Few phosphorylated target proteins for Pim-1, however, have been identified. In the present study, two-hybrid screening to clone cDNAs encoding proteins binding to Pim-1 was carried out, and a cDNA for heterochromatin protein 1γ (HP1γ) was obtained. Binding assays both in yeast and in vitro pull-down using the purified HP1γ and Pim-1 expressed in *Escherichia coli* showed that Pim-1 directly bound to the chromo shadow domain of HP1γ. HP1γ was also associated with Pim-1 in human HeLa cells and the serine clusters located at the center of HP1γ were phosphorylated by Pim-1 in vitro. Furthermore, a transcription repression activity of HP1γ was further stimulated by the deletion of the serine clusters targeted by Pim-1. These results suggest that Pim-1 affects the structure or silencing of chromatin by phosphorylating HP1.

Key words: Heterochoromatin protein 1; Phosphorylation; Pim-1

1. Introduction

The protooncogene *pim*-1 was identified as an activated gene in T cell lymphoma induced by provirus insertion of Molony murine leukemia virus [1]. Oncogenic activity of *pim*-1 was assessed by the results of transgenic mice harboring the *pim*-1 gene, that frequently brought about lymphoma [2]. Furthermore, synergistic oncogenecity between *pim*-1 and *myc* was suggested by the fact that double-transgenic mice containing both genes induced lymphoma more efficiently [3–6]. Pim-1, the product of *pim*-1, is highly expressed in bone marrow, spleen, thymus, lymph node and testis [3], and it is induced by cytokines such as IL-3 or GM-CFS through the Jak2/STAT5 pathway, thereby implying that it plays roles in proliferation or differentiation of blood cells [7–14]. Two other proteins of the Pim-1 family, Pim-2 [15,16] and Kid-1/Pim-3 [17,18], have been identified, and Pim-1 or its family members were also shown to be expressed in cells other than blood cells in a cell cycle-dependent manner [19]. Since Pim-1 knockout mice did not show significant disorders in tissues or cells, the physiological functions of Pim-1 may be compensated by other Pim-1 family proteins [20].

Pim-1 and its family proteins are a serine/threonine protein kinase [21–27] and a putative target sequence for Pim-1 phosphorylation was suggested: $(Arg/Lys)_3$-X-Ser/Thr*-X′ for human Pim-1 [28,29]. p100 [30] and cdc25A [31] were recently identified as target proteins for Pim-1 kinase. p100 is a nuclear transpiration coactivator that stimulates c-Myb transcription activity [30]. Although precise functions or other target proteins of Pim-1 have not been clarified, controversial roles of Pim-1 in apoptosis induction or suppression have been reported. Apoptosis induction was suppressed by Pim-1 in *pim*-1 transgenic mice with an *lpr*/*lpr* background [32], whereas ectopic introduction of Pim-1 to mouse NS-1-derived B cells induced apoptosis in vitro [33]. Furthermore, *pim*-1 stimulated c-*myc*-induced apoptosis in Rat-1 cells by concomitant phosphorylation of cdc25A [31,34]. Although the reasons for such discrepancy are not clear, it may be due to different cell types or systems: the NS-1-derived B cells and Rat-1 cells possess the intact Fas/Apo-1 gene, while the *lpr*/*lpr* mice lacked the gene.

In this study, we identified heterochromatin protein 1 (HP1) as a Pim-1 binding protein, and we found that HP1 was phosphorylated by Pim-1 and that the transcriptional repression function of HP1 was partially abrogated by the deletion of phosphorylation sites by Pim-1.

2. Materials and methods

2.1. Cells

Human 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

2.2. Plasmids

pGLex: *Hin*dIII–*Pst*I fragment of pHybLex/Zeo (Invitrogen) was inserted into the respective sites of pGBT9 (Clontech) in which the fragment containing the GAL4 DNA binding domain (GALBD) and multi cloning site had been deleted. The Cat-5 gene encoding a chloramphenicol-resistant gene from pKF3 (Takara) was then inserted into *Aat*II–*Bgl*II sites of the above DNA. Generally, the restriction enzyme site at the 5′ or 3′ end of Pim-1 or HP1 cDNA was changed to a site

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[1] The nucleotide sequence reported in this paper has been submitted to the EMBL/DDBJ/GenBank Data Bank with accession number AB030905.

Abbreviations: HP1, heterochoromatin protein 1; HA, hemagglutinin antigen; GST, glutathione-*S*-transferase

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suitable for vector plasmids by PCR. An *Eco*RI–*Xho*I fragment containing mouse Pim-1 was inserted into pGEX-6P-1 (pGEX-Pim-1), pGLex (pGLex-Pim-1) and pcDNA-FLAG [35] (pcDNA3-F-Pim-1). An *Eco*RI–*Pst*I fragment containing Pim-1Δ2 was inserted into pGLex (pGLex-Pim-1Δ2). An *Eco*RI–*Xho*I fragment containing HP1 or its deletion mutants was inserted into pGADGH (pGAD-HP1, pGAD-HP1(Δ83–94), pGAD-HP1-N or pGAD-HP1-C), into pGEX-6P-1 (pGST-HP1), into pcDNA3-hemagglutinin antigen (HA) [36] (pcDNA3-HP1-HA) or into pcDNA-GAL4 [36] (pGA4-HP1 or pGAL4-HP1(Δ83–94)). Details of the construction procedures are available upon request.

2.3. Cloning of HP1 by a two-hybrid system

Saccharomyces cerevisiae L40 cells were transformed first with pGLex-Pim-1Δ2, which did not activate *lacZ* transcription by itself. The transformant cells were subsequently transformed with HeLa MATCHMAKER cDNA (Clontech). Of approximately 4.7×10^5 colonies screened for lacZ expression, the plasmid DNAs in the lacZ-positive cells were extracted by the procedure described in the protocols from Clontech. The nucleotide sequence of plasmid derived from a positive colony was determined and its encoding protein was characterized.

2.4. In vitro binding assay

Glutathione-*S*-transferase (GST)-HP1 and GST were purified from a 1000 ml culture of *Escherichia coli* BL21(DE3) transformed with pGEX-HP1 and pGEX-6P-1, respectively, as described previously [37]. 2 μg of the purified GST-HP1 or GST was first applied to GST-Sepharose 4B (Amersham-Pharmacia) in a buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40 and 1 mM EDTA. The ^{35}S-labeled Pim-1 synthesized in vitro using pcDNA3-Pim-1 as a template in the coupled transcription/translation system (Novagen) was then applied to the column. After extensive washing of the column with the same buffer as above, the proteins bound to the resin were recovered, separated in a 12% polyacrylamide gel containing sodium dodecyl sulfate (SDS) and visualized by fluorography.

2.5. In vivo binding assay

1 μg of pCMV-FLAG-Pim-1 together with 1 μg of pCMV-HP1-HA was transfected to human 293T cells 60% confluent in a 10 cm dish using Lipofect Amine plus (Gibco BRL). 48 h after transfection, the whole cell extract was prepared by the published procedure [38]. Approximately 500 μg of the 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Kodak) in a buffer containing 50 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 1 mg/ml BSA, 150 μg/ml PMSF and 0.25% NP-40. After washing with the same buffer except for 0.05% NP-40 instead of 0.25%, the precipitates were separated in a 12% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, reacted with a mouse anti-HA antibody 12CA5 [39,40] or with the mouse anti-FLAG antibody.

2.6. Kinase assay

GST-HP1 or GST-HP1(Δ83–94) was expressed in *E. coli* and purified as described above. In vitro kinase reactions were carried out at 22°C for 20 min in a mixture containing 250 ng of either GST-HP1 or GST-HP1(Δ83–94), 2 μg of GST-free Pim-1 and 10 μCi (3000 Ci/mmol) of [γ-^{32}P]ATP in 200 μl of a kinase buffer (25 mM HEPES-KOH (pH 7.5), 30 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM dithiothreitol (DTT)). The reaction mixture was boiled in Laemmli buffer and phosphorylated proteins were separated in a 12% polyacrylamide gel containing SDS followed by autoradiography.

2.7. Luciferase assay

HeLa cells subconfluent in a 6 cm dish were transfected with various amounts of effector plasmids of pGAL4-HP1, pGAL4-HP1(Δ83–94) or pGAL4 in addition to 1 μg of pCMV-β-gal and 1 μg of p6×GAL4-SVP-Luc by the calcium phosphate precipitation method [41]. 48 h after transfection, cell extracts were prepared by adding a solution containing 25 mM Tris–phosphate (pH 7.8), 2 mM DTT, 10% glycerol, 1% Triton X-100 and 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (Promega). After standardization of the transfection efficiencies by β-galactosidase assays, the luciferase activity was examined in the luciferase assay mixture supplied by Promega.

3. Results and discussion

3.1. Identification of HP1 as a Pim-1 binding protein and determination of the Pim-1 binding region

To screen cDNAs encoding Pim-1 associating proteins, Pim-1Δ2, an N-terminal fragment containing amino acid numbers 1–93, was fused to the LexA binding domain and introduced to *S. cerevisiae* L40 cells. A human cDNA library prepared from HeLa cells and cloned in pGADGH was then introduced to the transformant yeast cells and the colonies resistant to *His* marker followed by β-galactosidase expression were selected. Among a total of 5×10^5 transformant cells, 18 colonies were *His*- and β-galactosidase-positive, and five of the 18 positive colonies were identified as HP1γ [42] after determination of their nucleotide sequences. Since the longest clone lacked three nucleotides, ATG for the first methionine, an oligonucleotide of ATG was attached to the N-terminus of this clone and used as a wild-type HP1γ. All the clones of HP1γ obtained, wt, Δ1, Δ10, Δ23 and Δ89, contained the same region from amino acid #90 to polyA (Fig. 1). Because the published sequence of HP1γ lacked the 3′ untranslated region [42], all the sequences of our clones were determined (accession number AB030905). HP1γ was comprised of at least three domains, chromodomain, serine cluster and chromo shadow domain, all of which were thought to be important domains for function (see review [43,44]).

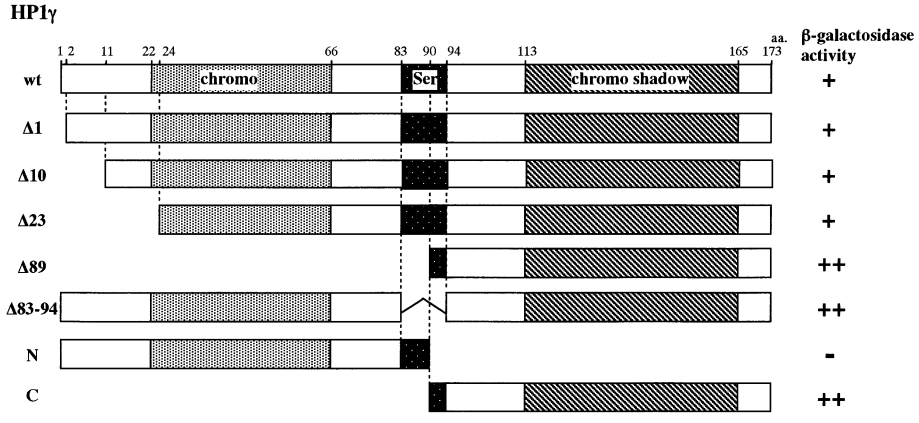

Fig. 1. Determination of the HP1γ region bound by Pim-1. The wild-type (wt) or various deletion mutants of HP1γ were fused to GALAD and used for the yeast two-hybrid assays in L40 cells pretransformed with LexBD-Pim-1Δ2. After incubation on filters, the β-galactosidase activity was assayed.

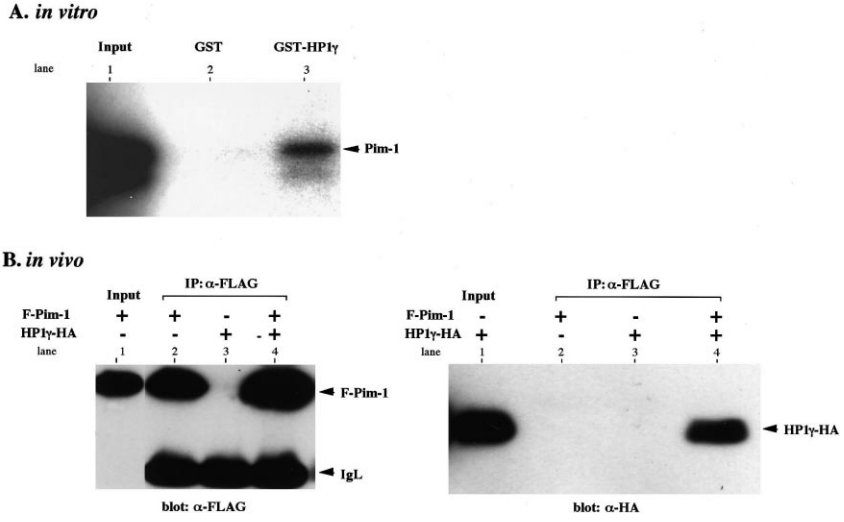

Fig. 2. In vitro and in vivo binding assays between Pim-1 and HP1γ. A: In vitro binding assay between Pim-1 and HP1γ. GST or GST-HP1γ was expressed in *E. coli* BL21(DE3) and applied to GST-Sepharose 4B. [^{35}S]Pim-1 synthesized in vitro in a coupled transcription/translation system was then applied to the column. The labeled proteins bound to the column were separated in a gel and visualized by fluorography. 1/50 Volume of the labeled Pim-1 used for the binding reaction was applied in the same gel (lane 1). B: Association of Pim-1 with HP1γ in human 293T cells. Pim-1 and HP1γ were tagged with either FLAG or HA, and their expression vectors alone or together were introduced to human 293T cells. Two days after transfection, cell extracts were prepared and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody. The proteins in the precipitates were separated in a 12% polyacrylamide gel and blotted with the anti-FLAG (left panel) or an anti-HA antibody (12CA5) (right panel). 1/10 Or 1/50 volume of the extract used for the binding reaction was applied in the same gel (Input, lane 1 in left and right panel, respectively).

To assess the Pim-1 binding region of HP1γ, various deletion constructs fused to the GAL4 activation domain (GAL-AD) were used for a two-hybrid assay with Pim-1Δ2 as a bait (Fig. 1). Except for HP1γ-N containing amino acid from #1 to 90, all other clones, including the original clones isolated, bound to Pim-1, and deletion of the region from #83 to 90 within the serine cluster gave a stronger signal for binding to Pim-1Δ2 than that of the wild-type. These results indicate that Pim-1 binds to the C-terminal half region of HP1γ including the chromo shadow domain. An in vitro binding assay was then performed by using purified GST-HP1γ and ^{35}S-labeled Pim-1 synthesized in vitro (Fig. 2A). Labeled Pim-1 bound to GST-HP1γ, but not GST, indicating a direct interaction between HP1γ and Pim-1. To see a complex formation of HP1γ with Pim-1 in vivo, expression vectors for FLAG-tagged Pim-1 and HA-tagged HP1γ alone or together were transfected to human 293T cells. 48 h after transfection, the cell extract was prepared and the proteins in the extract were first immunoprecipitated with an anti-FLAG antibody. The precipitates were divided in two aliquots, and each of the precipitates was immunoblotted against either the anti-FLAG antibody or an anti-HA antibody (Fig. 2B, left or right, respectively). The anti-FLAG antibody did precipitate FLAG-Pim-1 under the condition where the same levels of immunoglobulin light chain were precipitated in all the samples (Fig. 2B, left, lanes 2 and 3). HP1γ-HA, on the other hand, was only detected in the immunoprecipitate from cells transfected with both FLAG-Pim-1 and HP1γ-HA (Fig. 2B, right, lanes 2–3), indicating that HP1γ was associated with Pim-1 in ectopic-expressed 293T cells.

3.2. Phosphorylation of HP1γ by Pim-1

Since Pim-1 is a serine/threonine protein kinase and its consensus sequence is well-matched with the amino acid sequence around a serine cluster in HP1γ, ^{80}KRKSLSDSESDDSKS^{94}, it is possible that HP1γ is phosphorylated by Pim-1. To this end, GST, GST-wild-type HP1γ and GST-Pim-1 were purified from the expressed *E. coli* and then GST-free Pim-1 was prepared after the digestion of GST-Pim-1 with PreScission protease. Both GST-HP1γ and Pim-1 were incubated with [γ-^{32}P]ATP and the labeled proteins were separated on the gel (Fig. 3). Pim-1 was autophosphorylated irrespective of the presence or absence of substrate protein and GST-wild-type HP1γ, but not GST, was phosphorylated with Pim-1 (Fig. 3, lanes 1–5). A kinase-negative mutant of Pim-1 in which lysine at amino acid #67 was changed to methionine did not phosphorylate GST-HP1γ (data not shown). We then constructed mutant HP1γ by deleting serine clusters from amino acid #83–94 and used it for the kinase assay. The results clearly showed that GST-HP1(Δ83–94) was phosphorylated by Pim-1 at 1/20 the efficiency of that of wild-type HP1γ (Fig. 3, lanes 6 and 7). When each of six serines in this cluster of HP1γ was substituted for alanine and used for the kinase assay, phosphorylation of each mutant of HP1γ was marginally, but not completely, decreased (data not shown). These results suggest that

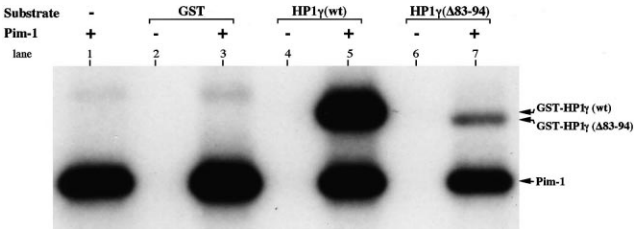

Fig. 3. Phosphorylation of HP1γ by Pim-1. GST by itself and GST fusion proteins of HP1γ or HP1γ(Δ83–94) were expressed in *E. coli*, purified and incubated in various combinations in the presence of [γ-^{32}P]ATP and GST-free Pim-1. Labeled proteins bound to the resin were then separated in a 12% polyacrylamide gel and autoradiographed.

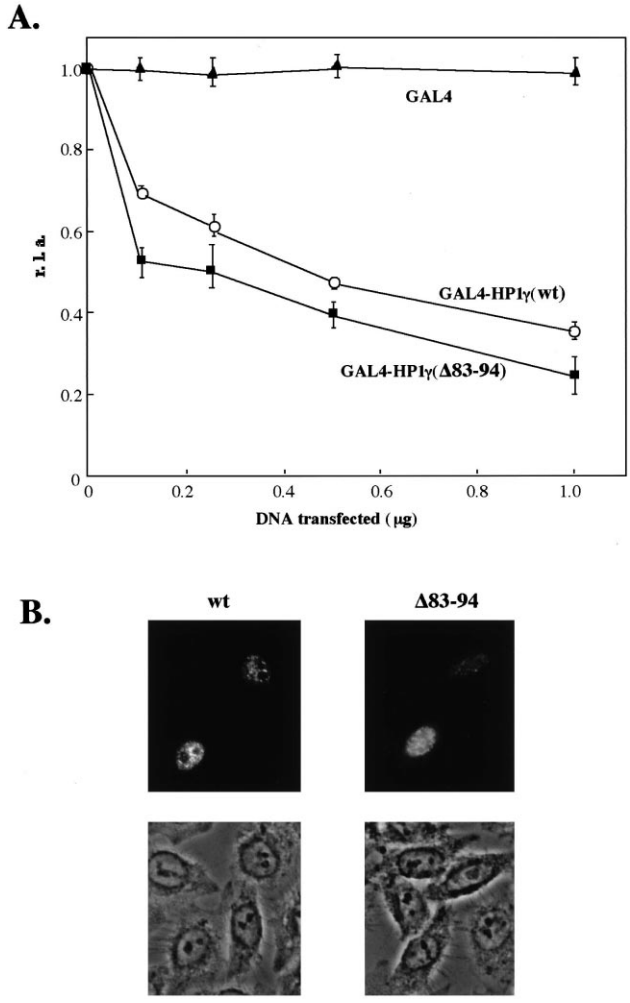

Fig. 4. Effect of a serine cluster on the transcriptional repression activity of HP1γ. A: HeLa cells were transfected with various amounts of the expression vector for the wild-type (wt), deletion mutant (Δ83–94) of HP1γ fused to GALBD, or GALBD, together with p6×GAL4-SVP-Luc as a reporter plasmid. Two days after transfection, the luciferase assay was carried out. Relative luciferase activities (r.l.a.) to that of p6×GAL4-SVP-Luc alone are shown. B: HeLa cells were similarly transfected as in A, and the localization of the introduced proteins was visualized by an anti-FLAG antibody followed by FITC-conjugated IgG. The stained cells were visualized under UV and phase-construct by fluorescence microscopy, upper and lower panels, respectively.

six serines in the cluster of HP1γ are target sites for phosphorylation by Pim-1 and that these serines mutually affect their phosphorylation states.

3.3. Effect of serine clusters on transcriptional repression activity of HP1γ

HP1 was identified as a protein involved in position-effect variegation in *Drosophila* and a component of a transcriptional repression complex [43–47]. Controversial results about DNA binding activity of HP1 have been reported [48,49]. We therefore tested the transcriptional repression activity of HP1 in the GAL4 system as reported [46,47]. Expression vector of HP1γ fused to GALBD was cotransfected to HeLa cells with 6×GAL4 fused to the SV40 promoter linking to the luciferase gene, and the luciferase activity was measured 48 h after transfection (Fig. 4). While the GAL4 DNA binding domain alone did not affect luciferase activity, GAL4-HP1γ repressed luciferase activity in a dose-dependent manner, indicating that this system was suitable to see the transcriptional repression activity of HP1. Then an HP1γ mutant in which a serine cluster had been deleted, HP1γ(Δ83–94), was fused to GALDB and its transcription activity was similarly tested. The result showed that transcriptional repression activity was enhanced by 15–20% compared to that of wild-type HP1γ (Fig. 4A). Since a putative nuclear localization signal of HP1 is present in a deletion containing amino acid 83–94, the effect of the deletion on the repression activity of HP1 might be due to different locations of two proteins fused to the GAL4 DNA binding domain. This possibility, however, was ruled out by the nuclear localization of both proteins after transfection (Fig. 4B). These results suggest that the transcriptional repression activity of HP1γ might be influenced by its phosphorylation state catalyzed by Pim-1.

HP1 is a heterochromatin-associated protein well-conserved from yeast to man. Genetic analysis of HP1 in yeast and *Drosophila* revealed that HP1 plays roles in position effect variegation and in silencing chromatin [43–45]. In the present study, we found that Pim-1 bound to the chromo shadow domain of HP1γ to be phosphorylated. The chromo shadow domain of human HP1 was reported to be essential for transcriptional repression activity of HP1, to which SP100, a component of a transcriptional repression complex, bound [46,47]. It is therefore worth testing whether Pim-1 is also involved in this transcriptional repression complex. HP1 is a highly phosphorylated protein and at least a cdc2-like kinase participated in HP1 phosphorylation in *Tetrahymena* [50]. After deletion of a serine cluster located at the center of human HP1γ, the amount of phosphorylated HP1γ by Pim-1 was reduced to 1/20 of that of wild-type of HP1γ, suggesting that other serine/threonine(s) are also phosphorylated by Pim-1. Although it is possible that kinases other than Pim-1 are also responsible for human HP1 phosphorylation, as in *Tetrahymena*, the fact that the deletion of a serine cluster from HP1γ stimulated a transcription repression activity of HP1γ suggests negative regulation of HP1γ function by Pim-1 phosphorylation. Taken together, these results suggest a new function of Pim-1 that may be involved in a certain aspect of chromatin dynamics.

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