Amyloid-β Protein Precursor (AβPP) Intracellular Domain-associated Protein-1 Proteins Bind to AβPP and Modulate Its Processing in an Isoform-specific Manner*

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The amyloid-β protein precursor (AβPP) is a type I transmembrane molecule that undergoes several finely regulated cleavage events. The physiopathological relevance of AβPP derives from the fact that its aberrant processing strongly correlates with the onset of Alzheimer’s disease (AD). AD is a neurodegenerative disorder characterized by neuronal cell death, loss of synapses, and deposition of misfolded protein plaques in the brain; the main constituent of these plaques is the amyloid-β peptide, a 40–42 amino acid-long protein fragment derived by AβPP upon two sequential processing events. Mutations in the genes encoding for AβPP and some of the enzymes responsible for its processing are strongly associated with familial forms of early onset AD. Therefore, the elucidation of the mechanisms underlying AβPP metabolism appears crucial to understanding the basis for the onset of AD. Apart from Aβ, upon processing of AβPP other fragments are generated. The long extracellular domain is released in the extracellular space, whereas the short cytoplasmic tail, named AβPP intracellular domain (AID) is released intracellularly. AID appears involved in several cellular processes, apoptosis, calcium homeostasis, and transcriptional regulation. We have recently reported the cloning and characterization of different isoforms of AID associated protein-1 (AIDA-1), a novel AID-binding protein. Here we further analyzed the interaction between several AIDA-1 isoforms and the cytoplasmic tail of AβPP. Our data demonstrated that the interaction between the two molecules is regulated by alternative splicing of the AIDA-1 proteins. Furthermore, we provide data supporting a possible function for AIDA-1a as a modulator of AβPP processing.

Alzheimer’s disease is a neuropathological disorder characterized by dementia, memory loss, neuronal apoptosis, and, eventually, death of the affected individuals (1). Studies on familial forms of Alzheimer’s disease revealed the crucial role played by mutations in genes encoding for AβPP1 and presenilins in the pathogenesis of the disease (2–9). Presenilins are part of the γ-secretase, an enzymatic complex responsible for the intramembranous proteolysis of several transmembrane receptors, among which is AβPP (10–20). Other enzymes, named β- and α-secretases, cleave AβPP in its extracellular region releasing soluble N-terminal fragments (21, 22). The above-mentioned mutations in presenilins and AβPP are the genetic basis for familial forms of Alzheimer’s disease, and they all result in aberrant processing of AβPP (10, 23–25). A lot of scientific interest has been more recently focused on studying potential functions for the intracellular domain of AβPP (AID), which is released upon cleavage by γ-secretase. AID has been shown to be a pro-apoptotic molecule (26), to play a role in intracellular calcium homeostasis (27), to inhibit Notch signaling (28), and to be required for the activation and potential transcriptional activity of the adaptor proteins Fe65 (29, 30) and Jun N-terminal kinase interacting protein (31). Many proteins have been described to interact with AID modulating its functions or interfering with AβPP processing and/or internalization (32). These are, for example, X11a (33–37), autosomal recessive hypercholesterolemia (38), Shc (39, 40), Growth Factor Receptor-Bound Protein 2 (41), Numb, and Numb-like (28), in addition to the previously mentioned Fe65 (42, 43) and Jun N-terminal kinase interacting protein (31, 44–47). We recently reported the cloning and initial characterization of novel AID-interacting proteins that we named AID-associated proteins-1 (AIDA-1) (48). Different isoforms of AIDA-1 proteins, deriving from alternative splicing of the same transcript, have been described, and the potential significance of this interaction has been speculated upon but not proved yet. In this paper we show that certain isoforms of AIDA-1 proteins do not bind to AβPP despite the presence of a phosphotyrosine binding domain in their C-terminal region and that the interaction is prevented by an inhibitory region encoded by exon 14 and expressed in an isoform-specific manner; more importantly, we demonstrate that AIDA-1a, the only AβPP-binding full-length isoform that we have cloned, can reduce AβPP processing by inhibiting activity of the γ-secretase, consequently diminishing Aβ secretion.

1 The abbreviations used are: AβPP, amyloid-β protein precursor; AID, intracellular domain of AβPP; AID-1, AID-associated proteins-1; HEK, human embryonic kidney; GST, glutathione S-transferase; Shc, Src homology/collagen protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; ECFP, enhanced cyan fluorescent protein; PTB, phosphotyrosine binding domain; SAM, Sterile α motif; PDZ, PSD-95, DGC, ZO-1/2.
MATERIALS AND METHODS

**cDNA Constructs**—The AβPP-Gal4 fusion construct was a kind gift of Dr. Tommaso Russo, University of Naples, Italy. Constructs encoding for full-length AIDA-1a, AIDA-1b, and AIDA-1bΔAnk have been described previously (48). AIDA-1bΔAnk-2 was cloned by reverse transcription-PCR using total RNA extracted from the pre-B cell line 697 as a template. To reduce the number of different products that could have been obtained because of the different splicing at the C terminus, we

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**Fig. 1.** Exon/intron organization of AIDA-1 isoforms and their expression in the brain. a, schematic representation of the basic AIDA-1 isoforms employed in this paper. Exons have been numbered progressively starting from a putative promoter region found in the genome. The coding region for each protein is represented by a pattern of diagonal lines. The relative position of ankyrin repeats, the sterile α motif (SAM) domains, and the PTB domain is shown at the top of the figure. Exons 14a, 15a, and 27a derive from exons 14, 15, and 27, respectively, upon splicing at internal splicing sites within the exons themselves. The difference in exon composition at the 3′-end of the cDNAs results in different C-terminal tails in the proteins. Especially interesting is the presence of a PDZ binding motif at the C terminus of AT13. This feature might allow isoform-specific interactions with PDZ-containing proteins. b, Western blot (WB) detection of anti-AIDA-1-reactive bands in brain lysates. Approximately 30–40 μg of mouse, human, and human Alzheimer’s disease brain lysate was analyzed by SDS-PAGE, and the membrane was blotted with anti-AIDA-1 antibody. Lysates of HEK293T cells transfected with different AIDA-1 isoforms were loaded for size comparison. To ensure antibody specificity, the AIDA-1 antibody was also preincubated with 50 μg/ml of AIDA-1 peptide or with the same amount of an irrelevant peptide.
Some AIDA-1 isoforms do not bind to the cytoplasmic tail of AβPP. HEK293T cells were transfected with different AIDA-1 constructs. 24 h after transfection cells were lysed and incubated with the indicated GST fusion proteins linked to glutathione-Sepharose beads (1 well of a 6-well plate/sample) for 2 h at 4 °C. After washing the beads, samples were loaded on a SDS-polyacrylamide gel and probed with anti-AIDA-1 antibody. The various symbols have been loaded on a SDS-polyacrylamide gel. Proteins were detected with an anti-AIDA-1 antibody. The various symbols at the C termini represent the different tails. The star symbol represents the EYFP moiety of EYFP-AIDA-1 PTB. The candidate regions for the binding inhibition are indicated by the open arrows. WB, Western blot.

![Diagram of AIDA-1 proteins](image_url)

**Fig. 3.** Different C-terminal tails of AIDA-1s do not affect their binding to AID. EYFP-tagged constructs encoding for AIDA-1 PTB domain followed by the different cytoplasmic tails described in the legend to Fig. 1 were generated and transfected in HEK293T cells. GST pull-down experiments were performed on the total cell lysates and loaded on a SDS-polyacrylamide gel. Proteins were detected with an anti-living color monoclonal antibody. The various symbols have been described in the legend to Fig. 2. WB, Western blot.

**Fig. 4.** The amino acid sequence encoded by exon 14 of AIDA-1bΔAnk-2 is responsible for inhibiting the binding to AβPP. Various constructs were generated progressively lacking one exon (exons are indicated with small rectangular boxes above the flat sequence line) at the N terminus of AIDA-1bΔAnk-2 and were used in GST pull-down experiments as described in the legends to Figs. 2 and 3. Samples were run on a SDS-polyacrylamide gel and detected by using an anti-AIDA-1 antibody. WB, Western blot.
Nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma). Pictures were taken using a 12-bit photometrics-cooled CCD camera mounted on an Olympus IX70 inverted microscope.

Antibodies—The anti-living colors monoclonal antibody was purchased from Clontech. The anti-AIDA-1 and the anti-AβPP C-terminal antibody were obtained from Zymed Laboratories Inc. The anti-AβPP N-terminal antibody 22C11 was purchased from Chemicon.

Luciferase Assay—cDNAs encoding for Aβ-galactosidase, luciferase, AβPP-Gal4, and AIDA-1s were transfected into HEK293T cells in a ratio of 1:6:7:7. A total of 0.21 g of DNA was transfected in 1 well of a 96-well plate. 24 h after transfection cells were lysed in reporter lysis buffer (Promega). Aβ-galactosidase and luciferase activity were assayed by using Aβ-galactosidase (Tropix, Bedford, Massachusetts) and luciferase (Promega) substrates, respectively.

Enzyme-linked Immunosorbent Assay—HEK293T cells stably expressing AβPP695, were transfected with different EYFP-tagged AIDA-1 constructs. 24 h after transfection the culture medium was replaced with fresh medium and incubated for 30'. Supernatants were analyzed by using a human amyloid-β (1–40) enzyme-linked immunosorbent assay kit (Immuno-biological Laboratories, Fujioka, Japan). The assay was performed according to the manufacturer’s protocol. Each value represents the average of three independent transfections and two readings/transfection.

Fluorescence Resonance Energy Transfer (FRET)—HEK293T were cotransfected with different combinations of ECFP/EYFP constructs (DNA ratio 3:1) and analyzed for FRET 24 h after transfection. Acquisition was performed on an LSR II instrument (BD Biosciences) using the BD-FacsDiva software. ECFP, EYFP, and FRET fluorescence was detected by using 470/20, 525/50, and 505 LP filters, respectively.

RESULTS

Exon/Intron Complexity of AIDA-1 Proteins—The cloning of several isoforms of AIDA-1 revealed the extraordinary complexity of the gene encoding for these proteins. The AIDA-1 gene maps on human chromosome 12 (12q21–23) and spans over 1 Mb on the chromosome. We could identify only one predicted promoter region, upstream of exon 1. It seems very likely, therefore, that the different isoforms derive from alternative splicing of the same transcript rather than from transcripts generated by different promoters. Although the regions encoding for the PTB domain and the two SAM domains are very well conserved among all the isoforms that we have cloned, all the other parts of the messenger RNA for AIDA-1 are potentially subjected to several splicing events. The different constructs that have been used in this paper are schematically represented in Fig. 1a. AIDA-1b, AIDA-1a, and AIDA-1bAnk are full-length clones that have been described previously (48). AT13 is a partial clone isolated with a yeast two-hybrid screening using AID as bait; AIDA-1bAnk-2 is partial clone of an alternatively spliced form of AIDA-1bAnk that was cloned by reverse transcription-PCR from the pre-B acute lymphoblastic leukemia cell line 697.

Three different C-terminal tails, downstream of the PTB domain, are predicted for AIDA-1a, AIDA-1bs, and AT13. In

FIG. 5. AIDA-1a, but not AIDA-1 proteins containing the exon 14 encoded sequence, interacts with AβPP in FRET experiments. HEK293T cells were co-transfected with various combination of EYFP-tagged AIDA-1s and ECFP-tagged AβPP and were analyzed 24 h after transfection for FRET. a, representative dot plot showing the distribution of the cells in a forward scatter (FSC)/side scatter (SSC) diagram. Live cells are gated in P1, h, to eliminate cellular aggregates, cells were plotted on an FSC-A/FSC-H diagram, and single cells were gated in P2. c, representative dot plot showing co-expression of EYFP and ECFP in the cell population. The cells displayed in this plot are present at the intersection of P1 and P2. Only the double transfected cells (intersection of Q2 and P6) were analyzed for FRET. d, dot plots showing double transfected cells in ECFP versus FRET diagrams. Cells present in Q2–3 were considered as positive for FRET. The numbers in the upper right corner of each plot indicate the percentage of double transfected cells exhibiting positive FRET signal.
was detected by fluorescence microscopy.

DAPI/6-diamidino-2-phenylindole (DAPI). Cellular localization followed by anti-rabbit Alexa 594 secondary antibody. Nuclei were transfection cells were fixed and stained with anti-AIDA-1 antibody. Cells were transfected with different AIDA-1 constructs, and 24 h after transfection cells were stained with anti-AIDA-1 antibody followed by anti-rabbit Alexa 594 secondary antibody. Nuclei were stained by 4’,6-diamidino-2-phenylindole (DAPI). Cellular localization was detected by fluorescence microscopy.

Interestingly, alternative splicing causes exon 27a-containing isoforms to have a PDZ binding motif at their C-terminals (as predicted by the ScanPro software), potentially allowing them to interact with PDZ-containing proteins (50).

In the N-terminal region, the ankyrin repeats predicted for AIDA-1b are spliced out in AIDA-1bΔAnk, and a large portion of the N-terminal part of the protein is not present in AIDA-1a. We recently reported (and we have reproduced these data in Fig. 6) how these diversities in the primary sequence correlate with differences in intracellular localization. In overexpression experiments AIDA-1b is excluded from the nucleus, whereas AIDA-1bΔAnk and AIDA-1a show a diffused localization with some nuclear accumulation (48). Western blot analysis of mouse brain, human brain, and human Alzheimer’s disease brain lysates (see Fig 1b) shows the presence of several bands specifically reacting with the anti-AIDA-1 polyclonal antibody. No band of the apparent size of AIDA-1b and AIDA-1bΔAnk could be detected, indicating that these proteins could be expressed below the level of detection of the available antibody. Several bands have the approximate size of AIDA-1a. Interestingly, even though more samples should be analyzed to give statistical significance to this observation, we noticed differences in AIDA-1 expression between the Alzheimer’s disease and the normal human brain. In fact many anti-AIDA-1 reactive bands that are clearly visible in the normal brain sample and the normal human brain. In fact many anti-AIDA-1 reactive bands that are clearly visible in the normal brain sample and the normal human brain.

Some Isoforms of AIDA-1 Do Not Bind to the Cytoplasmic Domain of AβPP—We have recently shown how overexpression of AβPP can modify AIDA-1a intracellular localization, causing its accumulation outside of the nucleus, where it is otherwise abundantly found (48). On the other hand, overexpression of AIDA-1bΔAnk, which shares the same intracellular localization of AIDA-1a, together with AβPP did not result in any modification in the distribution of these two proteins (data not shown). Even more surprising, the overexpression of AIDA-1b, which is excluded from the nucleus, did not have any detectable effect on the main nuclear localization of AIDA (data not shown).

We then decided to test several AIDA-1 isoforms for binding to AβPP in GST pull-down experiments. As shown in Fig. 2, some isoforms did not interact with GST-AID. This was extremely surprising because the PTB domain, which is sufficient for the interaction to occur (48), is conserved among the different isoforms. Consequently, we hypothesized that the lack of interaction was because of an inhibitory region contained specifically in certain isoforms.

The Sequence Encoded by Exon 14 Is Responsible for Inhibiting the Interaction between AIDA-1s and AID—By comparing the predicted protein sequences and the binding ability of these different AIDA-1 isoforms, we identified two possible candidate regions for the binding inhibition: the C-terminal tail, downstream of the PTB domain, and the stretch of amino acids comprised between the ankyrin repeats of AIDA-1b and the first SAM domain (Fig. 2, arrows). We generated EYFP-tagged constructs encoding for the PTB domains and the three different C-terminal tails, and we used them to perform GST pull-down experiments (Fig. 3). All of these fusion proteins interacted specifically with the C-terminal tail of AβPP fused to GST but not with GST3NPTY, a very efficient binding could be detected for the PTB domain also in the absence of any C-terminal tail. Thus we concluded that the C-terminal tails of AIDA-1s are not responsible for inhibiting the binding to AβPP.

To analyze the other candidate inhibitor region, we generated a series of deletion mutants of AIDA-1bΔAnk-2 by progressively removing exons from the N-terminal side of the cDNA (Fig. 4). Interaction with the cytoplasmic tail of AβPP could not be detected for any of these artificial constructs, suggesting that the shorter one (AIDA-1bΔAnk-2 exon 14) of these fusion proteins still contains the inhibitory sequence.

We decided to use FRET (51) as an alternative approach to confirm the in vitro binding data. FRET was detected only when EYFP-AIDA-1a was cotransfected with ECFP-AβPP (Fig. 5). Consistently with our GST pull-down data, no interaction was detected when ECFP-AβPP was co-expressed with EYFP AIDA-1bΔAnk-2 or AIDA-1bΔAnk-2 exon 14. None of the above-mentioned EYFP-tagged AIDA-1s gave a positive FRET signal when cotransfected with ECFP-Ncas, a form of AβPP lacking part of the C-terminal tail (downstream of the caspase-3 cleavage site). Ncas lacks the YENPTY motif, a short amino acid sequence that has been shown to be critical for binding to PTB domain containing proteins. Immunofluorescence experiments in HeLa cells show that the differences in binding ability do not depend on artifactual mistrafficking of the exon 14 containing constructs. EYFP-AIDA-1bΔAnk2 exon 14 shows a diffused localization with some nuclear accumulation, as expected. In fact, as we have demonstrated previously.
(48) and as it is shown here in Fig. 6, only AIDA-1 isoforms containing ankyrin repeats are prevented from entering the nucleus. Comparative analysis of the various AIDA-1 constructs employed in these experiments led to the conclusion that the stretch of amino acids encoded by exon 14 is responsible for inhibiting the binding of certain AIDA-1 isoforms to AβPP.

**Fig. 8. Overexpression of AIDA-1a causes a reduction in Aβ40 secretion.** HEK293T cells stably expressing AβPP were transfected with the indicated AIDA-1 constructs and control. After 24 h supernatants were harvested, and Aβ40 concentration was measured by enzyme-linked immunosorbent assay. Each bar represents the average of three independent transfections. The difference in Aβ levels between the samples transfected with AIDA-1a and the controls is ~30%. *, p < 0.05 relative to EYFP vector and p < 0.01 relative to EYFP AIDA-1bΔAnk-2 exon 14.

**Fig. 9. AIDA-1a acts through inhibition of γ-secretase.** HEK293T cells stably expressing AβPP were transfected (two transfections/sample) with the indicated AIDA-1 constructs and empty vector as a control in the presence of the γ-secretase inhibitor compound E or the same concentration of Me2SO. 24 h after transfection cell lysates were analyzed by SDS-PAGE, and membranes were probed with the anti-AβPP N-terminal antibody 22C11, anti-tubulin, and anti-AβPP C-terminal antibodies as indicated. Densitometric values were normalized for tubulin signal intensity. Histogram bars represent the average of two independent transfections. The weaker tubulin signal in the YFP vector samples treated with γ-secretase inhibitors is because of a difference in the amount of total proteins loaded. Despite this fact the ratio AβPP C-terminal fragment/tubulin remains unaffected.

**without γ-secretase inhibitors**

**with γ-secretase inhibitors**
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overexpression experiments, and we speculated that this might have some implications in vivo for the normal function of AIDA-1a. No effects on intracellular localization of AβPP or its processing-derived fragments were detected upon overexpression of different forms of AIDA-1. We decided to investigate a possible role for AIDA-1 in AβPP processing by taking advantage of a fusion construct encoding for the entire AβPP linked at its C terminus to the Gal4 transcription factor. This experimental approach has been successfully employed to clone and characterize modulators of AβPP processing (52). This construct can be cotransfected with a reporter plasmid in which the expression of luciferase is driven by a Gal4-dependent promoter. Upon physiological processing, an AID-Gal4 fragment is released and translocates to the nucleus, activating transcription of the reporter gene.

Cotransfection of EYFP-AIDA-1a with AβPP-Gal4 in HEK293T cells caused a dramatic reduction (70–80%) in luciferase activity when compared with a control transfection (Fig. 7). This outcome was specifically dependent on the binding of AIDA-1a to AβPP, because cotransfection with AIDA-1bΔAnk-2 exon 14 did not elicit any effect on luciferase activity.

AIDA-1a Inhibits Aβ40 Secretion through Inhibition of γ-Secretase Activity—The outcome of the above-described experiment with AβPP-Gal4 could have several explanations. 1) AIDA-1a might down-regulate the expression of AβPP in a nonspecific manner; 2) AIDA-1a might modulate AβPP processing; 3) AIDA-1a could destabilize and/or reduce nuclear translocation of the AID fragment released upon the processing of AβPP. When we analyzed by Western blot the same samples that we had used for the luciferase assay to verify the expression levels of the transfected proteins, we could not detect any difference that could account for the results seen in the reporter assay (data not shown). We therefore decided to approach the question of whether AIDA-1a could be involved in AβPP processing by employing an Aβ-40-specific enzyme-linked immunosorbent assay. HEK293T cells stably expressing AβPPp995 were transfected with AIDA-1a, AIDA-1bΔAnk-2 exon 14, or empty vector. 24–30 h after transfection the culture medium was replaced with fresh medium, and upon conditioning for 30’, the supernatants were tested for Aβ concentration. AIDA-1a could cause a statistically significant 30% reduction in Aβ production when compared with the controls (Fig. 8). To better determine how AIDA1a inhibits Aβ production, we have analyzed by Western blot the C-terminal fragments of AβPP upon transfection of AIDA-1a, AIDA-1bΔAnk2 exon 14, or empty vector in presence or absence of the γ-secretase inhibitor compound E (Fig. 9). Densitometric analysis demonstrated a consistent 30% increase of C-terminal fragments when AIDA-1a, but not the controls, was transfected in the absence of inhibitors; this difference was abolished by treatment with compound E proving that neither β- nor α-secretase are affected by overexpression of AIDA-1a. We concluded from these experiments that overexpression of AIDA-1a can down-regulate AβPP processing by γ-secretase, thus reducing Aβ secretion.

DISCUSSION

We have recently reported the cloning of three alternatively spliced forms (AIDA-1b, AIDA-1bΔAnk, and AIDA-1a) of a novel protein interacting with the intracellular domain of AβPP (48). In this paper we further analyzed the possible implications of this interaction providing evidence for a potential role for AIDA-1a on AβPP processing.

Comparative sequence analysis of the different isoforms of AIDA-1 proteins revealed an unusual complexity of their exon/intron organization. 26 exons are present in the cDNA sequence of AIDA-1b. Not only can many of these exons be spliced in or out generating different isoforms, but some of them also contain internal splicing sites (i.e. exons 14, 15, and 27). The combination of all these hypothetical splicing events exponentially increases the number of theoretically possible isoforms that can be generated from a single transcript.

We have previously shown how alternative splicing can affect the intracellular localization of AIDA-1 proteins (48). In this work we demonstrated that the binding of AIDA-1s to the cytoplasmic tail of AβPP can be inhibited by the presence, in the primary sequence of AIDA-1 proteins, of a short stretch of 24 amino acids encoded by exon 1. This observation is particularly noteworthy for two sets of reasons. 1) For the first time, we have shown that the interaction of a PTB domain with its elective binding motif can be inhibited by a portion of the PTB-containing protein itself, and 2) alternative splicing events can regulate the binding of AIDA-1s to AβPP, by removal of the inhibitory sequence, thus promoting isoform-specific interactions. At this point the mechanisms responsible for the binding inhibition are not fully elucidated. Does the 24-amino-acid sequence encoded by exon 14 bind to the PTB domain of AIDA-1s thus preventing interactions with other molecules, or does it simply cause a different folding of the protein that renders the PTB domain not accessible to potential interactors? We have not been able to show any interaction between exon 14-containing isoforms and the PTB domain of AIDA-1 by biochemical approaches (data not shown), and ultimately crystallographic studies will be required to address this question.

In this paper we have also shown data, from enzyme-linked immunosorbent assay, Western blotting, and luciferase assays, supporting a function for AIDA-1a as a modulator of AβPP processing. Other AβPP interactors have been previously shown to play roles in altering the cleavage of this transmembrane molecule (35–37, 53–55), but the mechanisms through which this effect is achieved have not been defined yet. AIDA-1a has revealed itself as potentially a very interesting molecule in Alzheimer’s pathogenesis. Not only, as we have previously shown, can it interact with AβPP at endogenous levels, but also the binding and the phenotypic effects resulting from it (such as altered processing of AβPP and change in subcellular localization of AIDA-1a) occur in an isoform-specific manner. The outcome of all these observations is a very complicated pattern of functional interactions between the AIDA-1 family of proteins and AβPP that will require genetics approaches to be elucidated.

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