Regulation of antigen-receptor gene assembly in hagfish

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Variable lymphocyte receptors (VLRs) are antigen receptors in the jawless vertebrates lamprey and hagfish. VLR genes are classified into *VLRA* and *VLRB*, and lymphocytes expressing *VLRA* are T-cell-like, whereas those expressing *VLRB* are B-cell-like in the sea lamprey. Diverse VLR genes are assembled somatically in lymphocytes; however, how the assembly is regulated is still largely unknown. Here, we analyse VLR gene assembly at the single-cell level in the inshore hagfish (*Eptatretus burgeri*). Each lymphocyte assembles and transcribes only one type of VLR gene, either *VLRA* or *VLRB*. In general, monoallelic assembly of VLR was observed, but diallelic assembly was found in some cases—in many of which, one allele was functional and the other was defective. In fact, all VLR-assembled lymphocytes contained at least one functional VLR gene. Together, these results indicate a feedback inhibition of VLR assembly and selection of VLR-positive lymphocytes.

Keywords: allelic exclusion; antigen-receptor gene; gene assembly; variable lymphocyte receptor

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INTRODUCTION

The jawless vertebrates, which are the most basal vertebrates known to exist, have non-immunoglobulin (Ig)-type antigen receptors called variable lymphocyte receptors (VLRs; Pancer *et al*, 2004, 2005; Cooper & Alder, 2006). Diverse VLR genes are generated by somatic assembly; the intervening sequence in an incomplete germline VLR gene is replaced with several leucine-rich repeat (LRR)-coding gene segments flanking the germline VLR gene (Pancer *et al*, 2004, 2005; Rogozin *et al*, 2007). Previously, we proposed a copy-choice/template-switching model for VLR assembly, in which the homologous sequence is used to prime the ordered insertion of LRR segments (Nagawa *et al*, 2007). A gene conversion-like mechanism has also been proposed for VLR

assembly in the sea lamprey (Alder *et al*, 2005; Rogozin *et al*, 2007). VLR gene diversity is achieved not only by allowing the insertion of several LRR segments in various combinations, but also by connecting LRR segments at different junctions (Nagawa *et al*, 2007; Rogozin *et al*, 2007). The VLR structure resembles the concave solenoid shape of the Toll-like receptor (TLR) ectodomain (Kim *et al*, 2007). VLR ligand affinity is comparable to conventional antigen receptors, and VLRs probably bind to antigens in the broad concave surface where sequence variation is greatest (Han *et al*, 2008; Herrin *et al*, 2008; Tasumi *et al*, 2009; Velikovskiy *et al*, 2009).

The conventional antigen receptors that are shared by all jawed vertebrates are the Ig receptor and the T-cell receptor (TCR), both of which contain the Ig-fold. Diverse antigen-receptor genes are generated somatically in lymphocytes through V(D)J recombination (Sakano *et al*, 1979; Tonegawa, 1983; Schatz, 2004). Multiple gene segments—V, D and J—are recombined in numerous combinations (combinatorial diversification), and nucleotide addition and/or deletion at the recombination junctions further diversifies the sequences (junctional diversification). V(D)J recombination is highly regulated to ensure that each lymphocyte expresses a single functional receptor (Schlissel, 2003; Jung & Alt, 2004; Jung *et al*, 2006; Krangel, 2009).

Jawless vertebrates, of which lampreys and hagfish are the only living descendants, also have two types of antigen receptor, *VLRA* and *VLRB* (Pancer *et al*, 2005; Rogozin *et al*, 2007), both of which undergo diversification in lymphocytes. The sea lamprey (*Petromyzon marinus*) has separate VLR-positive lymphocyte populations, each expressing *VLRA* or *VLRB* exclusively. Moreover, *VLRA*-positive lymphocytes are T-cell-like, whereas *VLRB*-positive ones are B-cell-like (Alder *et al*, 2008; Guo *et al*, 2009). However, how the VLR assembly is regulated remains unclear. Here, we analyse VLR gene assembly by single-cell PCR and single-cell reverse transcription-PCR (RT-PCR) in the inshore hagfish (*Eptatretus burgeri*).

RESULTS

Mutually exclusive assembly of *VLRA* and *VLRB*

To analyse *VLRA* and *VLRB* assembly at the single-cell level, approximately 1,000 lymphocyte-like cells were separated from buffy coat leukocytes of an adult hagfish (*E. burgeri*; hagfish-a)

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by single-cell sorting. Mature *VLRA* and *VLRB* genes are generated somatically at either locus (Fig 1A). Although the lengths of assembled VLR genes varied, they were longer than the germline gene for both *VLRA* and *VLRB* (Fig 1B). Among the lymphocytes analysed, approximately one-third of cells (335 cells) contained an assembled *VLRA* (*VLRA*⁺ cells), and about another one-third (303 cells) contained an assembled *VLRB* (*VLRB*⁺ cells; Fig 1C; Table 1). No lymphocytes contained assembled VLRs from both *VLRA* and *VLRB* genes (Table 1). These results indicate that *VLRA* and *VLRB* assembly is mutually exclusive.

Some *VLRA*⁺ lymphocytes contained identical *VLRA*s

Clonal expansion and somatic hypermutation of *VLRA* genes have been shown in the pacific hagfish (*Eptatretus stoutii*; Pancer et al, 2005). Therefore, PCR products of assembled VLR genes (316 *VLRA* and 280 *VLRB*) in each lymphocyte were isolated and directly sequenced from the 5' end to the middle of the variable region. All *VLRB* sequences were unique, but some *VLRA* sequences were identical; further analysis showed sequence identity throughout the variable region (supplementary Table S1 online), and there was no indication of somatic hypermutation. About 30% (86/316) of the *VLRA* genes analysed were present in more than one lymphocyte. As the diversity generated through VLR assembly is extraordinary (Alder et al, 2005; Pancer et al, 2005), it is unlikely that the same VLR genes are generated by independent assembly. Thus, proliferation of particular *VLRA*⁺ lymphocytes may have occurred after completion of VLR gene assembly in response to immunological stimulation.

VLR assembly is monoallelic, but in some cases diallelic

In the Japanese lamprey (*Lethenteron japonicum*), we previously showed that only one functional allele of the *VLRB* gene is assembled in a single lymphocyte (Nagawa et al, 2007). Monoallelic assembly has also been observed for the *VLRA* and *VLRB* genes in the sea lamprey (Guo et al, 2009). Here, single-cell PCR analysis of hagfish lymphocytes indicated monoallelic assembly of the VLR genes; PCR products from an assembled VLR as well as from the germline VLR were observed for most lymphocytes (Fig 1C; Table 1). Interestingly, in a small number of cells, no PCR product of the germline length was seen, but two products of the assembled *VLRA* or *VLRB* gene were observed and were confirmed by sequence analysis to be two distinct assembled VLRs (Fig 1D). Diallelic VLR assembly was identified in about 7% of *VLRA*⁺ cells and in about 4% of *VLRB*⁺ cells in hagfish-a (Table 1). The fact that the germline VLR was not detected in lymphocytes with two assembled VLRs indicates that there was no contamination from other lymphocytes in the single-cell PCR assay. Moreover, sequence analysis with maternal and paternal polymorphisms in the 3' constant region of the *VLRA* gene showed that when one *VLRA* was generated, VLR assembly was not restricted to either allele (supplementary Table S2 online). By contrast, when two assembled *VLRA* genes were generated, one was from the maternal allele and the other was from the paternal allele, supporting diallelic VLR gene assembly in a single lymphocyte (supplementary Table S2 online).

Both VLR alleles are transcribed

In addition to the single-cell VLR assembly analysis, VLR transcription was examined at the single-cell level. *VLRA*, *VLRB*

Fig 1 | Assembly of *Eptatretus burgeri* VLR genes in single lymphocytes. (A) A schematic representation of *VLRA* and *VLRB* assembly. Mature *VLRA* and *VLRB* genes are generated somatically at each locus, which are distant from each other but on the same chromosome (Kasamatsu et al, 2007). The intervening sequence of the germline VLR is replaced with several LRR gene segments. Gene segments are indicated as follows: signal peptide (grey), amino-terminal LRR plus the first LRR (blue), LRR (green), connecting peptide (red), 5' half of the carboxy-terminal LRR (orange) and 3' half of the C-terminal LRR (cream). The number of LRR modules in the middle (green) varies in the assembled VLR genes: one to nine in *VLRA* and one to seven in *VLRB*. (B) A schematic representation of single-cell PCR assay of *VLRA* and *VLRB*. Primer pairs (arrows) were designed to amplify the germline (shorter) and assembled (longer) VLR gene simultaneously. (C) Agarose gel electrophoresis of single-cell PCR products. Data reflect five representative *VLRA* or *VLRB* PCR products (hagfish-a). A germline product along with an assembled product was observed in most lymphocytes. (D) Diallelic VLR assembly. Data reflect eight representative PCR products of the minority of lymphocytes in which two assembled products of either *VLRA* or *VLRB* were amplified (hagfish-a). LRR, leucine-rich repeat; VLR, variable lymphocyte receptor; *VLRA*⁺/*VLRB*⁺ cells, lymphocytes with assembled *VLRA*/*VLRB*.

Table 1 | VLR assembly determined by single-cell PCR analysis

VLR assembly	Monoallelic		Diallelic	Lymphocyte total
	Germline+	Germline−		
VLRA	237	73	25	335
VLRB	250	41	12	303
VLRA and VLRB	0	0	0	0
None	—	—	—	280

VLRA and VLRB genes were amplified from 918 sorted lymphocytes of hagfish-a (*Eptatretus burgeri*). Most lymphocytes showed a single assembled VLR product as well as a germline VLR product (monoallelic, germline+), although some lymphocytes showed an assembled VLR product without a germline VLR product (monoallelic, germline−), probably owing to allele dropout that can occur in single-cell PCR analysis (Nagawa et al, 2007). A minority of lymphocytes showed two assembled VLR products (diallelic). No lymphocytes contained assembled VLRA from both VLRA and VLRB genes. In some cells, a germline VLR gene was amplified, but an assembled VLR gene was not, probably owing to the presence of non-lymphocytes in the sorted cells or to allele dropout.
VLR, variable lymphocyte receptor.

and control glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts were amplified from each lymphocyte by using single-cell RT-PCR analysis (Fig 2A). Assembled (longer than germline) and germline VLR transcripts were often detected together in a single lymphocyte and in comparable quantity (Fig 2B; Table 2; supplementary Table S3 online), indicating that both alleles, whether assembled or not, were expressed at similar levels. Transcription of both VLRA and VLRB in a single lymphocyte was not observed (Fig 2B; Table 2; supplementary Table S3 online). Moreover, two distinct assembled VLRA transcripts were seen in a small number of lymphocytes (Fig 2B; Table 2; supplementary Table S3 online), suggesting that VLRA generated by diallelic assembly are both expressed in a single lymphocyte.

Defective VLR genes in diallelic assembly

Analysis of VLRA generated by diallelic assembly showed a high occurrence of sequence defects such as frame shifts and in-frame stop codons. Many lymphocytes (7 out of 19 VLRA+ cells and 10 out of 12 VLRB+ cells) had one defective and one functional VLR gene (Table 3; supplementary Table S4 online). Although both assembled VLR genes were functional in some lymphocytes, no lymphocyte contained two defective VLRA genes (Table 3; supplementary Table S4 online). By contrast, in lymphocytes with monoallelic VLR assembly, all VLR genes were functional (Table 3; supplementary Table S4 online). Most defective VLR genes contained base deletions or additions (−2, −1, +1, +2 and +7) that resulted in a frame shift (Fig 3). Alignment of available LRR sequences indicated that frame-shift sites were not confined to any specific point in an LRR-coding module (Fig 3B). Except for a defective LRR module, LRR sequences in VLR genes were all functional and in order (Fig 3A), and therefore probably did not reflect the intermediates of incomplete VLR assembly (Alder et al, 2005; Nagawa et al, 2007). A small fraction of defective VLR genes contained an in-frame stop codon (Fig 3), which might have resulted from incorporation of a particular LRR segment containing an in-frame stop codon. The same LRR sequence containing a stop codon was found in different assembled VLR genes (VLRA #7

Fig 2 | Analysis of VLR transcription in single lymphocytes. (A) A schematic representation of single-cell RT-PCR assay of VLRA. VLRB was analysed similarly. The blue arrows represent the RT primers, the blue lines represent first-strand cDNA and the black arrows represent PCR primers designed to amplify the cDNAs of the germline (shorter) and assembled (longer) VLR gene. VLRA and VLRB forward PCR primers annealed upstream from introns (~6 kb for VLRA and ~0.2 kb for VLRB). (B) Agarose gel electrophoresis of single-cell RT-PCR products. Data reflect a total of nine representative VLRA or VLRB PCR products (hagfish-b, *Eptatretus burgeri*). VLRA (lanes 1–3, 7–9) or VLRB (lanes 4–6) transcripts were detected from both alleles. In some cases, lymphocytes contained transcripts from two assembled VLRA genes (lanes 7–9). GAPDH transcription was analysed as a control. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; VLR, variable lymphocyte receptor; VLRA+/B+, lymphocytes with assembled VLRA/B.

and #8, and VLRBs #8 and #9), and the stop codon was present in the genomic DNA (supplementary Fig S1 online).

DISCUSSION

We have shown that VLRA and VLRB are assembled and transcribed in a mutually exclusive manner in hagfish, indicating that VLRA+ and VLRB+ cells belong to distinct lymphocyte populations, as reported for the sea lamprey (Guo et al, 2009). VLR assembly was primarily monoallelic, but diallelic assembly was observed in some cases. In the case of the Ig and TCR genes, V(D)J recombination is regulated tightly at many levels (Schlissel, 2003; Jung & Alt, 2004; Jung et al, 2006; Krangel, 2009). V(D)J recombination activates the transcription of an antigen-receptor gene by placing the promoter and enhancer elements in close proximity. In hagfish, however, transcription was not linked with the assembled allele of the VLR gene; detection of assembled and germline transcripts in a single lymphocyte was common. It seems that VLRA and VLRB gene loci are activated in a mutually exclusive way regarding transcription and gene assembly, the latter of which is regulated primarily by monoallelic assembly. The mechanisms that ensure mutually exclusive assembly and transcription of the VLR gene have yet to be determined.

Table 2|Expression of VLR genes determined by single-cell RT-PCR analysis

VLR expression	One assembled VLR transcript		Two assembled VLR transcripts	Lymphocyte total
	Germline+	Germline−		
VLRA	33	11	3	47
VLRB	7	2	0	9
VLRA and VLRB	0	0	0	0
None	—	—	—	19

VLRA and VLRB transcripts were amplified by RT-PCR using 75 sorted lymphocytes of hagfish-b (*Eptatretus burgeri*). Transcripts were frequently detected from both alleles at the VLR locus. Most lymphocytes showed an assembled VLR transcript as well as a germline transcript. Some lymphocytes showed two assembled VLR transcripts. No lymphocytes contained both VLRA and VLRB transcripts. In some cells, no assembled VLR transcript was amplified. GAPDH transcription was analysed as a control, and was detected in 69 out of 75 (92%) lymphocytes.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; VLR, variable lymphocyte receptor.

Table 3|Sequence analysis of assembled VLRs in lymphocytes with monoallelic or diallelic VLR assembly

VLR assembly	Monoallelic		Diallelic			Lymphocyte total
	Defective	Functional	Both defective	One defective, one functional	Both functional	
VLRA	0	51	0	7	12	70
VLRB	0	56	0	10	2	68

The variable regions of assembled VLRA and VLRB genes were sequenced in 138 sorted lymphocytes of hagfish-a (*Eptatretus burgeri*). Defective VLR genes were frequently identified in lymphocytes with diallelic VLR assembly, whereas no defective VLR was found in lymphocytes with monoallelic VLR assembly (Table 1, monoallelic, germline+). VLR, variable lymphocyte receptor.

V(D)J recombination is regulated to ensure that each T cell or B cell expresses a unique antigen receptor (allelic exclusion). Production of a functional gene leads to feedback inhibition of further rearrangement (Jung et al, 2006; Krangel, 2009). If initial recombination generates an out-of-frame gene, rearrangement continues on the other allele. As a result, a large fraction of lymphocytes contain one functional and one defective allele. In hagfish, when VLR assembly was diallelic, there was often one defective and one functional VLR. By contrast, monoallelic assembly resulted only in functional VLR genes (Table 3). The fact that VLR assembly was generally monoallelic and that defective VLR genes were often found in lymphocytes with diallelic VLR assembly suggests a feedback inhibition mechanism by which production of functional VLR protein might inhibit further VLR gene assembly. When VLR assembly generates a defective VLR on one allele, assembly might continue on the other allele.

Occasionally, diallelic assembly resulted in two functional VLR genes that were transcribed simultaneously in a single lymphocyte. An example of diallelic VLRB transcription, in which the assembled genes were both functional, was previously reported in the sea lamprey (Pancer et al, 2004). In the present study, when two VLRA genes were detected in a single cell and both were functional, one was from the maternal allele and the other from the paternal allele (supplementary Table S2 online). This indicates that two functional VLR genes were assembled in a single lymphocyte. The result suggests that allelic exclusion might not be a strict rule in hagfish. It is possible that VLR assembly occurred simultaneously on both alleles in a small fraction of lymphocytes.

Another possibility is that the protein product of the VLR gene that assembled first was unable to participate in feedback inhibition of VLR assembly on the other allele. Notably, two productive Ig heavy-chain genes can also be generated in a single cell by V(D)J recombination; in such cases, one of them is usually unable to produce the feedback signal efficiently (ten Boekel et al, 1998; Schlissel, 2003).

The identification of defective assembled VLR genes indicates that VLR assembly might generate a non-functional VLR gene. All VLR-assembled lymphocytes had at least one functional VLR gene, however, suggesting that only those lymphocytes expressing a functional VLR protein enter the peripheral blood. This is supported by the observation that lymphocytes expressing only a non-functional VLR were not detected in the buffy coat leukocyte population. Conversely, VLR assembly could generate highly diverse VLR genes by combinatorial and junctional change, such that a harmful self-reactive VLR might be produced. Although thymus-like tissue has not been identified in jawless vertebrates (Bajoghli et al, 2009), the VLR-based immune system might contain a highly specialized site for lymphocyte selection based on the expression of a functional but non-self-reactive VLR.

As the hagfish genome sequence has not been completed, it is unclear whether the sequence defects observed in certain VLRs originated from a defective LRR segment in the genome or whether they were generated during VLR assembly. Some of the defective LRRs might have resulted from incorporation of a particular LRR segment containing an in-frame stop codon

Fig 3 | Sequence defects in VLR genes found in lymphocytes with diallelic VLR assembly. (A) A schematic representation of defective VLRA and VLRB genes from lymphocytes with diallelic assembly. All sequence defects were located in LRR (green) modules and are indicated as follows: arrowhead, frame shift; red X, in-frame stop codon. In VLRA #7, an in-frame stop codon (7-1) and a frame shift (7-2) were both found. VLRA #3 and #6 were identified by single-cell RT-PCR using hagfish-b (*Eptatretus burgeri*) and hagfish-c, respectively. The rest were identified by single-cell PCR using hagfish-a. The GenBank accession numbers are: VLRA #1–9, AB519982–AB519990; VLRB #1–10, AB520071–AB520080. (B) The nucleotide sequences around the defect sites shown in (A). The sequence defects are indicated as follows: open boxes, possible frame-shift sites; red boxes, in-frame stop codons. For sequences containing frame shifts, short repeats of a specific nucleotide near possible frame-shift sites are underlined, and the number of extra (+) or missing (–) nucleotides is indicated on the right. Consensus amino-acid residues (>90% identity) are indicated above the sequences. LRR, leucine-rich repeat; RT-PCR, reverse transcription-PCR; VLR, variable lymphocyte receptor.

(supplementary Fig S1 online). The sequences around possible frame-shift sites were all different in the 15 lymphocytes examined, and at some of those sites there was a short repeat of a specific nucleotide (AAA, for example; Fig 3). Slippage of DNA polymerase might occur around these repeat sequences during VLR gene assembly. It is also possible that frame shifts in assembled VLR genes are caused by incorporation of a particular germline LRR segment with a pre-existing frame shift.

At an early stage of vertebrate evolution, there seems to have been a demand for adaptive immunity, and jawless and jawed vertebrates developed distinct adaptive immune systems mediated by Ig-type or VLR-type antigen receptors (Cooper & Alder, 2006; Amemiya *et al*, 2007; Litman *et al*, 2007; Flajnik & Du Pasquier, 2008; Kasahara *et al*, 2008; Flajnik & Kasahara, 2010). Many

features seem to be shared in the two types of adaptive immune systems. For example, VLRA-positive lymphocytes are T-cell-like, whereas VLRB-positive lymphocytes are B-cell-like (Guo *et al*, 2009). For antigen-receptor gene assembly, diverse VLRA and VLRB genes are assembled somatically through combinatorial and junctional diversification mechanisms, and the assembly is mutually exclusive and monoallelic (Pancer *et al*, 2004; Alder *et al*, 2005; Nagawa *et al*, 2007; Rogozin *et al*, 2007; Guo *et al*, 2009). The data presented here suggest that VLR assembly is regulated by feedback inhibition and that VLR-positive lymphocytes are selected before entry into the peripheral blood. Examination of the VLR immune system in the jawless vertebrates will continue to offer valuable insight into the evolution of adaptive immunity.

METHODS

Animals and buffy coat leukocytes. Three adult hagfish (*E. burgeri*; hagfish-a, hagfish-b, hagfish-c) were captured in the Pacific Ocean off the coast of Misaki, Kanagawa Prefecture, Japan. The hagfish were sedated in 500 mg/l of MS222 (ethyl *m*-aminobenzoate methanesulphonate; Nacalai Tesque, Kyoto, Japan) and peripheral blood samples were collected from the caudal subcutaneous sinus into hagfish phosphate-buffered saline (Pancer *et al*, 2005) containing 30 mM of ethylenediaminetetraacetate. Buffy coat leukocytes were collected and lymphocyte-like cells were singly sorted as described (Pancer *et al*, 2004, 2005) using FACS Vantage (BD Biosciences, San Jose, CA, USA). All animal experiments were performed in accordance with the ethical guidelines of the University of Tokyo.

Amplification of germline and assembled VLRA and VLRB by single-cell PCR. Lymphocyte-like cells from hagfish-a were sorted and seeded at one cell per well into 96-well plates (0.2 ml, round bottom) containing 10 µl of LA-Taq PCR buffer (Takara Bio, Shiga, Japan). Single-cell PCR was performed as described by Nagawa *et al* (2007), with the following modifications: (i) primers were designed to anneal outside of the variable regions for amplification of both germline and assembled VLRA and VLRB; and (ii) VLRA and VLRB were amplified simultaneously in an initial PCR amplification reaction (30 cycles), and separately in a second (nested) PCR (30 cycles). When an assembled VLR was amplified without a germline VLR product, an alternative second (nested) PCR (30 cycles) was performed to amplify a germline VLR with a different primer set, of which a forward primer was designed within the intervening sequence (Malecek *et al*, 2008). All primer information is provided in supplementary Table S5 online.

Amplification of VLRA and VLRB transcripts by single-cell RT-PCR. Lymphocyte-like cells from hagfish-b and hagfish-c were sorted and seeded at one cell per well into 96-well plates (0.2 ml, round bottom) containing 10 µl of resuspension buffer and 1 µl of lysis enhancer (Invitrogen, Carlsbad, CA, USA). RT was performed according to the instructions in the CellsDirect Two Step qPCR kit (Invitrogen), with the following modifications: first-strand (cDNA) synthesis was performed by using three gene-specific reverse primers, and annealing buffer (included in SuperScript3 Supermix; Invitrogen) was added. The resulting cDNA was precipitated with ethanol and used for further experiments. In the same way as in single-cell PCR, VLRA, VLRB and GAPDH were amplified simultaneously in an initial PCR (30 cycles) and separately in a second (nested) PCR (20 cycles).

Data deposition. The VLR sequence data reported in this paper have been submitted to the GenBank database under accession numbers AB519822–AB520080.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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