Biochemical and Biophysical Research Communications 401 (2010) 53–57

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Torque generation by one of the motor subunits of heterotrimeric kinesin-2

Xiaoyu Pan, Seyda Acar, Jonathan M. Scholey *

Department of Molecular and Cell Biology, University of California at Davis, Davis, CA 95616, United States

ARTICLE INFO

Article history:
Received 2 September 2010
Available online 15 September 2010

Keywords:
Heterotrimeric kinesin-2
Torque generation
Intraflagellar transport

ABSTRACT

Heterotrimeric kinesin-2 motors [1,2] transport intraflagellar transport (IFT)-particles from the base to the tip of the axoneme to assemble and maintain cilia [3–10]. These motors are distinct in containing two non-identical motor subunits together with an accessory subunit [1,11–15]. We evaluated the significance of this organization by comparing purified wild type kinesin-2 holoenzymes that support IFT *in vivo*, with mutant trimers containing only one type of motor domain that do not support IFT *in vivo*. In motility assays, wild type kinesin-2 moved microtubules (MTs) at a rate intermediate between the rates supported by the two mutants. Interestingly, one of the mutants, but not the other mutant or the wild type protein, was observed to drive a persistent counter-clock-wise rotation of the gliding MTs. Thus one of the two motor domains of heterotrimeric kinesin-2 exerts torque as well as axial force as it moves along a MT, which may allow kinesin-2 to control its circumferential position around a MT doublet within the cilium.

1. Introduction

Intraflagellar transport (IFT), the motor-dependent movement of IFT particles along the axoneme, is critical for the assembly, maintenance, and function of motile and sensory cilia, and, consequently, this process underlies ciliary motility, cilium-based signaling, and ciliopathies [3,4]. A large body of evidence supports the hypothesis that members of the kinesin-2 family of microtubule (MT)-based motors play critical roles in IFT by driving the anterograde transport of IFT particles, together with associated cargo, from the base of the axoneme to the distal tip [1–17]. It has been proposed that heterotrimeric kinesin-2 serves as the "core" anterograde IFT motor that builds the axoneme foundation in many cell-types, and that this motor's activity can be augmented by "accessory" kinesins that function to confer cilia-specific properties [18].

For example, the simple nervous system of the nematode *Caenorhabditis elegans* contains 60 sensory neurons whose dendritic endings use cilia of distinct morphology and molecular composition to detect a variety of sensory inputs [19]. In the head of the animal, bundles of amphid channel cilia on chemosensory neurons detect hydrophilic molecules in the environment, whereas the adjacent "wing" cilia detect volatile, hydrophobic odorant molecules [20,21]. Based on the use of time-lapse fluorescent microscopy-based transport assays in living animals, combined with *in vitro* motility assays with purified motor proteins, it has been proposed that two members of the kinesin-2 family, heterotrimeric kinesin-II and homodimeric OSM-3 cooperate in a partially redundant fashion to assemble the channel cilia [9,16,20–29]. Specifically, the "middle" or "initial" segment of the axoneme, which serves as the cilium foundation consisting of 9 doublet MTs, is built by the kinesin-II and OSM-3-directed movement of IFT particles, with either motor being dispensable for this process. OSM-3 alone assembles 9 singlet MTs which are required for cilium-based signaling on the distal endings of the axoneme foundation [9,16]. These pathways of IFT appear to be modulated in a cilia-specific fashion to produce distinct types of cilia in *C. elegans* itself [20,24] although there is evidence that the extension of distal singlets on the endings of sensory cilia by OSM-3 homologs may be a general phenomenon [30].

In previous work we studied cooperative motility between purified heterotrimeric kinesin-II and homodimeric OSM-3 and we replicated, *in vitro*, the rates of IFT observed along the sensory cilia of living animals. We observed that kinesin-II moves MTs at a slow rate of 0.5 μm/s, OSM-3 moves MTs at a fast rate of 1.3 μm/s, and mixtures of the two motors move MTs at intermediate rates. The results support a simple mechanical competition model in which the two motors move the same IFT particle at an intermediate rate of 0.7 μm/s along the axoneme middle segment [16,18]. Thus, this work supported the hypothesis that these two distinct kinesin-2 holoenzymes cooperate in a partially redundant manner to drive two sequential IFT pathways, but did not address the functional significance of the presence of two non-identical motor subunits within the kinesin-II holoenzyme itself [28].

A distinct property of all heterotrimeric kinesin-2 motors is the presence of two non-identical motor subunits, together with an accessory KAP subunit. Heterodimerization of the two motor

* Corresponding author. Address: Department of Molecular and Cell Biology, University of California at Davis, One Shields Avenue, Davis, CA 95616, United States. Fax: +1 530 752 3085.
E-mail address: jmscholey@ucdavis.edu (J.M. Scholey).

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doi:10.1016/j.bbrc.2010.09.007

subunits appears to be favored over homodimerization but the functional significance of this organization is unknown [31–34]. This problem was previously addressed in vertebrates using comparative *in vitro* motility assays in which engineered chimeric motor subunit heterodimers versus homodimers were compared in terms of their motility rates; significant differences were observed in one study but not in the other [34,35]. In the current study, we addressed this by investigating possible functional differences between the two non-identical motor subunits present in the *C. elegans* heterotrimeric kinesin-2 motor, kinesin-II [9,16].

2. Materials and methods

2.1. Cloning, expression, and purification of recombinant kinesin-II mutants

The KLP-20 motor/KLP-11 stalk–tail and the KLP-11 motor/KLP-20 stalk–tail genes, containing stop codons in the pDONR-221 vectors, were inserted into pDEST8 by Gateway LR recombination (Invitrogen) and were subsequently cloned into Bacmid, transfected into Sf9 cells for expression and purification as described previously [16]. The only exception is that the kinesin-II(homo-20), unlike kinesin-II and kinesin-II(homo-11), could not be purified by Talon column affinity chromatography using a C-terminal 6xHis-tagged KAP-1 together with untagged KLP-20 motor/KLP-11 stalk–tail and KLP-20 (the reason for this is unknown). Instead, we cloned and expressed C-terminal 6xHis-tagged KLP-20 and untagged KAP-1 using the same baculovirus system and successively purified the kinesin-II(homo-20) by co-infection of the viruses containing the two genes mentioned above together with untagged KLP-2011.

2.2. Hydrodynamic analysis and motility assays

The *in vivo* motility assays and *in vitro* microtubule gliding assays were performed as previously described [16], details of the assay can be found in [40]. A Superose 6 10/300 (GE Healthcare) was used for gel filtration analyses. The assay buffer contained 80 mM PIPES, pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA, 200 mM NaCl.

2.3. Strains

The wild type strain was N2 Bristol. The following mutant alleles were used in this study: *kap-1(ok676)*, *klp-11(tm324)*, *osm-3 (p802)*, *dpy-20(e1282)*, *osm-6(p811)*, *mnIs17 [osm-6::GFP, unc-36(+)]*. All strains were grown at 20 °C.

2.4. Expression vectors and construction of transgenic worms

The expression vector pCJF8 containing an *osm-5* promoter region, followed by polycloning sites and CFP coding sequences was generously provided by [41]. A Gateway cassette (Invitrogen) was inserted into the SmaI site. KLP-2011 containing the head domain of KLP-20 and stalk–tail region of KLP-11, was generated using a PCR fusion strategy, and the same strategy was used to create the KLP-11 motor/KLP-20 stalk–tail [42]. The PCR products were confirmed by sequencing after cloning them into plasmid pDONR221 (Invitrogen). The stop codons between target genes were removed by site-directed mutagenesis using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The target genes were inserted into the Gateway cassette in modified pCJF8 by Gateway recombination (Invitrogen). Worms were injected with plasmid constructs at 2–10 ng/μl along with markers containing 100 ng/ul of *rol-6* and/or *dpy-20*. Transgenic worms carrying the injected plasmids were identified by their right-handed roller or dumpy phenotype [43].

2.5. Fluorescence microscopy

IFT was assayed as described previously [9,25,26]. For an extended description of the methods used please see Hao et al. [40].

3. Results and discussion

C. elegans kinesin-II consists of the two motor subunits, KLP-20 and KLP-11, and the accessory subunit, KAP-1 (former names CeKRP85, CeKRP95, CeKAP) [16,28]. Wild type subunits co-assemble into active, heterotrimeric complexes that can be purified using the baculovirus system (Table 1 and Fig. 1B–E) [16] and they can complement loss-of-function mutants *in vivo* (Table 2). To compare the properties of native kinesin-II containing the two non-identical motor domains with kinesin-II complexes containing two KLP-20 motor domains or two KLP-11 motor domains, we adapted the strategy of [34] as modified by [35], involving the co-expression of chimeric and wild type motor subunits, but we also included the KAP subunit to permit testing of heterotrimer formation (Fig. 1A). Chimeric motor subunits expressed in ciliated chemosensory neurons fail to complement loss-of-function mutants (Table 2), but they do co-assemble into active heterotrimeric complexes *in vitro* (Fig. 1B–E, Table 1). For example, we used the baculovirus system to express and purify (i) "kinesin-II(homo-20)", a complex of KLP-20: KLP-20 motor/KLP-11 stalk–tail: KAP-1; (ii) "kinesin-II(homo-11)", a complex of KLP-11: KLP11 motor/KLP-20 stalk–tail: KAP-1; and (iii) "kinesin-II(double chimera)" a complex of KLP-11 motor/KLP-20 stalk–tail: KLP-20 motor/KLP-11 stalk–tail:KAP-1. Based on hydrodynamic analysis and gel densitometry, kinesin-II(homo-20), kinesin-II(homo-11) and kinesin-II(double chimera) all behave as heterotrimeric complexes of two motor subunits and a KAP subunit, like "wild type" kinesin-II (Fig. 1B–E, Table 1). This reveals that heterotrimeric kinesin-2 motors do not require two non-identical motor domains in order to co-assemble with the KAP subunit.

Purified kinesin-II, kinesin-II(homo-20), kinesin-II (homo-11) and kinesin-II(double chimera) were all active in MT motility assays under standard assay conditions (Table 1; Fig. 1F and G). We previously noted that, in order to replicate the rate of motility displayed by kinesin-II along cilia, it was necessary to vary the PIPES buffer concentration slightly [16]. Using this strategy we optimized conditions for each motor and observed slight differences in the maximal rates of MT gliding driven by these motors (Fig. 1F). The different maximal rates observed for kinesin-II, kinesin-II(homo-20), kinesin-II(homo-11) and kinesin-II(double chimera) under these conditions (i.e. 0.55, 0.4, 0.2, and 0.3 μm/s, respectively) were somewhat smaller than those published for the vertebrate KIF3 heterodimers, KIF3A/3B, KIF3A/3A and KIF3B/3B (i.e. 0.2, 0.04, and 0.4 μm/s, respectively [35]), but it is unknown if this is due to the influence of the KAP subunit, which is present in our preparations (Fig. 1B–E). We also noted that, under standard assay conditions, all the motors drove motility according to simple Michaelis–Menten kinetics with respect to MgATP, displaying linear double reciprocal plots (Fig. 1G). However, both kinesin-II(homo-20) and kinesin-II(homo-11) displayed significantly lower K_m values than the wild type and double chimera, indicating a higher apparent affinity for MgATP (Fig. 1G, Table 1). Thus heterotrimeric kinesin-II motors containing identical versus non-identical motor domains are able to drive MT-based motility, but display subtle differences in this activity.

Unlike wild type kinesin-II, the three mutant complexes were inactive at low PIPES concentrations, but within the range of PIPES concentrations which supported motility (using 5 mM [Mg–ATP] as the fuel), the rate of MT gliding driven by kinesin-II was consistently found to be the average of the faster rate driven by kinesin-II(homo-20) and the slower rate driven by

Table 1
Biochemical properties of recombinant kinesin-II and its mutants.

	Wild type	Homo-20	Homo-11	Double chimera
S value	7.6	7.2	7.6	7.8
Stokes radius (nM)	7.4	9.0	9.0	8.2
Molecular weight (Da)	2.3×10^5	2.7×10^5	2.8×10^5	2.6×10^5
Velocity (μm/s)[a]	0.55 ± 0.04	0.39 ± 0.02	0.21 ± 0.07	0.27 ± 0.06
V_{max} (μm/s)	0.26	0.28	0.15	0.2
K_m (mM)	0.28	0.084	0.051	0.54
Rotation pitch (μm)	None	None	1.31 ± 0.51	None

[a] These velocities were measured under optimized conditions (PIPES and MgATP) for each molecule.

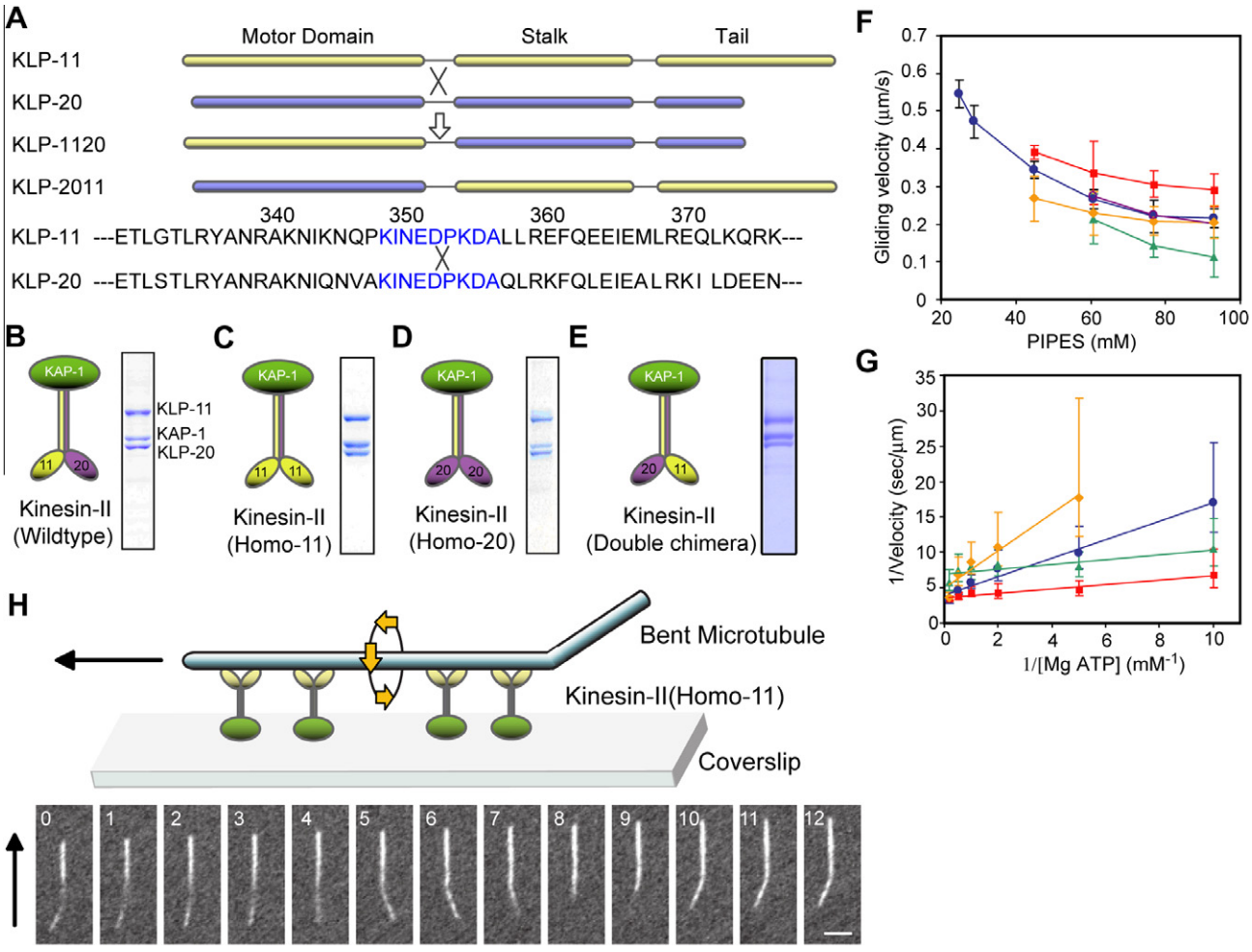

Fig. 1. Recombinant kinesin-II chimeras form heterotrimeric complexes. (A) Diagram showing the sequences of KLP-11, KLP-20, and the regions of exchange for chimeric KLP-20 motor/KLP-11stalk–tail (KLP-2011) and KLP-11 motor/KLP-20stalk–tail (KLP-1120). (B–E) The components and SDS gels of purified recombinant kinesin-II (B), kinesin-II(homo-11) (C), kinesin-II(homo-20) (D), and kinesin-II(double chimera) (E). (F–H) *In vitro* motility assay results of recombinant kinesin-II chimeras. (F) Velocities of MT gliding driven by kinesin-II (blue circle), kinesin-II(homo-20) (red), kinesin-II(homo-11) (green), and kinesin-II(double chimera) (yellow) in 5 mM [Mg–ATP] and different PIPES concentrations. The kinesin-II chimeras did not bind to microtubules at PIPES concentrations below 40 mM for kinesin-II(homo-20) and kinesin-II(double chimera) or 60 mM for kinesin-II(homo-11). The velocities of gliding driven by kinesin-II were close to the average of the velocities of the kinesin-II(homo-11) and kinesin-II(homo20) motors. (G) Double reciprocal plots of the rates of MT gliding driven by kinesin-II (blue), kinesin-II(homo-20) (red), kinesin-II(homo-11) (green), and kinesin-II(double chimera) (yellow) versus [Mg-ATP]. (H) Rotation of gliding microtubules by kinesin-II(homo-11). The numbers show the time in seconds and the black arrows indicate the direction of translocation of the microtubules. Bar: 2.5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

kinesin-II(homo-11) (Fig. 1F). The kinesin-II(double chimera) displayed very similar motility to the wild type protein at 80 or 100 mM PIPES, but became more similar to the kinesin-II(homo-11) at lower PIPES concentrations (Fig. 1F). The results are generally consistent with the idea that the rate of motility of kinesin-2 complexes represents a composite of the activity of its two constituent motor subunits.

The most striking difference observed in these motility assays was the induction of MT rotation by one of the two homo-motor complexes (Fig. 1H). Specifically, kinesin-II(homo-11) drives the rotation of sliding MTs in motility assays, exerting torque that rotates the MTs counter-clockwise with pitch 1.3 μm as they are translocated axially with their minus-ends leading (Table 1). In contrast, wild type kinesin-II, kinesin-II(double chimera) and

Table 2
Velocity of IFT particles and kinesin-II in kinesin-2 mutant animals.

Anterograde motility of	Strain	Average velocities (μm s^{-1})			
		Middle segment	*n*	Distal segment	*n*
IFT Particle OSM-6::GFP	*klp-11*	1.23 ± 0.17	101	1.17 ± 0.18	146
	klp-11; KLP-11(wt)	0.76 ± 0.13	185	1.27 ± 0.23	109
	klp-11; KLP-2011	1.18 ± 0.13	137	1.23 ± 0.14	91
	kap-1	1.15 ± 0.14	202	1.14 ± 0.18	173
	kap-1; KAP-1(wt)	0.77 ± 0.14	142	1.17 ± 0.17	106
Kinesin-II subunit KAP-1::GFP	*klp-11*	None		None	
	klp-11; KLP-11(wt)	0.65 ± 0.09	125	None	
	klp-11; KLP-2011	None		None	
	kap-1; osm-3; KAP-1(wt)	0.58 ± 0.11	105	None	
	klp-11; osm-3; KLP-11(wt)	0.45 ± 0.10	97	None	
	klp-11;osm-3; KLP-2011	None		None	

C. elegans loss-of-function *klp-11* and *kap-1* mutant animals have full length amphid channel cilia which are built by OSM-3 in the absence of kinesin-II function, and they display abnormally fast OSM-3 driven transport of IFT particles at 1.1–1.3 μm/s along the ciliary middle segments [9]. The slower, wild type rate of transport of the IFT particle protein, OSM-6::GFP (0.7 μm/s) along ciliary middle segments was restored by the wild type KLP-11 and KAP-1 subunits but not by the chimeric KLP-2011 subunit. Similarly, KLP-11(wt), but not mutant KLP-2011, also restored kinesin-II activity as monitored by KAP::GFP transport in *klp-11* mutants. Double mutants lacking both KLP-11 and OSM-3 function lack the entire ciliary axoneme. Wild type KLP-11 and KAP-1 subunits restored the assembly of axoneme middle, but not distal, segments and slow IFT at rates characteristic of kinesin-II alone, but the chimeric subunit did not. (wt, wild type; n, number of fluorescent particle.)

kinesin-II(homo-20) did not drive rotation of translocating MTs. A similar rotation of gliding MTs has been observed in assays of the mitotic kinesin-14, Ncd [36] and of monomeric kinesin-1 constructs [37] and is proposed to be a general property of non-processive motors. The counter-clockwise rotation mediated by kinesin-II(homo-11) is of the same handedness as that driven by the kinesin-1 constructs [37] whereas the minus-end-directed kinesin-14 mediates clock-wise rotation of MTs [36]. Our observation that the KLP-11 motor domain rotates a moving MT whereas the KLP-20 motor domain does not, suggests functional differentiation between the two non-identical motor domains within the wild type kinesin-II heterotrimer. Thus it is possible that the KLP-11 head may be a low processivity motor that exerts torque as well as axial force on a MT whereas the more processive KLP-20 motor domain may counteract the torque component to prevent rotation of kinesin-II relative to the MT track, possibly facilitating processive motility of IFT particles *in vivo*. Interestingly, optical trap studies done independently by Okten and colleagues [38] support the idea that KLP-11 is non-processive, whereas KLP-20 can drive persistent processive motility. Conversely it is possible that a KLP-11 homodimer can actually move processively along a MT, but in the optical trap, its rotational motion around the circumference of the immobilized MT may hinder its translational movement, giving rise to low processivity.

Thus, chimeric motor subunits are capable of translocating MTs and forming heterotrimers *in vitro*, but appear to be non-functional *in vivo* (Table 2). Based on fluorescence microscopy, the KLP-2011 chimera is expressed at similar levels to the wild type subunit in ciliated chemosensory neurons (not shown), and based on the *in vitro* data, it is plausible to think that KLP-2011 can co-assemble with KLP-20 and KAP-1 in transgenic animals. However, this would create heterotrimeric kinesin-II(homo-20) complexes with different motility and transport properties from those of wild type kinesin-II, which could account for the failure of kinesin-II(homo-20) to function properly. To contribute to IFT, kinesin-II must execute a complex sequence of events, including entry into the cilium, docking onto an IFT particle, then cooperating with the homodimeric kinesin-2, OSM-3 to move an IFT particle to the distal tip of the axoneme [9]. For faithful execution of these processes, the rates and other motility properties of the two motor domains of kinesin-II may need to be optimal, with one motor domain, but not the other, exerting torque on the MT track. In this context it is tempting to speculate that KLP-11 exerts torque in order allow kinesin-2 to adjust its circumferential position around MT doublets within the axoneme and maintain its association with the B tubule [39]. For example, in *C. elegans* sensory cilia, kinesin-II and OSM-3 motors cooperate to move IFT particles along the axoneme, yet there is evidence that kinesin-II moves along B tubules of the doublets whereas OSM-3 moves on the A-tubules [9]. Perhaps an IFT particle being moved by both motors must be confined to protofilaments located at the junction of the A and B tubules, with KLP-11 generating torque in order to resist kinesin-II being pulled too far around the doublet from the B tubule onto the A tubule by OSM-3. Thus our finding that one of the two motor subunits of heterotrimeric kinesin-2 can exert both torque and axial force on a MT raises fascinating questions about its role in IFT and ciliogenesis.

Acknowledgments

This work has been supported by NIH grant GM50718. We thank Dr Guangshou Ou for teaching X.P. the *in vivo* transport assays. We thank Drs Ou, Gul Civelekoglu-Scholey, Limin Hao, Mel Thein and Frank McNally for critically reading the manuscript and them, together with Dr Rob Cross and the UCD "worm supergroup" for valuable discussion.

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