Clinical Endocrinology (2008) **69**, 906–910 doi: 10.1111/j.1365-2265.2008.03368.x

RAPID PUBLICATION

SDHC mutation in an elderly patient without familial antecedents

Elena López-Jiménez*, José M. de Campos†, Elena M. Kusak‡, Iñigo Landa*, Susanna Leskelä*, Cristina Montero-Conde*, Luis J. Leandro-García*, Luis A. Vallejo§, Beatriz Madrigal¶, Cristina Rodríguez-Antona*, Mercedes Robledo*,** and Alberto Cascón*,**

**Hereditary Endocrine Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, †Neurosurgery Department, Fundación Jiménez Díaz, Madrid, Spain, ‡Radiosurgery, Hospital Ruber Internacional, Madrid, Spain, §ENT Department, and ¶Pathological Anatomy Service, Hospital Universitario Rio Hortega, Valladolid, Spain, and **Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain*

Summary

Head and neck paragangliomas are usually asymptomatic and benign tumours arising mainly from the carotid body and the vagal, tympanic or jugular glomus. The majority of patients develop sporadic masses, and around 30% of cases harbour germline mutations in one of the succinate dehydrogenase genes: *SDHB*, *SDHC* or *SDHD*. In these hereditary cases, the presence of familial antecedents of the disease, multiplicity/bilaterality, young age at onset, and more recently, presence of gastrointestinal stromal tumours, are main factors to be considered. Here we describe a new mutation (c.256–257insTTT) affecting the *SDHC* gene in a 60-year-old-patient with a single head and neck paraganglioma, and without familial antecedents of the disease. *In silico* splice site analysis showed that this variant created a cryptic splice acceptor site and loss of heterozygosity (LOH) supported the pathogenic role of the mutation. Control population analyses did not detect this variant but revealed a novel *SDHC* polymorphism that exhibited a frequency of 0·3% (3/1020). This latter finding highlights the importance of assessing the clinical relevance of variants of unknown significance by means of analysing sufficient controls. Despite having found a germline mutation in an older, apparently sporadic patient, we consider that the high costs of analysing all susceptibility genes related to the disease support the recommendation of screening for mutations only in patients fulfilling the above criteria.

(Received 30 January 2008; returned for revision 10 March 2008; finally revised 18 March 2008; accepted 31 July 2008)

Introduction

Paraganglia are chemoreceptor structures of the diffuse neuroendocrine system with sensory innervation and a common embryonic origin in the neural crest.[1] Tumours arising within the covering of the carotid artery in the neck (carotid body), or from anatomically dispersed paraganglia of the jugulotympanic, vagal, laryngeal and aortic regions, are called head and neck paragangliomas (H & N PGLs) and are closely associated with the parasympathetic nervous system.[1] These tumours are usually asymptomatic and benign masses, but, depending on their location, local expansion of these slow growing tumours can cause cranial nerve deficit, occlusion of blood flow of the jugular veins, invasion of the skull base and erosion of bone, and eventually compression of the brain stem.[2]

Recently, mutations in three of the four succinate dehydrogenase genes (*SDHB*, *SDHC* and *SDHD*) were described as being involved in hereditary H & N PGLs. While mutations in the *SDHD* and *SDHB* genes have been widely found in patients both with H & N PGLs and with tumours located in the thoraco-abdominal region, only four cases amongst all the *SDHC* patients reported to date[3–9] developed PGLs outside the H & N region.[3,9,10] In addition, the number of different mutations described affecting the *SDHC* gene is very low (14 according to the SDH mutation database registries http://chromium.liacs.nl/lovd_sdh/), compared with the *SDHD* (73 mutations) and the *SDHB* (109 mutations) genes which encode the other structural subunit and the iron-sulphur protein subunit of the succinate dehydrogenase, respectively. Moreover, the limited knowledge of this protein and its recently reported link with gastrointestinal tumour (GIST) development[3] call for a detailed characterization of any new variant found. In this article, we describe a new in-frame mutation affecting *SDHC* in a patient with H & N PGL and without familial antecedents of the disease.

Subjects and methods

Subjects

A 60-years-old male patient presented with progressive dysphonia in 2003. A deficit of cranial nerves IX to XI was observed. Image studies with MRI (Fig. 1a) showed two lesions: one was an intrasellar pituitary macroadenoma, the other a highly vascularized tumour with a diameter of two centimetres located in the foramen jugularis, suggestive of PGL. Hyperprolactinaemia of 600 μg/l and testosterone at the lower limit of normal values were found in the blood hormone

Correspondence: Alberto Cascón, Hereditary Endocrine Cancer Group, Human Genetics Program, Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro 3, E-28029 Madrid, Spain.
Tel.: +34 91 224 69 47; Fax: +34 91 224 69 23; E-mail: acascon@cnio.es

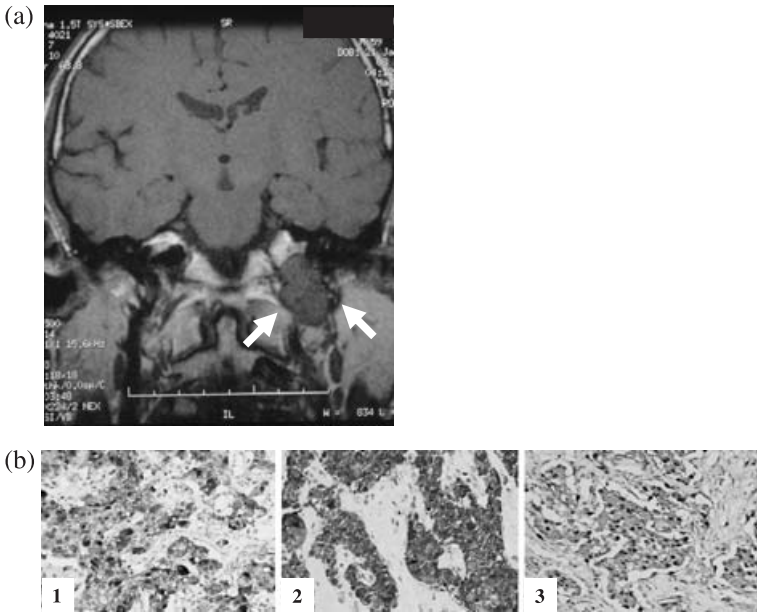

Fig. 1 (a) Magnetic resonance image; and (b) immunohistochemical chromogranin (1), synaptophysin (2) and sustentacular S-100 staining (3) of the jugular PGL.

tests. All other basal hormone levels were normal, as were blood and urine catecholamine levels. There was no history of neoplastic disease in his ancestors or in his four daughters.

In July 2003, a first surgical procedure was performed, with total resection of the pituitary tumour using a transsphenoidal approach. Normalization of prolactin and testosterone blood levels followed. Histopathological analysis showed a pituitary adenoma that was immunohistochemically positive for prolactin. Three months later, a second operation took place. An infratemporal approach was used, and a well-encapsulated lesion adherent to the wall of the jugular vein was apparently completely resected. Histopathological analysis confirmed the initial diagnosis of PGL. Clumps of epithelioid tumour cells with positive chromogranin and synaptophysin immunoreactivity and sustentacular S-100 positive cells could be seen (Fig. 1b). MIB-1 proliferation index was less than 2%.

Clinical and imaging controls performed to date have shown complete remission of the pituitary macroadenoma, with partial persistence of dysphonia because of residual malfunction of the right Xth cranial nerve related to the PGL surgery. An image of tumour persistence or recurrence in the foramen jugularis was treated with radiosurgery with subsequent tumour control.

Informed consent was obtained from the patient and his four children. Genomic DNA was extracted from blood samples following a standard method.[11] We used DNA from 1020 unrelated and unaffected individuals as a control population.

Amplification and sequence analysis

Sequence analysis was carried out on amplified genomic DNA from peripheral blood leukocytes. *SDHC* comprises five introns and six exons. Exon-specific polymerase chain reaction (PCR) and direct sequencing analyses were performed on exons 1–6 of the *SDHC* gene (ENSG00000143252). The primer pairs for exon amplification were designed based on the genomic sequence.

Multiplex method

To investigate the presence of loss of heterozygosity (LOH) affecting *SDHC*, we performed Multiplex-PCR in blood and tumour (PGL) samples from the patient. The primer pairs for *SDHC* amplification were also designed on the basis of the genomic sequence of the gene and were as previously described.[12] Five control fragments (C1 to C5) from chromosomes 1, 3 and 11 were used as internal controls of the assay. We amplified genomic DNA of the patients by means of a multiplex PCR kit, following the manufacturer's recommendations (Qiagen GmbH, Hilden, Germany). Briefly, multiplex amplifications were performed in 25 μl of a mixture containing 1X multiplex PCR master mix, 0·2 μM of each primer, and 100–200 ng of genomic DNA. The PCR program started with an initial step at 95 °C for 15 min. to activate HotStartTaq DNA polymerase. Amplification was for 20 cycles: 30 s at 94 °C, 90 s at 60 °C and 90 s at 72 °C, followed by 10 min. of final extension at 72 °C. PCR amplification products obtained from the *SDH* deletion tests were used for fragment analysis on an ABI PRISM™ 310 capillary sequencer (Applied Biosystems, PerkinElmer) and analysed using GeneScan v3·1 (Applied Biosystems). Normalization was performed by overlapping blood and tumour samples from the patient, determining the peak surface of all fragments and calculating the normal peak fractions.[13]

Splice site analysis

To evaluate changes in splice site strength and prospective splicing mutations, we used splice site analysis software (https://splice.cmh.edu/).

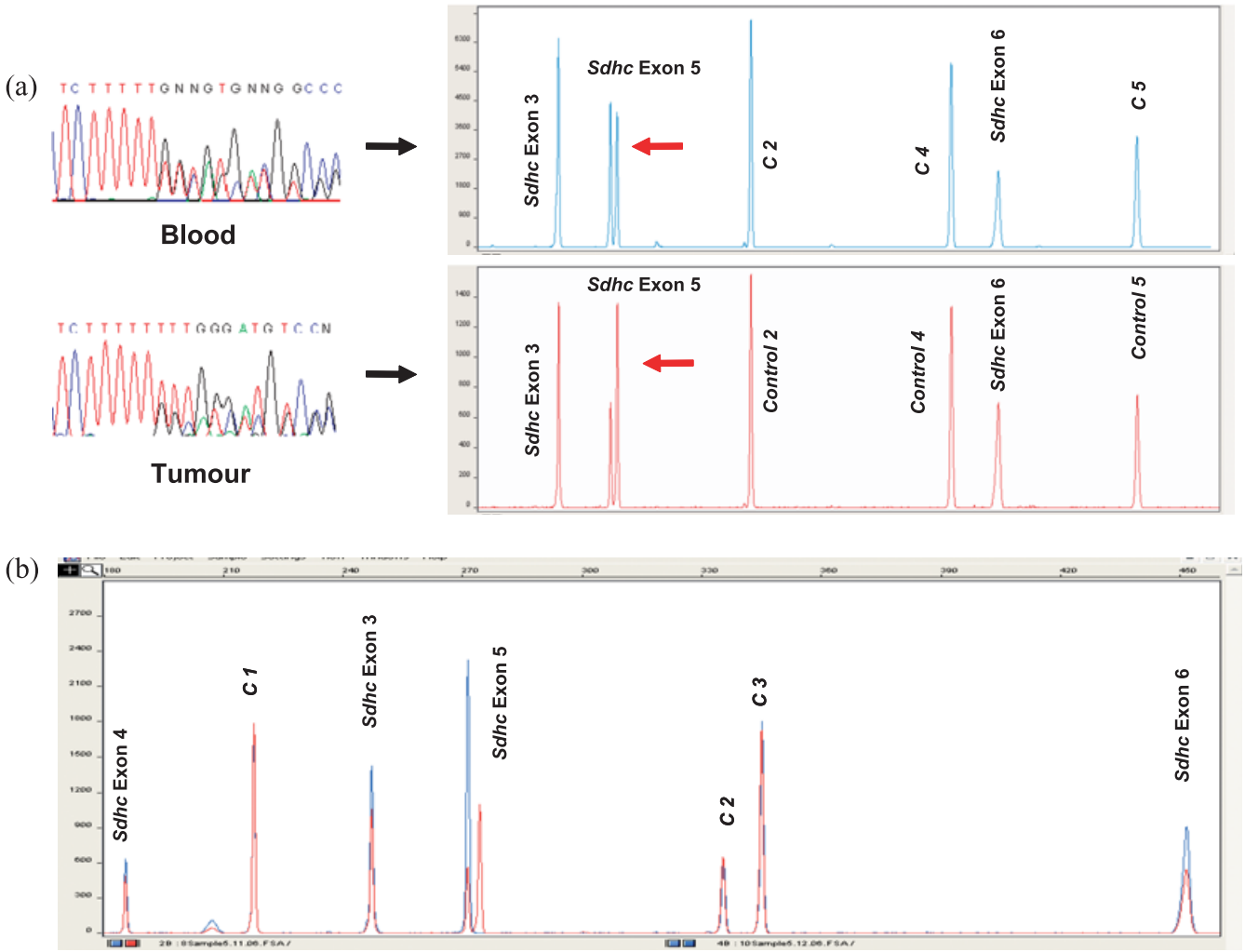

Fig. 2 (a) *SDHC* mutation sequences from blood and tumour samples. GeneScan analysis of amplified PCR fragments using multiplex-PCR was performed on blood (blue), and tumour (red) samples from the proband. Fragments corresponding to mutant alleles are marked with red arrows; (b) GeneScan analysis performed on control DNA (blue), overlapped with the tumour sample from the proband (red).

The information content (*Ri*, in bits) describes the degree to which a member of a sequence family contributes to the conservation of the entire family, and is defined as the number of choices needed to describe a sequence pattern using a logarithmic scale in bits.[14,15]

Results

The diagnosis of the proband with PGL and pituitary macroadenoma led to an initial analysis for the presence of mutations affecting the *MEN1* gene; no alterations were found. Next, *SDHD* was considered as the most plausible candidate gene involved in the disease, and germline alterations were discarded by means of both point mutation and gross deletion analysis. Finally, we performed genetic screening for mutations in all the exons of *SDHC* and found an in-frame insertion (c.256–257insTTT) in exon 5 of the *SDHC* gene (Fig. 2a). This mutation was not previously described; therefore, to exclude this alteration being a polymorphism, we analysed a panel of 1020 control DNAs corresponding to healthy Spanish people and found no other case carrying the alteration. A not previously described polymorphic change of a valine to a phenylalanine (c.244G > T) was found in three out of the 1020 controls analysed for exon 5. Subsequent analysis of the PGL revealed the same insertion and probable loss of the normal allele, suggesting LOH. This latter was confirmed by means of multiplex-PCR comparing blood and tumour samples from the patient (Fig. 2b).

In silico splice site analysis showed that the presence of this new codon in position p.85 (c.256–257insTTT) led to the creation of a cryptic splice acceptor site, 15 nucleotides downstream from the natural exon 5, intron 4 site (Fig. 3a). When we performed the same analysis for the insertion of either one or two instead of the three thymines, no cryptic site was created (Fig. 3b,c).

No blood sample could be obtained for the deceased parents of the patient, but molecular analysis performed in the four children of the proband revealed two more carriers in the pedigree. Both carriers, aged 29 and 34 years, were studied clinically when their father was genetically diagnosed and were asymptomatic and lacked any feature of the disease. Image studies with cranial and abdominal MRI were negative, and the patients have refused any other studies.

(a)

c.280insTTT

Binding Sites on + Strand

Type of change	Genomic Coordinate	Position Relative to Natural Site	Closest Natural Site	Initial(Ri)	Final(Ri)	Δ Ri	Fold change	% Binding (Final/Initial)	Initial(Z)	Final(z)	Δ Z
No change	**158104340**	**0**	158104340	**10·8**	**10·8**	**0·0**	**1·0**	**100**	**0·4**	**0·4**	**0·0**
No change	158104341	1	158104340	0·6	0·6	0·0	1·0	100	-1·8	-1·8	0·0
INCREASED	**158104355**	**15**	158104340	**1·3**	**3·4**	**2·1**	**4·3**	**433·0**	**-1·7**	**-1·2**	**0·5**
Increased	158104359	19	158104340	2·1	3·7	1·6	3·1	310·8	-1·5	-1·1	0·4
No change	158104379	39	158104340	1·0	1·0	0·0	1·0	100	-1·7	-1·7	0·0
Increased	158104356	16	158104340	-0·1	1·7	1·8	3·2	323·0	-2·0	-1·6	0·4
Increased	158104363	23	158104340	-0·6	1·3	1·8	2·4	237·8	-2·1	-1·7	0·4

(b)

c.280insTT

Binding Sites on + Strand

Type of change	Genomic Coordinate	Position Relative to Natural Site	Closest Natural Site	Initial(Ri)	Final(Ri)	Δ Ri	Fold change	% Binding (Final/Initial)	Initial(Z)	Final(z)	Δ Z
No change	**158104340**	**0**	158104340	**10·8**	**10·8**	**0·0**	**1·0**	**100**	**0·4**	**0·4**	**0·0**
No change	158104341	1	158104340	0·6	0·6	0·0	1·0	100	-1·8	-1·8	0·0
Increased	158104355	15	158104340	1·3	2·6	1·3	2·5	246·9	-1·7	-1·4	0·3
Increased	158104359	19	158104340	2·1	3·7	1·6	3·0	302·0	-1·5	-1·1	0·3
No change	158104379	39	158104340	1·0	1·0	0·0	1·0	100	-1·7	-1·7	0·0
Increased	158104356	16	158104340	-0·1	1·2	1·3	2·3	227·7	-2·0	-1·7	0·3
Increased	158104363	23	158104340	-0·6	0·6	1·2	1·5	154·7	-2·1	-1·8	0·3

(c)

c.280insT

Binding Sites on + Strand

Type of change	Genomic Coordinate	Position Relative to Natural Site	Closest Natural Site	Initial(Ri)	Final(Ri)	Δ Ri	Fold change	% Binding (Final/Initial)	Initial(Z)	Final(z)	Δ Z
No change	**158104340**	**0**	158104340	**10·8**	**10·8**	**0·0**	**1·0**	**100**	**0·4**	**0·4**	**0·0**
No change	158104341	1	158104340	0·6	0·6	0·0	1·0	100	-1·8	-1·8	0·0
Increased	158104355	15	158104340	1·3	2·1	0·9	1·8	181·0	-1·7	-1·5	0·2
Increased	158104359	19	158104340	2·1	3·2	1·2	2·2	223·7	-1·5	-1·2	0·3
No change	158104379	39	158104340	1·0	1·0	0·0	1·0	100	-1·7	-1·7	0·0
Increased	158104356	16	158104340	-0·1	0·4	0·4	1·3	129·8	-2·0	-1·9	0·1

Legend: Site Abolished; Leaky Site; Cryptic Site created; Strengthened Pre-existing site; Pre-existing Site; Weakened Pre-existing Site

Fig. 3 Splice site analysis (https://splice.cmh.edu/) results simulating the insertion of three (a), two (b), or one (c) thymine after nucleotide position c.255 of the SDHC gene. Position Relative to Natural Site: The relative distance of the base from the closest natural site; Closest Natural Site: The genomic coordinate number of the closest natural splice site; Initial(Ri): Initial information content measured at the base before the mutation is made; Final(Ri): Final information content measured at the base after the mutation is made; ΔRi: Final(Ri) – Initial(Ri); change of information content obtained at the site because of a mutation or variant; Fold change: A single bit difference in Ri value corresponds to at least a two-fold difference in binding site strength; Fold change indicates the change in binding affinity of two sites; Fold Change = 2ΔRi, where ΔRi = difference between the respective individual information contents of two sites (wild type, mutant type);% Binding (Final/Initial): Indicates the change of binding energy: calculated as a percentage; Initial(Z): Z score for this evaluation, assuming that individual information values form a Gaussian distribution; Final(Z): Z score after mutation; ΔZ: Change in Z score obtained at the site due to a mutation or variant.

Discussion

By means of splice site analysis, we found that the new codon inserted into the *SDHC* sequence led to the creation of a cryptic splice acceptor site. The use of a new cryptic splice site will either alter the reading frame, or insert or delete one or more codons.[16] We were not able to confirm altered transcription of the *SDHC* gene, but it seems that this new variation, not found in controls, could lead to aberrant splicing. The introduction of a new phenylalanine into helix 2 L of SDHC could affect the ubiquinone binding site in the matrix in which this protein region is involved,[17] even if splicing were not altered. In addition, we confirmed LOH in the PGL supporting the relevance of this mutation in the clinical phenotype. The study carried out in Spanish controls allowed us to identify another variant that exhibited a frequency of 0·3% (3/1020). This variant affected a nonconserved amino acid located inside the same helix, so it is likely to be a rare polymorphism. This is the first coding polymorphism described for the *SDHC* gene. This finding highlights the importance of assessing the clinical relevance of variants of unknown significance by means of analysing sufficient controls. This is even more important in the case of diseases with a very low incidence.

In our patient, it was not possible to determine whether the mutation was inherited or *de novo*, however, we confirmed the pathogenicity of the variant by LOH analysis. The two asymptomatic carriers should be followed clinically despite the apparently low penetrance of this mutation because, to date, no factors affecting the penetrance of the *SDH* genes mutations have been reported.

The presence of a second endocrine tumour, a pituitary macroadenoma, arising in the same patient could support a pathological role of the *SDHC* germline mutation. Nevertheless, as we were not able to obtain this second tumour to confirm the LOH, and because this is the first time that such a tumour has been reported in a *SDH* mutation carrier, we can not be sure about an association between pituitary adenoma and familial PGL. Further studies are needed to study this hypothesis.

Little is known about the clinical characteristics of *SDHC*-mutation carriers, as only 15 cases (nine familial and six isolated cases) have been described to date. It seems that the presence of head and

neck tumours is the main clinical feature associated with the disease. However, a recent report not only describes two retroperitoneal PGL patients as carriers of a mutation affecting the *SDHC* gene, but also reports a syndromic association between PGLs and gastrointestinal stromal tumours.[3] It has been described that the prevalence for mutations affecting *SDHC* in PGL patients is 4%, which is the lowest presentation amongst those found in the SDH genes.[4] Multiplicity or malignancy, features associated with *SDHD* and *SDHB* mutations, respectively, seem to be absent or clearly reduced in *SDHC* patients. In addition, apart from the four mentioned exceptions,[3,9,10] no other location for PGLs than head and neck has been related to the presence of *SDHC* mutations. In fact, it has been suggested that clinical features of *SDHC* patients are closely related to those found in patients with sporadic presentation of the disease. Indeed, amongst 11 consecutive patients molecularly characterized in our lab developing apparently sporadic head and neck PGLs, we found 10 cases developing a single head and neck tumour. The mean age at diagnosis for these sporadic cases was 48 years (from 28 to 73 years), compared with 45 years described for *SDHC* mutation carriers,[4] and 27 and 34 years for *SDHD* and *SDHB* mutation index cases from our series, respectively. In addition, only 4·5% (3/68) of *SDH* mutation carriers developed a PGL or a pheochromocytoma after the age of 50 years (data not shown). Thus, the finding of a single benign jugular tumour in a 60-year-old patient can be easily confused with a sporadic presentation of the disease. The need to study patients with H & N PGLs for *SDHD* or *SDHC* alterations has been questioned a number of times, because of the low risk of developing malignant disease. In fact, routine screening for germline *SDHC* mutations is not recommended.[18] In summary, even though we describe here an *SDHC* mutation found in a late-diagnosed patient, screening for mutations should be performed only in patients at risk, that is, those with familial antecedents of H & N PGL, an early age of onset or the presence of GIST.

Acknowledgements

This work was supported in part by the Fondo de Investigaciones Sanitarias, projects PI061477 and PI042154, and the Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII. Alberto Cascón and Cristina Rodríguez-Antona hold FIS and 'Ramon y Cajal' contracts, respectively, from the Spanish government. We especially thank Rocio Letón for her excellent technical assistance.

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