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ORIGINAL ARTICLE

Expression of smooth muscle calponin in synovial sarcoma

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Abstract

Purpose. Histogenesis of synovial sarcoma remains controversial and reliable molecular markers for diagnosis are necessary. Expression of basic calponin, a smooth muscle differentiation-specific actin-binding protein, was studied in synovial sarcoma.

Subjects and Methods. The basic calponin gene and the gene product were analyzed by reverse transcription PCR analysis (RT-PCR) and immunohistochemistry in 14 synovial sarcomas and a human synovial sarcoma cell line (HS-SY-II).

Results and Discussion. Immunoreactivity for basic calponin was detected in the cytoplasm of 6 synovial sarcomas (43% positive). In the basic calponin-positive tumors and the HS-SY-II cells, expression for smooth muscle-specific genes, including basic calponin and SM22α, was detected by RT-PCR, suggesting a lineage relationship between synovial sarcoma cells and smooth muscle-like mesenchymal cells.

Conclusions. A subset of synovial sarcomas expressing the basic calponin gene and the gene product were identified. The basic calponin may have potential utility as a novel molecular marker identifying certain synovial sarcomas.

Key words: *calponin*, *synovial sarcoma*, *myoepithelium*, *smooth muscle.*

Introduction

Synovial sarcomas, which account for approximately 10% of soft tissue tumors, arise most commonly in the para-articular regions in adolescents and young adults.[1] The lineage of differentiation or histogenesis still remains controversial,[2,3] including the synovial,[4] epithelial,[5] neural,[6] and primitive mesenchymal[1] cells in origin. Positive immunostaining for cytokeratin and/or epithelial membrane antigen as well as vimentin has been applied for diagnosis,[1] but histological differential diagnosis may be difficult in the poorly differentiated cases. Therefore, additional genetic or phenotypic markers seem to be necessary to identify synovial sarcoma and predict the biological behaviors of the tumors.

Basic calponin is an actin-, tropomyosin- and calmodulin-binding protein originally isolated from smooth muscle.[7,8] Structural analysis of cDNAs encoding calponin isoforms has revealed the presence of three types of genes with distinct expressional regulation.[7,9,10] Each of the three calponin genes encodes distinct classes of isoforms categorized into basic (p*I* 8 to 10), neutral (p*I* 7 to 8) and acidic (p*I* 5 to 6) calponins on the basis of their isoelectric points. Although the neutral and acidic calponins are expressed in both smooth muscle and non-smooth muscle tissues,[9,11] the basic calponin gene is one of the earliest markers of differentiated smooth muscle cells.[12–15]

Recently, expression of basic calponin has proved not to be restricted solely to smooth muscle cells, and has been detected also in cells that present with certain smooth muscle-like phenotypes, i.e. myofibroblasts and myoepithelial cells expressing a set of other smooth muscle markers.[16,17] With regard to tumors, expression of basic calponin was detected in leiomyomas,[18] leiomyosarcomas,[19,20] and a subset of osteosarcomas.[21]

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In the current study, we extended the analysis of basic calponin expression to synovial sarcoma using the tumor samples and a cultured cell line (HS-SY-II).[22] We identified a subset of synovial sarcomas expressing the basic calponin gene and the gene product.

Materials and methods

Tumor tissues

Tumor samples listed in Table 1 were obtained from primary tumors in 14 patients with synovial sarcoma and in 51 patients with other soft-tissue sarcomas at the Department of Orthopaedic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases. The histologic diagnoses and subtypes were established through routine pathologic evaluation according to the published criteria.[1] Diagnosis of synovial sarcoma was confirmed by expression of the SYT-SSX fusion gene resulting from the chromosomal translocation.[23,24] The SYT-SSX fusion gene was detected previously for 8 of 14 synovial sarcomas,[25] and for the remaining 6 synovial sarcomas in this study. Clinical and pathological data of the patients with synovial sarcoma are summarized in Table 2. All synovial sarcomas were considered high grade and deep-seated. Histologic subtypes were as follows; monophasic type: 10 cases, biphasic type: 4 cases. The specimens were fixed in 10% formalin/PBS and embedded in paraffin. Sections of 4 μm thickness were prepared for staining with hematoxylin-eosin, and for immunohistochemical examination.

Synovial sarcoma cell line

A human synovial sarcoma cell line (HS-SY-II),[22] which showed a characteristic chromosomal translocation t(X;18)(p11;q11), was cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Tech., Gaithersburg, USA) supplemented with 10% (v/v) fetal bovine serum (Upstate Biotech., Waltham, USA) and 1% penicillin/streptomycin at 37°C under a humidified atmosphere containing 5% (v/v) CO_2. Cells in a logarithmic phase of proliferation were collected and served for detection of mRNAs.

Immunohistochemistry

Monoclonal antibodies against basic calponin (clone hCP) and α-smooth muscle actin (α-SMA)(clone 1A4) were obtained from Sigma Chemicals (St Louis, USA). The specificity of the clone hCP monoclonal antibody to basic calponin isoform was verified as described previously.[11,21] The sections were mounted on poly-L-lysin coated microslides, deparaffinized in xylene, dehydrated through graded alcohol, and immersed in 70% methanol with H_2O_2 to block endogenous peroxidase. Antigen retrieval for basic calponin was performed using a 400-W microwave oven (TOSHIBA ERT 330) for 5 min (4 times) in a 10 mM citrate buffer (p*H* 7.0). The sections were incubated with 1% (v/v) goat serum/PBS for 1 h at room temperature, washed in PBS, and incubated with the antibody in 2% (w/v) BSA/PBS overnight at 4°C. They were then washed 5 times with 0.005% (v/v) Tween 20/PBS, followed by incubation with the biotinylated goat anti-mouse IgG (TAGO Immunologicals, Camarillo, USA) in 2% (w/v) BSA/PBS for 1 h at room temperature and avidin–biotin–horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After being washed in 0.005% (v/v) Tween 20/PBS, the final reaction product was visualized with diaminobenzidine (WAKO Chemicals, Osaka, Japan), and the sections were counterstained with hematoxylin. Negative control study with mouse non-immune IgG was included to assess non-specific staining. All assays were scored by three independent observers; staining levels were graded with regard to the number of positive cells in a given tumor sample (++; 50–95% positive cells, +; 10–45% positive cells; –; less than 10% positive cells).

Reverse transcription-PCR analysis

Tumor tissues were frozen immediately after surgical removal and stored at –80°C until extraction of RNA. Total RNA was extracted from tumor tissues using the RNA extraction kit (Nippon Gene, Toyama, Japan). Reverse transcription (RT) of 2 μg of total RNA was carried out using reaction mixture of *Ready-To-Go* You-Prime First-Strand Beads (Pharmacia

Table 1. *Immunohistochemical analyses of smooth muscle calponin and α-smooth muscle actin in soft tissue sarcomas*

		Calponin positive				α-SMA positive			
Diagnosis	No.	2+	1+	–	rate (%)	2+	1+	–	rate (%)
Synovial sarcoma	14	3	3	8	43	0	0	14	0
Malignant fibrous histiocytoma	11	0	1	10	9	1	4	6	45
Leiomyosarcoma	8	4	2	2	75	5	3	0	100
Liposarcoma	8	0	1	7	13	0	0	8	0
Malignant schwannoma	7	0	1	6	14	0	0	7	0
Rhabdomyosarcoma	5	0	0	5	0	0	0	5	0
Alveolar soft-part sarcoma	5	0	0	5	0	0	0	5	0
Dermatofibrosarcoma	3	0	0	3	0	0	1	2	33
Angiosarcoma	2	0	0	2	0	0	0	2	0
Epithelioid sarcoma	2	0	0	2	0	0	0	2	0

Table 2. *Clinicopathological data of the patients with synovial sarcoma*

No.	Calponin expression*	Age	Sex	Location	Tumor size**	Histologic subtype
1	(++)	26	F	leg	1	biphasic
2	(++)	45	M	hand	1	monophasic
3	(++)	45	M	inguinal	2	biphasic
4	(+)	20	F	thigh	3	monophasic
5	(+)	51	F	chest wall	1	monophasic
6	(+)	57	M	foot	2	monophasic
7	(–)	61	M	forearm	2	monophasic
8	(–)	12	F	inguinal	2	monophasic
9	(–)	25	F	chest wall	1	biphasic
10	(–)	27	F	thigh	1	monophasic
11	(–)	14	M	popliteal	2	monophasic
12	(–)	13	F	buttock	3	monophasic
13	(–)	37	F	calf	1	biphasic
14	(–)	36	M	buttock	2	monophasic

*(++); 50–95% positive cells, (+); 10–45% positive cells; (–); less than 10% positive cells.
**1; <5 cm, 2; 5–10 cm; 3; >10 cm.

Biotech., Uppsala, Sweden) in the presence of 0.2 μg of the random hexaprimers pd$(N)_6$. After 60 min incubation at 37°C, 0.5 μM of each of the forward and reverse primers, 200 μM of each dNTP mix and 2.5 U of Taq DNA polymerase (Pharmacia Biotech.) were added to 8 μl of the first strand reaction mixture and then, total volume was adjusted to 50 μl with water. The parameters used for the amplification were 30 cycles of denaturation (94°C, 40 s), annealing (60°C, 30 s) and polymerization (72°C, 90 s). Sequences of the selected forward and reverse primers used, and predicted products size were as follows: SYT-SSX, CAACAGCAAGATGCATACCA (forward), CACTTGCTATGCACCTGATG (reverse), 585 bp;[25] basic calponin, GAGTGTGCAGACGGAACTTCAGCC (forward), GTCTGTGCCCAACTTGGGGTC (reverse), 671 bp;[21] neutral calponin, CTGCAGAGCGGGGTGGACATTGGC, (forward) GCCGGCCTCCTCCTGGTAGTAAGG (reverse), 519 bp;[21] acidic calponin, GGAAGCGAAGTGCGAGAGACC (forward), CTGTGTGGATCTAATAATCAATGC (reverse), 1061 bp;[21] SM22α, CGCGAAGTGCAGTCCAAAATCG (forward), GGGCTGGTTCTTCTTCAATGGGG (reverse), 928 bp;[26] glyceraldehyde 3-phosphate dehydrogenase (GAPDH), CCCATCACCATCTTCCAGGA (forward), TTGTCATACCAGGAATGAGC (reverse), 731 bp.[21] The linearity of the PCR products for SYT-SSX, calponins, and SM22α was obtained between 25–30 cycles and for GAPDH between 20–25 cycles. After agarose gel (1%) electrophoresis in the presence of 0.5 mg/ml of ethidium bromide, the PCR products were revealed by UV irradiation, and the image captured, digitized and quantitated by Eagle Eye II Still Video System (Stratagene, La Jolla, USA). Variations in signal intensities between different agarose gels were corrected by using signal intensities of the molecular weight markers in every gel analyzed. Negative results were repeated at least twice.

Results

Immunohistochemical analyses for basic calponin

Table 1 summarizes the data on immunohistochemistry of basic calponin and α-SMA in soft tissue sarcomas. Among 65 malignant tumors, 6 of 14 (43%) synovial sarcomas showed cytoplasmic immunoreactivity of basic calponin. The basic calponin expression was also detected in 6 of 8 (75%) leiomyosarcomas, 1 of 11 (9%) malignant fibrous histiocytomas (MFHs), 1 of 8 (13%) liposarcomas, and 1 of 7 (14%) malignant schwannomas. The fractions of calponin-positive tumor cells were ranged from more than 90% to as low as 10–20% in synovial sarcoma and leiomyosarcoma samples. Calponin-positive tumor cells in synovial sarcomas showed a spindle cell feature (Fig. 1(a,b)), with no immunoreactivity to epithelial markers such as cytokeratin and epithelial membrane antigen (data not shown). The remaining eight synovial sarcomas lacked immunoreactivity for calponin (Fig. 1(c)). There were no apparent differences in tumor size or histologic subtype between calponin-positive and calponin-negative synovial sarcomas (Table 2).

Calponin-positive leiomyosarcomas tended to show an orderly fascicular pattern and little pleomorphism of tumor cells (Fig. 1(d)). The histology of two calponin-negative leiomyosarcomas was an anaplastic type with less fascicular pattern than the calponin-positive tumors, but they showed immunoreactivity for α-SMA (10–45% positive cells). One MFH, one liposarcoma, and one malignant schwannoma, showed heterogeneous staining for basic calponin (10–20% positive cells). Rhabdomyosarcoma (Fig. 1(e)), alveolar soft-part sarcoma, dermatofibrosarcomas protuberans (Fig. 1(f)), angiosarcoma, epithelioid sarcoma, and the majority of MFHs, liposarcomas, and malignant schwannomas lacked basic calponin immunoreactivity. Notably, in contrast to MFH and

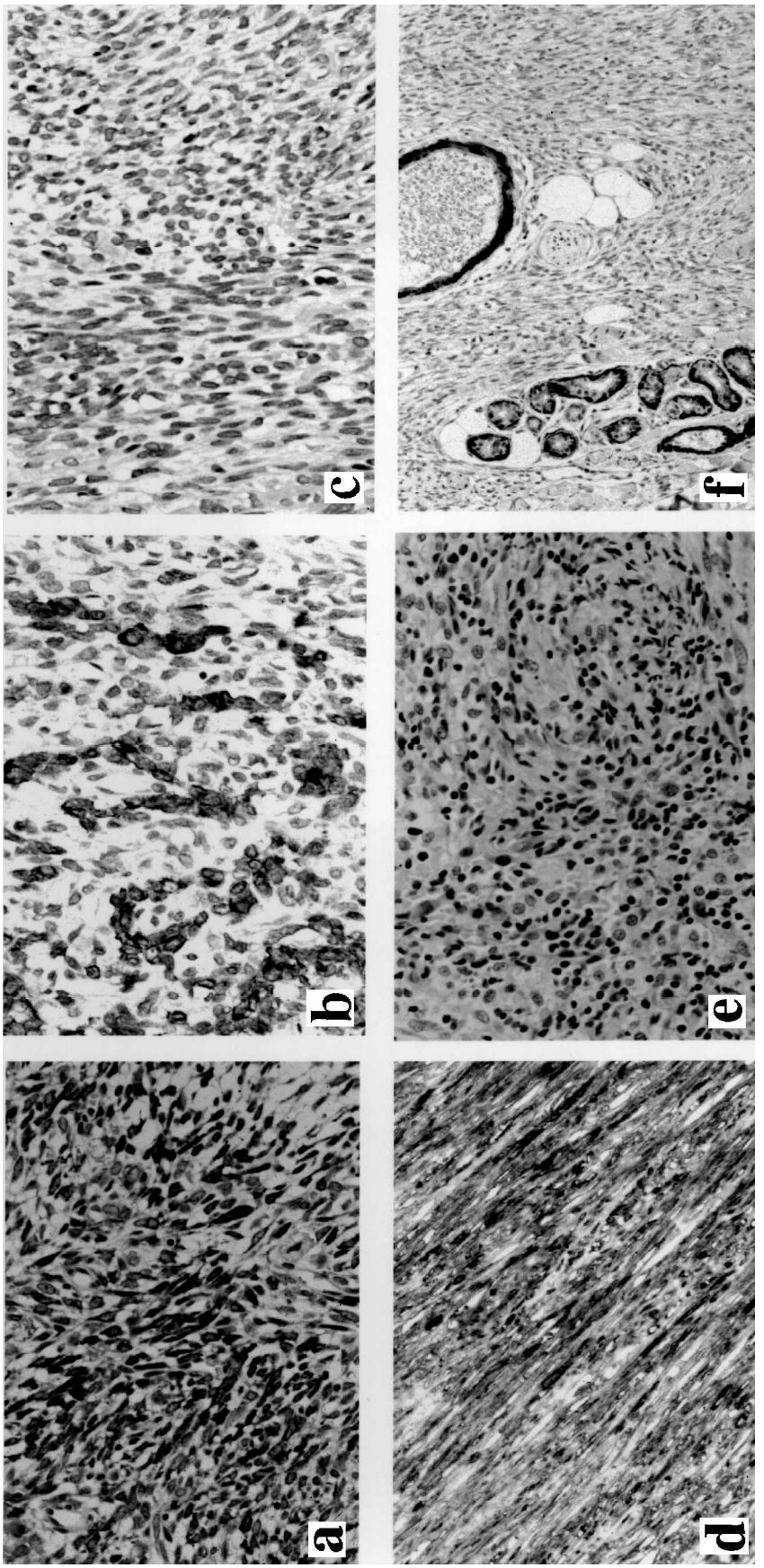

Fig. 1 *Immunohistochemical detection of basic calponin expression in soft tissue tumors. Immunostaining was localized in the cytoplasm of spindle tumor cells. The sections were counterstained with hematoxylin. (a) Calponin positive synovial sarcoma (diffuse expression pattern), (b) calponin positive synovial sarcoma (focal expression pattern), (c) calponin negative synovial sarcoma, (d) calponin positive leiomyosarcoma, (e) calponin negative rhabdomyosarcoma, (f) calponin negative dermatofibrosarcoma protuberans (tumor cells: negative; smooth muscle cells of a mature vessel and myoepithelial cells of the glands: positive). Original magnification: (a–e); ×50, (f); ×25*

leiomyosarcoma, immunoreactivity for α-SMA was not detected in any calponin-positive synovial sarcomas.

Expression of calponin isoforms and other smooth muscle markers in synovial sarcomas

Figure 2 shows the results of RT-PCR analyses for the representative synovial sarcomas with negative and positive immunoreactivity for basic calponin, and for a clonal synovial sarcoma cell line (HS-SY-II). The mRNA transcript of the SYT-SSX fusion gene was detected in both of the two synovial sarcoma tissues. The mRNA transcript of the basic calponin gene was expressed in the case with positive immunoreactivity for basic calponin, but reduced in the case with negative immunoreactivity for basic calponin, indicating a correlation between the intensity of immunohistochemical staining of basic calponin and the expression levels of its mRNA transcript. The HS-SY-II cells also expressed the basic calponin mRNA transcript. The mRNAs for neutral calponin and SM22α were uniformly expressed in the two synovial sarcomas, but acidic calponin mRNA transcript was reduced. In HS-SY-II cells, all calponin isoforms and SM22α were expressed.

Discussion

Expression of smooth muscle calponin in a subset of synovial sarcomas provides the first evidence that this gene can be expressed in soft-tissue tumors that lack the features of smooth-muscle or myofibroblast differentiation. Synovial sarcomas also expressed the mRNA transcript of another smooth muscle-specific gene, SM22α, suggesting a lineage relationship between synovial sarcoma cells and smooth muscle-like mesenchymal cells.

Recently, we have also detected expression of basic calponin and smooth muscle-specific genes in a subset of human osteosarcomas,[21] suggesting a lineage relationship between smooth muscle-like cells and osteoblasts. Calcification, an unlikely event in other soft-tissue sarcomas, is a well-known feature of synovial sarcoma occurring in approximately 30% of the cases.[27] Furthermore, Milchgrub *et al.* found that synovial sarcoma may produce tumorous osteoid and bone formation rather than the metaplastic bone and proposed a close relationship between osteoblasts and synovial sarcoma cells.[28] The fact that smooth muscle specific genes are commonly expressed in osteosarcoma and synovial sarcoma may support their hypothesis.

Another unique feature of synovial sarcoma is expression of epithelial markers.[1] Expression of both epithelial and smooth muscle markers suggests that histogenesis of synovial sarcoma may be related to myoepithelial cells which present both phenotypes.[16,17] Basic calponin expression holds promise as a new diagnostic marker that enables differential diagnosis of soft tissue sarcomas, particularly in distinguishing certain cases of calponin-producing synovial sarcomas from undifferentiated spindle cell sarcomas. In other words, a basic calponin-positive and α-SMA-negative spindle cell tumor in immunohistochemistry may favor a diagnosis of synovial sarcoma.

Basic calponin is not only a smooth muscle specific gene, but also a cell proliferation-related gene involved in the regulation of cell shape via the actin cytoskeleton.[29,30] Of particular interest is the finding that the N-terminal region of basic calponin shares a characteristic domain structure termed 'calponin homology' or CH-domain with the molecules essential for signal transduction of the Ras *superfamily* of

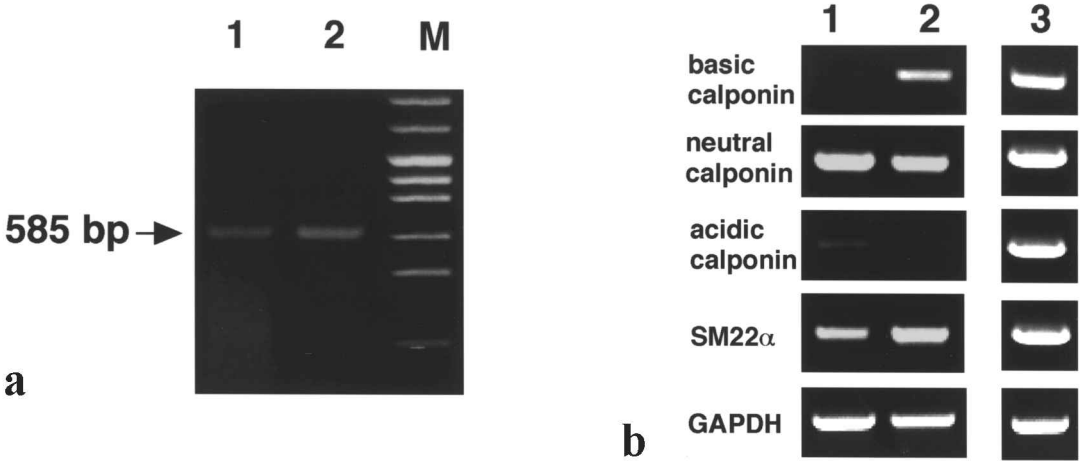

Fig. 2 *(a) Detection of SYT-SSX fusion gene expression in synovial sarcoma by RT-PCR analysis. Lane 1: synovial sarcoma (Case 8) (basic calponin immunostaining; –. Lane 2: synovial sarcoma (Case 1) (basic calponin immunostaining; ++), PCR product of the fusion gene was 585 bp in size. (b) Detection of basic, neutral and acidic calponins, and SM22α expression in synovial sarcomas by RT-PCR analysis. Lane 1: synovial sarcoma (Case 8) (basic calponin immunostaining; –). Lane 2: synovial sarcoma (Case 1) (basic calponin immunostaining; ++). Lane 3: HS-SY-II cells during proliferation.*

GTPase proteins such as Vav proto-oncogene product and IQGAP1.[31,32] Removal of the N-terminal 67 residues of the Vav protein, which contains a region of the CH-domain, is sufficient to activate the transforming potential of the Vav proto-oncogene product.[33,34] These findings suggest that basic calponin may control actin cytoskeleton, and therefore may exert influence on proliferation, the transformed phenotype and the metastatic potential of the tumor cells. It is also possible that loss of the inhibitory action of calponin on actin–myosin interaction may promote the migration and cytokinesis of the tumor cells, and thus may also influence their proliferation and metastatic potential.

With regard to osteosarcoma patients, a poorer clinical outcome was correlated with loss of basic calponin expression.[21] Therefore, analyses on expression of basic calponin in synovial sarcomas seem to be important for understanding the biological behaviors.

In conclusion, basic calponin expression in a subset of synovial sarcomas may provide us with some leads to solve the histogenesis of these tumors. Further research will examine whether the status of basic calponin expression in synovial sarcomas is a new and independent prognostic factor that may help in the subclassification of these tumor types.

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