Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1

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Abstract cDNA for a novel proton/organic cation transporter, OCTN1, was cloned from human fetal liver and its transport activity was investigated. OCTN1 encodes a 551-amino acid protein with 11 transmembrane domains and one nucleotide binding site motif. It is strongly expressed in kidney, trachea, bone marrow and fetal liver and in several human cancer cell lines, but not in adult liver. When expressed in HEK293 cells, OCTN1 exhibited saturable and pH-dependent [^{3}H]tetraethyl ammonium uptake with higher activity at neutral and alkaline pH than at acidic pH. Furthermore, treatment with metabolic inhibitors reduced the uptake, which is consistent with the presence of the nucleotide binding site sequence motif. Although its subcellular localization and detailed functional characteristics are not clear at present, OCTN1 appears to be a novel proton antiporter that functions for active secretion of cationic compounds across the renal epithelial brush-border membrane. It may play a role in the renal excretion of xenobiotics and their metabolites.

Key words: Cation transport; Proton antiport; Tetraethyl ammonium; ATP binding site; Human kidney

1. Introduction

Many xenobiotics that bear ionizable groups exist as anions or cations at physiological pH. It seems reasonable to suppose that they are transported across membranes via specialized carrier-mediated transport mechanisms to be excreted into urine or bile, and the existence of closely related transporters in both organs has been suggested [1,2]. The transport systems have been empirically classified into three groups specific to anionic, cationic and neutral compounds and functionally subdivided in more detail. It has been established that organic cationic compounds are actively secreted from blood to urine across the renal tubular epithelial cells via multiple transport mechanisms, including a membrane potential-dependent transporter and a proton/organic cation antiporter in basolateral and brush-border membranes, respectively [1,3]. The application of molecular biological techniques has been helpful in characterizing the transporters. Two renal membrane transporters, OCT1 and OCT2, which exhibit membrane potential dependence, have been cloned from rat, porcine kidney-derived LLC-PK1 cells and human [4–9]. Although Gründemann et al. [6] suggested that OCT2 is an apical-type transporter on the basis of its susceptibility to specific transport inhibitors, Okuda et al. [5] found a negligible pH dependence of rat OCT2 and suggested that it is a basolateral – but not an apical – membrane transporter. The human counterpart of OCT2 was also cloned recently and was implicated in pH-independent, membrane potential-dependent transport at the luminal surface of distal tubules [9]. Accordingly, human OCT2 was presumed to function for the reabsorption of endogenous organic cations such as choline, as well as xenobiotics. So far, however, molecular identification of a proton/organic cation antiporter in the renal apical membrane, which would be important for the efficient elimination of organic cations into urine, has not been reported.

Our interest has been focused on the regulation of drug disposition in the body and the targeting of drugs to specific tissues by utilizing specialized transporter functions in the tissues [10–12]. In the present study, we cloned from human fetal liver a cDNA for a novel organic cation transporter (OCTN1), which carries a nucleotide binding site sequence motif. It is strongly expressed in kidney and transports organic cations such as tetraethyl ammonium (TEA) in a pH-dependent and metabolic inhibitor-susceptible manner, strongly suggesting that OCTN1 is a renal proton/organic cation antiporter functioning at the renal epithelial apical membrane.

2. Materials and methods

2.1. Construction of cDNA library and isolation of human OCTN1 and Northern blot analysis

A subtracted cDNA library was constructed by suppression subtractive hybridization [13] using a PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA, USA). Double-stranded cDNAs synthesized from human fetal and adult liver polyA$^+$ RNA were used as tester and driver, respectively. After two rounds of hybridization, cDNA fragments derived from differentially expressed genes were amplified by suppression polymerase chain reaction, and cloned into pT7-Blue-T (Novagen, Madison, WI, USA) by the TA cloning method. The resulting cDNA clones were subjected to random sequencing to generate ESTs. BLAST analysis identified one EST, OCTN1, which has an open reading frame with significant sequence similarity to known organic cation transporters. A cDNA insert of this clone was then used as a probe to screen a human fetal liver cDNA library (Clontech). Eight overlapping positive clones were isolated from about 8×10^5 plaques and the whole sequence of OCTN1 was determined by sequencing cDNA inserts of these clones. Multiple tissue Northern blots containing mRNA from human fetal and adult tissues and cancer cell lines (Clontech) were probed with cDNA fragments corresponding to the carboxyl-terminal half of OCTN1 cDNA labeled with [^{32}P]dCTP by the random primer method. The probes were hybridized in ExpressHyb Hybridization Solution (Clontech) at 68°C for 1 h. The blots were then washed in 2×SSC containing 0.1% SDS at

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Abbreviations: TEA, tetraethyl ammonium

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room temperature for 60 min with several changes of the buffer, and twice for 20 min in 0.1×SSC containing 0.1% SDS at 50°C.

2.2. Transport experiments by transient expression in HEK293 cells

Human OCTN1 cDNA was subcloned into *Eco*RI sites of the expression vector pcDNA3 (Invitrogen, San Diego, CA, USA). The construct pcDNA3/OCTN1 was used to transfect HEK293 cells (Japanese Cancer Research Resources Bank, Tokyo, Japan) according to the calcium phosphate precipitation method [14]. HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco, Grand Island, NY), penicillin, and streptomycin in a humidified incubator at 37°C and 5% CO_2. After 24 h cultivation of HEK293 cells in 10-cm dishes, pcDNA3/OCTN1 or pcDNA3 vector alone was transfected by adding 10 μg of the plasmid DNA per dish. At 48 h after transfection, the cells were harvested and suspended in transport medium containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM HEPES (pH 7.4). This suspension and a solution of a radiolabeled test compound in the transport medium were each incubated at 37°C for 10 min, then transport was initiated by mixing them. At appropriate times, 200-μl aliquots of the mixture were withdrawn and the cells were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicon oil and liquid paraffin with a density of 1.03. Each cell pellet was solubilized in 3 N KOH, and neutralized with HCl, and the associated radioactivity was measured by means of a liquid scintillation counter. When transport was measured at an acidic or alkaline pH, MES and Tris were used instead of HEPES to maintain the desired pH, respectively. Cellular protein content was determined according to the method of Lowry et al. [15].

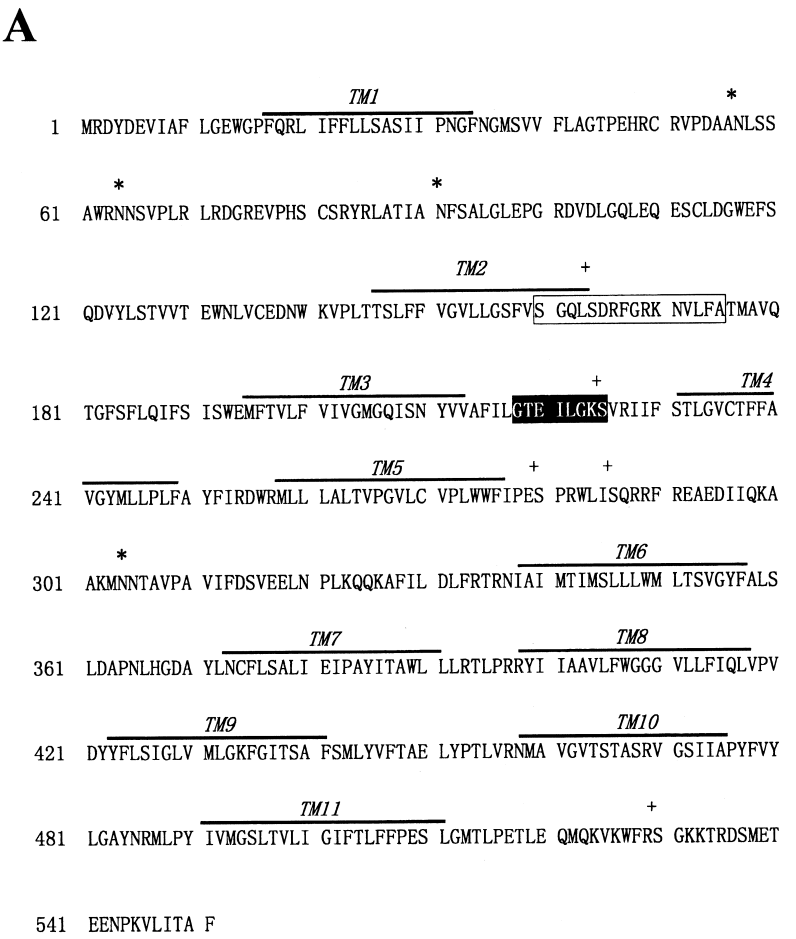

Fig. 1. Amino acid sequence (A) and hydropathy plot (B) of human OCTN1. Potential *N*-glycosylation sites and protein kinase C phosphorylation sites are indicated by asterisks and plus signs, respectively. Putative transmembrane domains (TM) are indicated by lines and are numbered. The sugar transporter protein signature and nucleoside binding site sequence motif are boxed and highlighted, respectively.

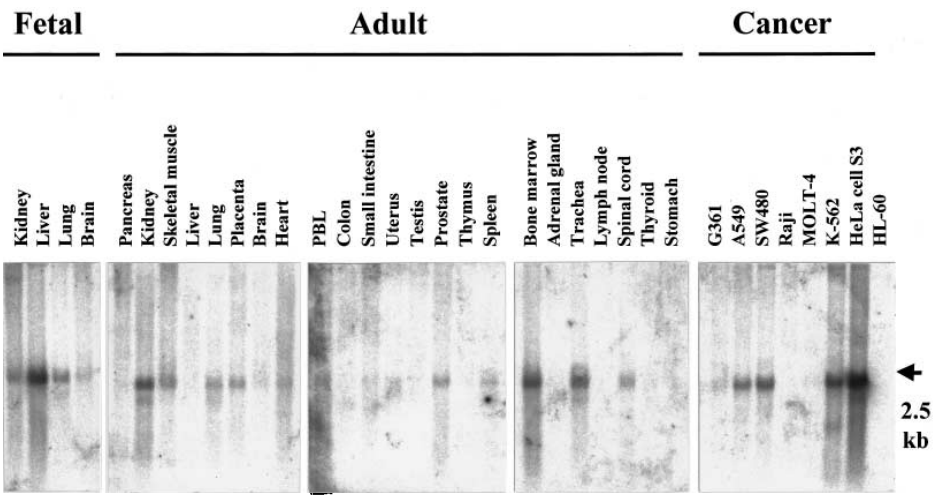

Fig. 2. Northern blot analysis of human OCTN1. Commercially available hybridization-ready polyA$^+$ RNA blots were probed with an internal fragment of OCTN1 cDNA. The sizes of hybridizing bands were determined using RNA standards. PBL, peripheral blood leukocyte; G361, melanoma; A549, lung carcinoma; SW480, colorectal adenocarcinoma; Raji, Burkitt's lymphoma; HeLa cell S3, HeLa cell line; HL-60, promyelocytic leukemia.

2.3. Materials

[1-^{14}C]Tetraethyl ammonium bromide (0.124 GBq/mmol), [^{14}C]inulin (185 MBq/g), ^3H_2O (37 MBq/g), and [α-^{32}P]dCTP were purchased from New England Nuclear (Boston, MA, USA). pcDNA3 was obtained from Invitrogen. Multiple tissue Northern blots and the PCR-Select cDNA subtraction kit were purchased from Clontech. All other enzymes and reagents for molecular biology were purchased from Takara (Otsu, Japan), Toyobo (Osaka, Japan) and Wako Pure Chemical Industries (Osaka).

3. Results

3.1. Sequence and tissue distribution of OCTN1

Fig. 1A shows the predicted amino acid sequence of the human organic cation transporter, OCTN1. The cloned OCTN1 cDNA consists of 2135 nucleotide and contains an open reading frame encoding a 551-amino acid residue protein (GenBank accession number AB007448). Hydropathy analysis according to Kyte and Doolittle [16] predicted that OCTN1 has 11 putative membrane-spanning domains, four *N*-glycosylation sites, and five protein kinase C phosphorylation sites (Fig. 1B). OCTN1 is unique in having a nucleotide binding site sequence motif and a sugar transport protein signature. Similarities of human OCTN1 with rat OCT1 [4], rat OCT2 [5], human OCT1 [8] and human OCT2 [9] in amino acid sequence were 32%, 33%, 31%, and 33%, respectively. Accordingly, human OCTN1 is considered to be a member of the organic cation transporter family.

The tissue distribution of human OCTN1 was studied by Northern blot analysis as shown in Fig. 2. Human OCTN1 was originally cloned from a human fetal liver cDNA library as a fetal liver-specific clone. It was also detected in several other fetal tissues, including kidney and lung. In adult, strong signals were observed in kidney, trachea, and bone marrow and weak signals in skeletal muscle, prostate, lung, pancreas, placenta, heart, uterus, spleen, and spinal cord. Interestingly, specific bands which correspond to human OCTN1 were observed in several human cancer cell lines, including lung carcinoma A459, colorectal adenocarcinoma SW480, myelogenous leukemia K-562, and HeLa cell S3.

3.2. Transport activity of OCTN1 in HEK293 cells

The time course of uptake of [^{14}C]TEA by HEK293 cells transfected with OCTN1 or expression vector pcDNA3 alone was studied (Fig. 3). The uptake of [^{14}C]TEA at pH 7.4, expressed as the cell/medium ratio obtained by dividing the amount of [^{14}C]TEA taken up by the cells by its concentration in the transport medium, increased time-dependently in OCTN1-transfected cells, whereas the uptake by the cells transfected with pcDNA3 vector alone (designated Mock) exhibited a negligible increase with time. In OCTN1-transfected cells, the uptake of TEA was linear up to 5 min and reached a steady-state. The OCTN1-derived steady-state uptake is more than 20 μl/mg protein. Since the intracellular space of HEK293 cells obtained as the difference between the uptakes of ^3H_2O and [^{14}C]inulin was determined to be 6.3 μl/mg protein in the present study, [^{14}C]TEA is apparently accumulated concentratively within the cells.

Fig. 4 shows the concentration dependence of TEA uptake by OCTN1 obtained after subtraction of the Mock uptake. The Eadie-Hofstee plot (Fig. 4, inset) gave a single straight line, suggesting the participation of a single functional binding site for TEA on OCTN1. By non-linear least-squares analysis, values of the maximum uptake rate (V_{max}) of 6.68 ± 0.345

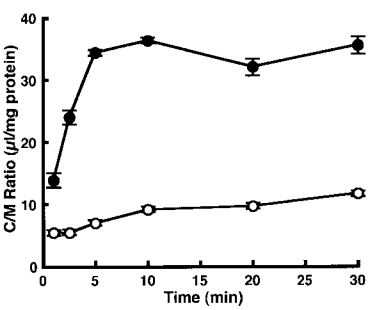

Fig. 3. Time course of [^{14}C]TEA uptake by OCTN1-transfected HEK293 cells. Uptake of [^{14}C]TEA (60 μM) by OCTN1- (closed circles) and pcDNA3 vector-transfected (open circles) HEK293 cells was measured at pH 7.4 and 37°C. The results are shown as means and S.E.M. of three determinations.

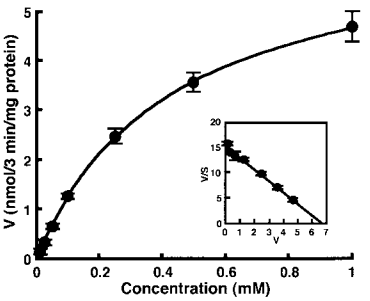

Fig. 4. Concentration dependence of TEA uptake by OCTN1-transfected HEK293 cells. Uptake at various concentrations of TEA was measured at pH 7.4 and 37°C for 3 min. The results show the values after subtraction of the Mock uptake. Inset: Eadie-Hofstee plot of the saturable uptake of TEA.

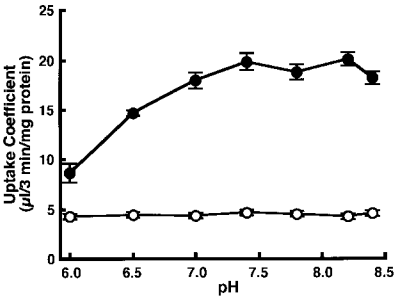

Fig. 5. Effect of pH on [^{14}C]TEA uptake by OCTN1-transfected HEK293 cells. Uptake of [^{14}C]TEA (60 μM) by OCTN1 (closed circles) and pcDNA3 vector-transfected (open circles) HEK293 cells was measured at 37°C for 3 min. The results are shown as means and S.E.M. of three determinations.

nmol/3 min/mg protein and half-saturation concentration (K_m) of 0.436 ± 0.041 mM were obtained.

Fig. 5 shows the pH dependence of [^{14}C]TEA uptake by OCTN1-transfected cells. When the pH in the transport medium was acidic (pH 6.0), OCTN1-mediated uptake of [^{14}C]TEA was decreased to about 30% of those at neutral and alkaline pH, whereas no significant change in the uptake of [^{14}C]TEA was observed in Mock. Furthermore, comparable uptakes were observed at neutral and alkaline pH. When cellular ATP was depleted by pretreatment with metabolic inhibitors, sodium azide, sodium fluoride and 3-*O*-methylglucose, which can decrease cellular ATP level to about 10% of the control [17], the uptake of [^{14}C]TEA by OCTN1 obtained after correction of Mock uptake was decreased to 52–62% of the control at all pH values tested (Table 1). Accordingly, transport by OCTN1 is suggested to occur at least partially through a primary active process, which is consistent with the nucleotide binding site motif expected from its amino acid sequence. In ATP-depleted cells, a more marked pH dependence of OCTN1 activity was observed compared with that of uptake by the normal non-ATP-depleted cells.

4. Discussion

In 1994, the first renal organic cation transporter OCT1 was isolated from rat kidney by expression cloning [4]. As a homologue of OCT1, OCT2 from rat and their counterparts from human kidney and liver and a porcine kidney-derived cell line, LLC-PK1, were isolated [5–9]. Rat OCT1 is present in several tissues, rich in kidney, liver and intestine, and likely to function at the basolateral membrane of renal tubular epithelial cells, considering its pH-independent and membrane potential-dependent transport characteristics. The distribution of rat OCT2 is limited to kidney and, to a minor extent, brain. Gründemann et al. [6] suggested that rat OCT2 may be an apical-type transporter on the basis of its susceptibility to specific cation transport inhibitors and pH-dependent transport activity. However, Okuda et al. [5] reported a discrepant result, i.e. pH independence of rat OCT2, so further study on the localization within the kidney and the functional characteristics of rat OCT2 is needed. As for human OCT1 and OCT2, they are strongly expressed in liver and kidney, respectively, and both of them exhibit membrane potential-dependent transport activity [8,9]. Although an immunohistochemical study indicated that OCT2 is present at the apical membrane of kidney, it is expected to have a role in reabsorption from the renal tubular lumen, since it exhibits membrane potential-dependent transport activity [9]. Thus, no apically expressed organic cation/proton antiporter has yet been clearly identified.

In the present study, a novel cDNA clone which was expected to encode an organic cation transporter, because of the relatively high similarity of the predicted amino acid sequence to those of known rat and human organic cation transporters, OCT1 and OCT2. We transiently expressed OCTN1 in HEK293 cells and the transport activity was examined using TEA as a model organic cation. As can be seen in Figs. 3–5, OCTN1 exhibited saturable, metabolic energy-dependent, and pH-sensitive transport of [^{3}H]TEA with higher activity at neutral and alkaline pH than at acidic pH. The Michaelis constant obtained at pH 7.4, 436 μM, is high compared with those of previously cloned OCT family members, for which the K_m values range from 20 to 100 μM, including human [4,6,8,9], and the inhibition constant K_i (163 μM) of TEA for the transport of 1-methyl-4-phenylpyridinium by human OCT1 [7]. Accordingly, OCTN1 has lower affinity for TEA than those of previously isolated transporters and seems to be a novel organic cation transporter. The K_m value of 0.21 mM

Table 1
Effect of ATP depletion on [^{14}C]TEA uptake by OCTN1-transfected HEK293 cells

		Uptake of [^{14}C]TEA (μl/2 min/mg protein)			
		OCTN1-transfected		Mock	
		Normal	ATP-depleted	Normal	ATP-depleted
pH	6.0	9.33 ± 0.35	6.46 ± 0.30*	4.63 ± 0.19	4.00 ± 0.21
	7.4	14.51 ± 0.11	11.2 ± 0.93*	4.45 ± 0.43	4.64 ± 0.19
	8.4	14.0 ± 0.36	10.4 ± 0.22*	4.83 ± 0.37	4.71 ± 0.49

Uptake of [^{14}C]TEA (60 μM) by OCTN1- and pcDNA3 vector-transfected HEK293 cells was measured at 37°C for 2 min. The procedure for ATP depletion is described in the text. Each value represents the mean ± S.E.M. of three determinations. *Significantly different from normal not ATP-depleted cells.

for pH-dependent TEA uptake was obtained by using isolated renal brush-border membrane vesicles from rat [18], and this is close to the K_m value obtained in the present study. Although no K_m value for human renal transport of TEA is yet available, it is worth noting that the available K_m values for renal basolateral transporters so far identified are distinct from that obtained in the present study. TEA uptake by HEK293 transfected with OCTN1 was clearly pH-dependent, whereas the uptake of TEA by Mock showed little variation at pH values between 6.0 and 8.4. This observation strongly suggests that OCTN1 is a novel organic cation transporter, which may operate at the apical membrane of renal epithelial cells. Uptake at pH 8.4 was comparable with that at pH 7.4. Maegawa et al. reported that there is an optimal pH for a proton/organic cation antiporter of around pH 7 and the activity of the antiporter is regulated by pH as well as proton gradient as the driving force [19]. The observed pH dependence of OCTN1 may be explained by the presence of such an optimal pH. Both an outward proton gradient and the pH in the uptake medium may be important, as a driving force for OCTN1 and for regulation of transport activity, respectively.

OCTN1 possesses a nucleotide binding sequence motif, which suggests it may be an ATP-dependent transporter. As shown in Table 1, by depleting ATP with metabolic inhibitors, uptake of [^{14}C]TEA was partially decreased. The effect of ATP depletion was most evident at pH 6.0, where only 52% of the activity of the control remained, whereas approximately 62% of the control activity remained at neutral and alkaline pH. These results might suggest that ATP dependence was minimal in the presence of a proton gradient, as observed at pH 7.4 and 8.4, whereas at acidic pH the transport largely depends on ATP. ATP-dependent transport of organic cations at the apical membrane of kidney has been reported to function for reabsorption of endogenous cations and some xenobiotics [20]. It was suggested that the ATP binding site of the transporter is localized at the apical surface, since supply of ATP, for which the membrane permeability is negligible, from the extravesicular transport medium is effective to energize TEA uptake by renal brush-border membrane vesicles which are oriented right-side out. From our hydropathy analysis and the location of possible *N*-glycosylation sites of OCTN1, the putative nucleotide binding site may be located at the extracellular portion between transmembrane domains 3 and 4 of OCTN1. Accordingly, OCTN1 may be the transporter responsible for the ATP-dependent reabsorption, as previously suggested. However, it is still necessary to rule out the possibility that the apparent metabolic energy dependence observed in the present study may be ascribed to a reduced ability to maintain a constant intracellular pH owing to the lowered ATP content.

The presence of OCTN1 in several human cancer cell lines is interesting, since it may suggest that OCTN1 can regulate uptake or efflux of chemotherapeutic compounds in these cells. To clarify the clinical significance of OCTN1, it will be necessary to elucidate the precise substrate specificity and transport directionality of OCTN1.

In conclusion, we have cloned a cDNA of a novel human organic cation transporter OCTN1 which is sensitive to pH, suggesting that it may be a proton/organic cation antiporter. Characteristic features of OCTN1 include a nucleotide binding sequence motif and the partial dependence of its transport activity on metabolic energy. It was found in several human cancer cell lines as well as normal kidney, bone marrow and trachea, although its physiological role in these tissues remains to be established. Further studies, including subcellular localization using antibody and elucidation of the mechanism of the pH dependence and metabolic energy-sensitive activity, as well as the substrate specificity, are needed to establish the physiological importance of this transporter.

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