Isolation and characterization of the circulating form of human endostatin

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Abstract Recently, fragments of extracellular proteins, including endostatin, were defined as a novel group of angiogenesis inhibitors. In this study, human plasma equivalent hemofiltrate was used as a source for the purification of high molecular weight peptides (10–20 kDa), and the isolation and identification of circulating human endostatin are described. The purification of this C-terminal fragment of collagen α1(XVIII) was guided by MALDI-MS and the exact molecular mass determined by ESI-MS was found to be 18 494 Da. N-terminal sequencing revealed the identity of this putative angiogenesis inhibitor and its close relation to mouse endostatin. The cysteine residues 1–3 and 2–4 in the molecule are linked by disulfide bridges. In vitro biological characterization of the native protein demonstrated no anti-proliferative activity on different endothelial cell types. These data indicate that human endostatin, which is a putative angiogenesis inhibitor, is present in the circulation.

Key words: Hemofiltrate; Endostatin; Angiogenesis; Mass spectrometry; Endothelium; Blood plasma; Collagen XVIII

1. Introduction

A crucial step in tumor progression and metastasis is the vascularization of the tumor and its immediate surroundings [1,2] through the interaction of pro- and anti-angiogenic factors. The induction of angiogenesis is mediated by angiogenic factors such as the fibroblast growth factors, and the factors related to vascular endothelial growth factor/vascular permeability factor [3,4]. In addition, several negative regulators of angiogenesis have been identified [5], e.g. platelet factor 4, interferon α, thrombospondin, and angiopoietin 2 [6]. Moreover, polypeptide angiogenesis inhibitors generated by a primary tumor were shown to inhibit its metastasis [7,8]. In this respect, angiostatin or endostatin was isolated from cell culture medium [9,10], and shown to specifically inhibit proliferation of endothelial cells and consequently tumor growth and metastasis in different animal models. Mouse endostatin, which entails a C-terminal 20 kDa fragment of collagen XVIII [11], in combination with angiostatin, the kringle-containing fragment of plasminogen [10], exhibited potent tumor inhibitory activity.

Using a human peptide bank including at least 300 000 peptide components generated from hemofiltrate of patients with chronic renal diseases [12] we were able to isolate and characterize different bioactive peptides. Most of these peptides identified so far are proteolytic products of plasma components [13]. For example, bioactive RGD peptides from vitronectin and fibrinogen [14,15], proteolytic fragments of plasma albumin, haptoglobin or β_2-microglobulin were purified in high amounts. During the course of defining and characterizing high molecular weight peptides of this human peptide bank (in the range 10–20 kDa), which strongly bind to cation exchangers, we isolated the circulating form of human endostatin which was previously described from mouse origin and was shown to be a potent angiogenesis inhibitor in vivo [10]. Our findings indicate that endostatin is present in the circulation of patients not bearing any detectable tumor.

2. Materials and methods

2.1. Isolation of endostatin from human hemofiltrate

Human blood ultrafiltrate (hemofiltrate, HF) was obtained from patients with chronic renal insufficiency. Hemofilters with a cut-off of 20 kDa were used and the filtrate was immediately acidified with HCl to pH 3.0 and cooled to inhibit proteolysis. Peptides from 2500 l HF were extracted and processed as described [12]. In brief, the extracts were pooled for the first separation step using a 10 l cation exchange column. Stepwise batch elution was performed using seven buffers with different pHs (increasing from 3.6 to 9.0; pools 1–7). An additional pool 8 was generated by washing the column with water, pH 7.0. The resulting eight pools (15–25 l each) were further separated using reverse phase chromatography. Pool 8 was applied to a RP-C18 column (15–20 μm, 30 nm, 4.7×30 cm; Vydac, Hesperia, USA), and separation was performed at a flow rate of 35 ml/min with a gradient from 100% A to 50% B in 30 min (A: water, 10 mM HCl; B: 100% methanol, 10 mM HCl). Fractions of 1.4 min were collected, monitoring the absorbance at 280 nm. Aliquots were subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The next purification step was carried out with an analytical RP-C18 column (5 μm, 30 nm, 1.0×25 cm; Vydac; flow rate: 2 ml/min) using the following gradient and buffers: from 100% A (water, 0.1% trifluoroacetic acid) to 60% B (80% acetonitrile, 0.1% trifluoroacetic acid) in 50 min. Fractions of 1 min were collected and those peaks containing the peptide with a molecular mass of 18.5 kDa were rechromatographed at a flow rate of 0.6 ml/min by analytical RP-C18 (5 μm, 30 nm, 0.46×25 cm; YMC, Schernbeck, Germany) using the same RP buffers. A gradient from 30% to 80% B in 150 min was applied to obtain highly purified endostatin. To check the purity of the isolated peptides, capillary zone electrophoresis was carried out as described [16].

2.2. Peptide analysis and synthesis

Mass determination of the purified peptides was carried out on a Sciex API *III*+ quadrupole mass spectrometer (Sciex, Thornhill, Can-

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Abbreviations: ESI-MS, electrospray mass spectrometry; HF, hemofiltrate; HUVEC, human umbilical vein endothelial cells; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; RP, reverse phase

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ada) with an electrospray interface (ESI-MS) as described [16]. Collision-induced dissociation of peptides obtained from proteolytic digests (ESI-MS/MS analysis) was performed with argon gas and interpreted with the help of 'Sherpa' [17]. The peptides were further analyzed by a Procise 494 gas-phase sequencer (Applied Biosystems, Weiterstadt, Germany). For MALDI-MS, aliquots of 1 μl of the samples from the reverse phase chromatography, representing a 5 ml hemofiltrate equivalent, were applied on a stainless steel multiple sample tray and mixed with either sinapinic acid or α-cyanohydroxycinnamic acid using the dried drop technique as described [18]. For analysis of cysteines, lyophilized peptides were reduced with 2-mercaptoethanol and alkylated at cysteine residues with iodoacetamide using standard conditions. The carboxamido-methyl-alkylated peptides ($AcNH_2$ peptides) and generated tryptic fragments were purified by RP-HPLC and analyzed by mass spectrometry. Proteolytic cleavage of aliquots containing about 250 pmol native and $AcNH_2$ peptides was performed by thermolysin, chymotrypsin, Glu-C, and trypsin, respectively (Boehringer Mannheim, Germany) using standard conditions as recommended by the manufacturer. Successful digestions were separated in a linear acetonitrile gradient by analytical RP-C18 column. Peptide synthesis of HTHQDFQPVLHL amide was carried out on a 9050 peptide synthesizer using a preloaded Rink-amid resin (PerSeptive Biosystems, Wiesbaden, Germany) using conventional Fmoc chemistry. Purity and identity of the peptide were checked by HPLC, capillary zone electrophoresis, and electrospray mass spectrometry.

2.3. Endothelial cell proliferation assays

Bovine brain capillary endothelial cells were isolated as described [19] and kindly provided by Dr. L. Schweigerer (Essen, Germany). Human umbilical vein endothelial cells (HUVEC) were isolated as described [20]. Both cell types were cultured in endothelial cell medium MCDB 131 from PromoCell (Heidelberg, Germany), containing 2.5% fetal calf serum, and basic fibroblast growth factor (2 ng/ml) on gelatin-coated plates. Cells (30 000/well) were seeded in gelatin-coated 48-well plates in complete medium, and the next day the medium was replaced with fresh medium without any basic fibroblast growth factor. Endostatin (0–50 nM) was added to cells for 30 min before the addition of 4 ng/ml basic fibroblast growth factor. After incubation for 4 days the cells were trypsinized and counted in an electronic counter (Schärfe System, Reutlingen, Germany). A total of five experiments were performed each using three separate preparations of endostatin.

3. Results and discussion

3.1. Peptide isolation and biochemical characterization of endostatin

An established large-scale method for peptide preparation from human blood, generating a bank of circulating human peptides, was used to isolate endostatin [12]. Chromatographic methods in combination with MALDI-MS were used to purify high molecular weight basic peptides within a molecular range of 10–20 kDa and the isolation of highly purified endostatin from a crude mixture was performed via a four-step purification scheme (Fig. 1). Using MALDI-MS, peptides occurring in the lower picomolar range were detectable in very complex mixtures and are suitable for purification as demonstrated previously [18]. Initially, hemofiltrate was fractionated via cation exchange chromatography into pH pools containing peptides with different basicity and/or p*I* (Fig. 1A). Further fractionation of pool 8 was performed by RP chromatography and yielded several fractions which were examined by MALDI-MS. Major components of the marked fraction (Fig. 1B) had molecular masses of 25, 21, 11.7, and 10.5 kDa, while signals at 18.5 and 12.5 kDa were just detectable (Fig. 1C). Purification of the high molecular weight pep-

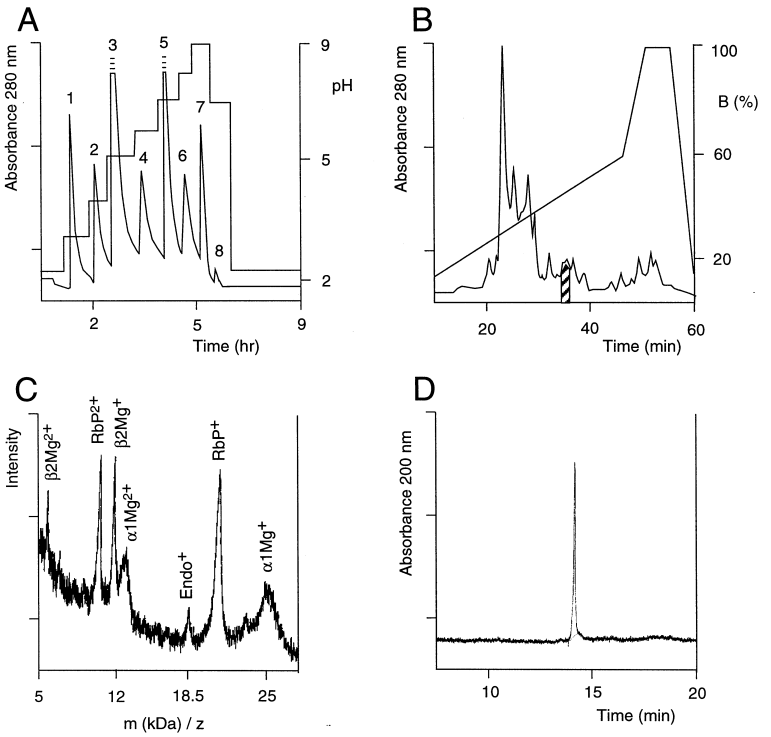

Fig. 1. Purification of human circulating endostatin from hemofiltrate guided by MALDI-MS. A: Preparative cation exchange chromatography and pH pool fractionation of 2500 l human hemofiltrate. In pool 8 high molecular weight peptides were detected and subsequently purified by several chromatographic steps. B: RP-HPLC fractionation of pH pool 8. High molecular weight peptides were detected in the marked fraction using MALDI-MS. C: MALDI-MS scan of the fraction marked in B. α_1-Microglobulin (α1Mg), retinol binding protein (RbP), endostatin (Endo), and β_2-microglobulin (β2Mg) were detected. D: Purification of human circulating endostatin was performed using two additional RP-HPLC steps. The purity of the peptide was demonstrated by capillary zone electrophoresis.

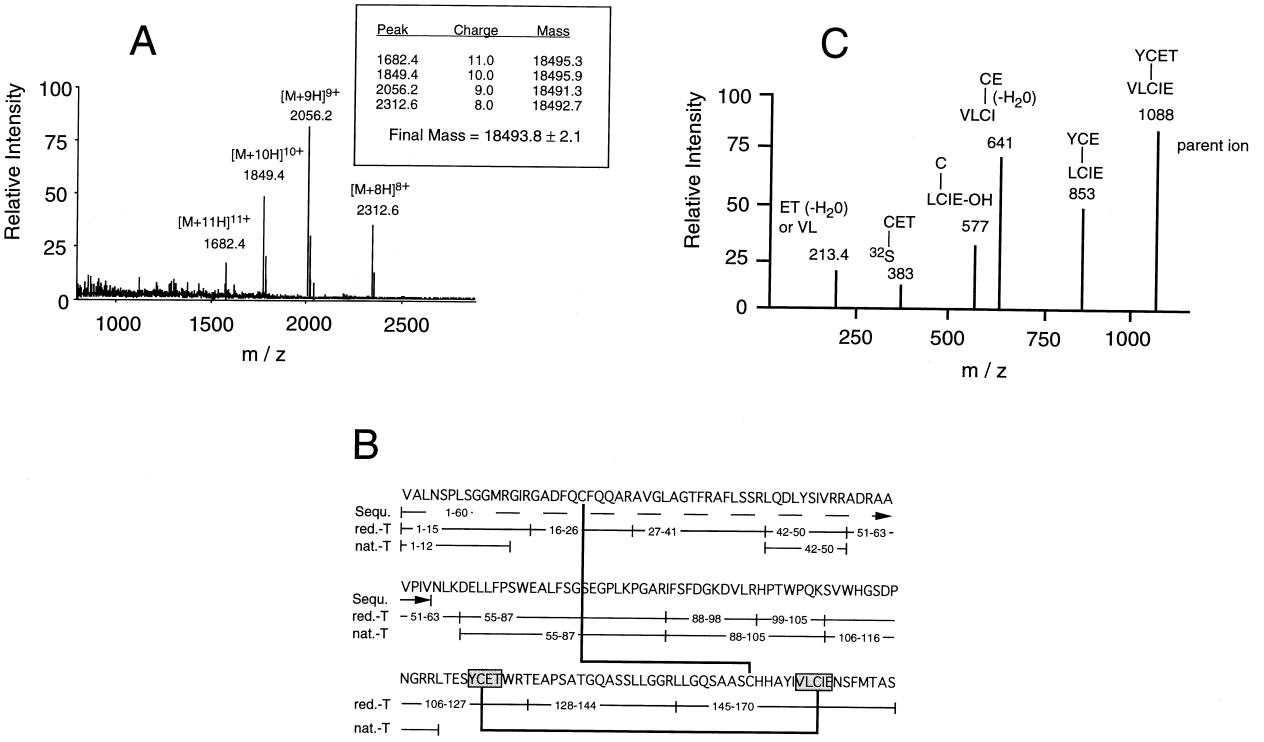

Fig. 2. Identification of human circulating endostatin as collagen α1(XVIII) 514–683. A: Mass determination of the native endostatin using electrospray mass spectrometry. The mass spectrum consists of four different ion species with *m/z* ratios of 1682.4, 1849.4, 2056.2, and 2312.6 representing multiple charged ions of the peptide. The molecular mass of the native peptide was determined to be 18 494 ± 2.1 Da. B: Sequence analysis and enzymatic digest of human endostatin. Sixty residues of the N-terminus were sequenced (broken line). Native and reduced $AcNH_2$ peptide were cleaved by trypsin (T). The resulting fragments were analyzed by MALDI-MS (solid lines). The cysteine containing regions in the native molecule show high stability against trypsin. Determination of the two disulfide bonds (bold lines) was performed by ESI-MS/MS analysis of peptides derived from thermolytic digest of the native endostatin molecule. C: Analysis of disulfide bonds of endostatin by ESI-MS/MS. Peptides from thermolytic digest were separated by RP-HPLC, and subsequent ESI-MS/MS analysis of the peptide with M_r 1088 (parent ion, amino acids boxed and shaded in B) and some fragment ion signals are shown with their deduced sequences. The parent ion mass and its fragment pattern were in accordance with the amino acid sequence corresponding to residues 122–125 and 159–163 of human endostatin from hemofiltrate. In the region of the immonium ion and dipeptides, all single amino acid (except Y and C) residues and several dipeptides were detected with the proposed sequence (data not shown).

tides was performed by subsequent RP chromatography (data not shown) and was guided by MALDI-MS. Substances were isolated with high purity, as evidenced by capillary zone electrophoresis (Fig. 1D). Sequencing of the purified peptides derived from one single fraction of RP chromatography (Fig. 1B) revealed the existence of human circulating endostatin (M_r 18.5 kDa), β_2-microglobulin (M_r 11.7 kDa), retinol-binding protein (M_r 21 kDa), and partially deglycosylated α_1-microglobulin (M_r 25 kDa). These polypeptides exhibit a basic or neutral p*I* and their masses were confirmed by ESI-MS (exemplified in Fig. 2A).

For a more detailed characterization, circulating human endostatin was isolated in larger amounts from 2500 l of hemofiltrate which yielded 200 μg of pure polypeptide. Sequencing of the N-terminal 60 residues (Fig. 2B) as well as proteolytic digestion of the native and amido-alkylated peptide in conjunction with MALDI-MS, ESI-MS, and MS/MS analysis revealed high homology to the recently described mouse endostatin. Native endostatin showed high resistance against endoproteases Glu-C and chymotrypsin, whereas trypsin led to partial degradation. A tryptic map of the reduced and amido-alkylated peptide (Fig. 2B) clearly demonstrated that human endostatin comprises amino acids 514–683 of the human collagen α1(XVIII) sequence deduced from

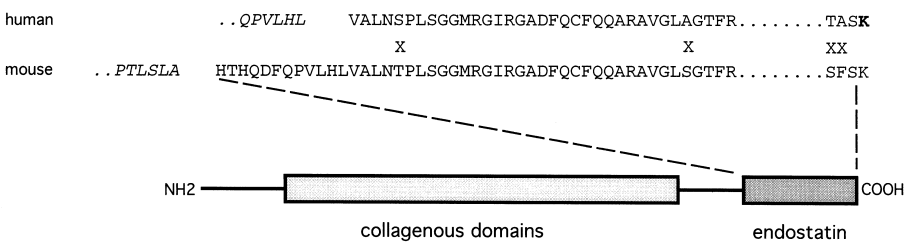

Fig. 3. N-terminal sequences of human circulating endostatin and mouse endostatin. The sequences from the non-triple-helical C-terminal regions of collagen α1(XVIII) (schematic drawing) indicate high homology, mismatches between the mouse [10] and human sequences are denoted by X. The pre-N-terminal sequences are in italics. The lacking C-terminal lysine (K) in the human sequence is in bold. Note that the human endostatin isolated from hemofiltrate lacks 12 N-terminal amino acids, as compared to the mouse counterpart.

cDNA [11] with a C-terminal Ser residue. One fragment (M_r 2881) of the tryptic map exactly corresponded to the C-terminal amino acids 145–170 of the molecule (Fig. 2B). In comparison to the full cDNA sequence, the C-terminus of human endostatin lacks a single lysine residue as deduced by mass spectrometry, indicating possible processing by carboxypeptidase(s) (Fig. 3). The alignment with mouse endostatin, isolated from mouse hemangioendothelioma cell medium [10], indicates that the N-terminus of circulating human endostatin is 12 amino acids shorter than endostatin from mouse tumors (Fig. 3). This difference might be explained by different releasing mechanisms from the parent collagen α1(XVIII) by as yet unknown protease(s). The pre-N-terminal regions of processed mouse and human endostatin (Fig. 3) consist of hydrophobic amino acids indicating a chymotryptic-like cleavage site. The human and mouse protein sequences show 86% identity and >90% similarity, demonstrating a high structural (and possibly functional) relationship. Comparison of the tryptic digests of native and alkylated endostatin consisting of 170 amino acids indicated stabilization by two disulfide bonds. Thermolytic digest revealed a fragment with a molecular mass of 1087.4 Da identified as (^{122}YCET^{125})-S-S-(^{159}VLCIE^{163}) by ESI-MS/MS (Fig. 2C). This indicates that disulfide bridges are linked between Cys-123/Cys-161 and Cys-21/Cys-153. The molecular mass of the native molecule determined by electrospray mass spectrometry (Fig. 2A) was 18 494 ± 2 Da and is thus exactly in accordance with the calculated mass of amino acids 514–683 from collagen α1(XVIII) sequence (18 496 Da) deduced from cDNA (supposing that the cysteines are bridged).

Collagen α1(XVIII) consists of multiple collagenous domains which are separated and flanked by non-triple-helical regions [21,22]. The endostatin sequence comprises the C-terminal non-triple-helical region of the collagen molecule (Fig. 3). For the mouse endostatin region one potential glycosylation site was proposed [21]. However, there is no consensus sequence (XXSer-GlyXX) for attachment of glycosaminoglycans within the corresponding human sequence. A similar structure and about 60% identity at the amino acid level were shown for the mouse collagen α1(XVIII) and human collagen α1(XV) sequences, with the conserved pattern of four cysteine residues in the C-terminal (endostatin) region [21], suggesting the possible existence of corresponding endostatin molecules.

Collagen α1(XVIII) was classified as a class of non-fibrillar proteins of the extracellular matrix and was shown to be localized in vascular basement membrane zones of various organs [22]. In this context the release of an angiogenesis inhibitor like endostatin from vessel sites refers to a paracrine regulation of neovascularization. In contrast, our findings provide evidence for circulating endostatin functioning in an endocrine fashion.

3.2. Functional characterization of endostatin

Based on the experience with several other peptide isolations from hemofiltrate carried out previously, the recovery during purification was estimated to be in the range of 20% resulting in a concentration of $>10^{-11}$ M in hemofiltrate. Considering the cut-off (20 kDa) of the hemofilters used, the concentration of endostatin in (patient) plasma was in the range of 10^{-10} M or higher. It remains to be established whether an additional pool of tissue-bound endostatin exists,

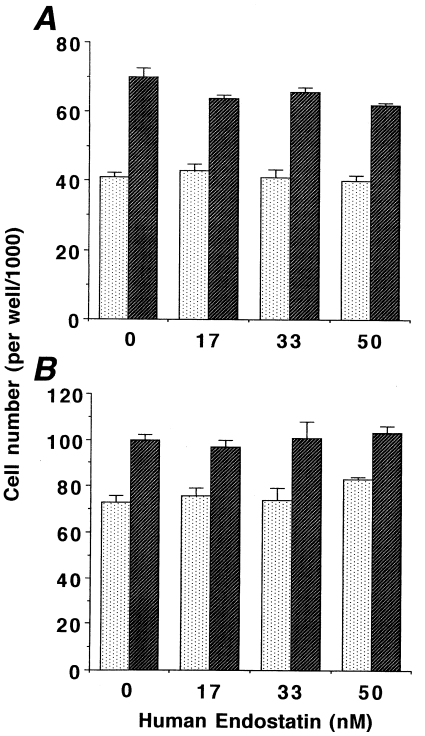

Fig. 4. The effect of human endostatin on endothelial cell proliferation. Bovine brain retinal capillary endothelial cells (A) or human umbilical vein endothelial cells (B) were pretreated with the indicated concentrations of human endostatin for 30 min. To these pretreated cells was added either 4 ng/ml basic fibroblast growth factor (dark bars) or saline (light bars) as a control. After 4 days, the wells were trypsinized and the cell number was determined. Results are expressed as number of cells per well divided by 1000 (mean ± S.E.M.). Similar results were obtained in three separate experiments with each cell type.

as was proposed for angiostatin [23]. A specific ELISA or RIA is needed to determine this parameter more precisely. The reported concentrations for anti-proliferative effects of mouse endostatin on endothelial cells are in the range of $>3\times10^{-9}$ M or higher [10].

Native endostatin was tested in proliferation assays using endothelial cells from human umbilical vein or bovine brain capillaries. Basic fibroblast growth factor induced stimulation of cell proliferation on both cell types (Fig. 4). Human endostatin showed no anti-proliferative effect on microcapillary endothelial cells (Fig. 4A), and no effect of endostatin was detected on HUVEC (Fig. 4B). Since the human peptide is 12 amino acids shorter than the mouse peptide, the dodecapeptide mouse sequence was synthesized by solid phase chemistry and tested in both systems. In both assays, no effect of the peptide on endothelial cell proliferation was observed (data not shown). The conditions of the assays were identical to those used to test endostatin in the original report [10].

Some differences between our results and previously published data in the mouse system are apparent and need to be addressed: (i) mouse and human endostatin were isolated from different sources and thus may have different properties regarding selectivity or specificity. Moreover, the efficacy of mouse endostatin in vivo or in vitro may not be duplicated by the human counterpart. (ii) Mouse and human peptides may undergo different posttranslational processing events that are crucial for their respective biological activity. (iii) Human en-

dostatin may not necessarily inhibit endothelial cell proliferation but can (indirectly) influence other cellular functions needed for angiogenesis which are only prominent in a complex in vivo system. Additional experiments also including production of recombinant forms of human endostatin are being currently undertaken to characterize the cellular interactions of this putative angiogenesis inhibitor.

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