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Activity and stability of human kallikrein-2-specific linear and cyclic peptide inhibitors

MIIKKA PAKKALA,[a] CAN HEKIM,[b] PASI SOININEN,[a] JARI LEINONEN,[b] HANNU KOISTINEN,[b] JANNE WEISELL,[a] ULF-HÅKAN STENMAN,[b] JOUKO VEPSÄLÄINEN[a] and ALE NÄRVÄNEN[a]*

[a] Department of Chemistry, University of Kuopio, P.O. Box 1627, FIN-70211, Kuopio, Finland
[b] Department of Clinical Chemistry, University of Helsinki and Helsinki University Central Hospital, Finland

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Abstract: Human glandular kallikrein (KLK2) is a highly prostate-specific serine protease, which is mainly excreted into the seminal fluid, but part of which is also secreted into circulation from prostatic tumors. Since the expression level of KLK2 is elevated in aggressive tumors and it has been suggested to mediate the metastasis of prostate cancer, inhibition of the proteolytic activity of KLK2 is of potential therapeutic value. We have previously identified several KLK2-specific linear peptides by phage display technology. Two of its synthetic analogs, A R R P A P A P G (KLK2a) and G A A R F K V W W A A G (KLK2b), show specific inhibition of KLK2 but their sensitivity to proteolysis *in vivo* may restrict their potential use as therapeutic agents. In order to improve the stability of the linear peptides for *in vivo* use, we have prepared cyclic analogs and compared their biological activity and their structural stability. A series of cyclic variants with cysteine bridges were synthesized. Cyclization inactivated one peptide (KLK2a) and its derivatives, while the other peptide (KLK2b) and its derivatives remained active. Furthermore, backbone cyclization of KLK2b improved significantly the resistance against proteolysis by trypsin and human plasma. Nuclear magnetic resonance studies showed that cyclization of the KLK2b peptides does not make the structures more rigid. In conclusion, we have shown that backbone cyclization of KLK2 inhibitory peptides can be used to increase stability without losing biological activity. This should render the peptides more useful for *in vivo* applications, such as tumor imaging and prostate cancer targeting.

Keywords: cancer; human kallikrein-2; prostate; synthetic peptides; resistance against proteolysis; stability

INTRODUCTION

Kallikreins are serine proteases that belong to the human tissue kallikrein family, which comprises 15 members (KLK1–KLK15) [1]. Two highly prostate-specific kallikreins, KLK2 and KLK3 (PSA), are widely used as tumor markers [1–4]. Although PSA is a very useful marker for prostate cancer, the expression level of PSA is higher in normal prostate epithelium than in the tumor tissue and it is further reduced in poorly differentiated tumors. In contrast, the expression of KLK2 is increased in aggressive tumors and is suggested to be involved in tumor spread [5,6].

Proteases have been suggested to play an important role in aggressive tumors mediating invasion and metastasis. Therefore, inhibition of proteases that promote tumor spread may be of therapeutic value in the treatment of cancer. Synthetic low-molecular-weight inhibitors to matrix metalloproteinases (MMP) have been extensively studied clinically as cancer therapeutics. However, side effects due to their broad specificity have limited their clinical utility. Therefore, it would be advantageous if the protease inhibitors to be used for therapy are specific. Proper identification and specific inhibition of proteases require specific probes. Short synthetic peptides corresponding to the amino acid sequences of the *N*- and *C*-termini of human immunodeficiency virus protease (HIV-1 PR) have been shown to inhibit the proteolytic activity of HIV-1 PR by preventing association of the inactive subunits to the active dimer [7,8]. Furthermore, specific inhibition of MMP-2 and MMP-9 has been reported to decrease the size of tumors in mice by inhibiting angiogenesis [9,] and a specific inhibitor of human tissue kallikrein has been reported to suppress cancer cell invasiveness of human adenocarcinomas [10].

We have identified KLK2- and KLK3-specific peptides by using the phage display methodology. These peptides are enzyme-specific and do not inhibit other proteases [11–14]. We modified the biological activity of two linear KLK2-specific peptide inhibitors by alanine substitution to optimize their inhibitory properties [11].

The proteolytic stability is an important factor for the use of peptides *in vivo*. Most peptides have to be modified to prevent enzymatic degradation. Several approaches including the use of D-amino acids, unusual amino acids, peptidomimetics or cyclization have been used to improve peptide stability [3,15]. In this study we have prepared cyclic variants of KLK2 inhibitory peptides and compared their inhibitory activity to the original peptides. Furthermore, we

* Correspondence to: A. Närvänen, Department of Chemistry, University of Kuopio, FIN-70211, Kuopio, Finland; e-mail: ale.narvanen@uku.fi

studied the effect of cyclization on the peptide stability against digestion by trypsin and human plasma. Also, possible conformational constraints of the peptides were studied by proton NMR spectroscopy.

MATERIALS AND METHODS

Peptide Synthesis

The peptides (Table 1) were synthesized using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY) with Fmoc strategy and TBTU/DIPEA as the coupling reagent. Fmoc-Gly-Wang and Rink resins were used as the solid phase (Novabiochem, Läufelfingen, Switzerland). The side-chain protecting groups used in synthesis were Trt for Asn, Gln and His; OtBu for Glu and Asp; Boc for Trp; and tBu for Ser, Thr and Tyr. For Cys, both Acm and Trt proctetion groups were used. During cleavage from the resin with 96% TFA, the Acm protection group remains in the Cys side chain. Allyl-protected aspartic acid (Fmoc–Asp(OH)–OAll) was used for head-to-tail cyclization. Carboxy terminal-protected aspartic acid was coupled to the Rink resin via the free side chain. For stability tests, one of the peptides, KLK2b, was acetylated on the resin. The free amino group was treated with 20% acetic anhydride in DMF twice for 10 min before cleavage. The peptides were purified by HPLC (Shimadzu, Kioto, Japan) on a C_{18} reverse-phase column (xTERRA, Waters, Milford, MA) using an acetonitrile (ACN) gradient (0.1%TFA in H_2O/0–60% ACN gradient for 60 min) and verified by mass spectrometry on an ABI QSTAR XL hybrid mass spectrometer using the MALDI interface (Applied Biosystems, Foster City, CA). The purity was determined by analytical HPLC on a 240 × 1.4 mm C_{18} column (xTERRA, Waters) eluted with 0–60% ACN for 30 min.

Cyclization of the Peptides

Peptides containing cysteines with Acm were cyclicized by the iodination method. Briefly, the lyophilized peptide was dissolved in 50% acetic acid (AcOH) in H_2O at a concentration of 2 mg/ml. HCl (1 M; 0.1 ml/mg of peptide) was added and immediately followed by the addition of 0.1 M iodine solution in 50% AcOH in H_2O (5 equiv./Acm). The solution was stirred vigorously at room temperature for 40 min and the reaction was stopped with 0.1 M sodium thiosulfate. After filtering (0.45 μm), the peptides were purified by HPLC as described above.

Head-to-tail cyclization was performed on the resin. Deprotection of the allyl ester was performed manually with Pd(0) under argon. The catalyst dissolved in a small amount of dry DCM was transferred to a sealed vial containing the Fmoc–peptidyl(resin)–OAll and $PhSiH_3$. The reaction mixture was stirred for 2.5 h at room temperature under argon. The resin was washed sequentially with the catalyst-dissolving mixture, 0.5% DIEA in DMF, 0.5% sodium diethyl dithiocarbamate in DMF and DCM, and dried. The *N*-terminal Fmoc group was removed by treatment with 20% piperidine in

Table 1 Activities of linear KLK2 peptides and the corresponding cyclic forms (c = cyclic form with cysteine bridge, ht = head-to-tail cyclic peptide). Activities indicate the degree of inhibition of KLK2 activity

Peptide	Sequence	MW	KLK2 inhibition (%)	±SD (%)
KLK2a linear	A R R P A P A P G	891.30	72	1.6
KLK2a(c1)	C A R R P A P A P C G	1095.35	−4	0.0
KLK2a(c2)	C A A R R P A P A P C G	1166.56	23	1.2
KLK2a(c3)	C A A A R R P A P A P C G	1237.36	3	0.1
KLK2a(c4)	C R R P A P A C G	927.49	−9	0.1
KLK2a(c5)	C A R R P A P A C G	1098.34	1	0.2
KLK2a(c6)	C A R R P A P A A C G	1069.13	−4	0.0
KLK2a(c7)	C A A R R P A P A A C G	1140.28	7	0.2
KLK2a(c8)	C A A A R R P A P A A C G	1211.42	0	0.0
KLK2b linear	G A A R F K V W W A A G	1318.53	74	0.3
KLK2b(c1)	G C A A R F K V W W A A C G	1522.60	97	0.0
KLK2b(c2)	C A A R F K V W W A A C	1408.60	95	0.2
KLK2b(c3)	G C A R F K V W W A C G	1380.59	94	1.7
KLK2b(c4)	G C A A R F K V W W A A C G	1522.80	90	1.8
KLK2b(c5)	C R F K V W W C	1124.40	72	1.4
KLK2b(c6)	G C R F K V W W C G	1238.50	70	1.2
KLK2b linear	G A A R F K V W W A A G	1318.53	72	1.2
KLK2b linear R4K mutation	G A K F K V W W A A G	1219.60	82	1.0
KLK2b ht1	A R F K V W W N	1087.69	93	0.8
KLK2b ht2	A R F K V W W A N	1158.62	94	0.6
KLK2b ht3	A A R F K V W W A N	1229.66	94	0.8
KLK2b ht4	A A R F K V W W A A N	1300.69	96	0.3
KLK2b ht5	A A A R F K V W W A A N	1371.73	95	2.1
KLK2b ht6	A R F K V W N	901.48	68	0.7

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DMF. Head-to-tail cyclization of the peptides was performed twice by the addition of HBTU/DIEA (8 equiv., 1 : 1, mol/mol) in DMF for 2 × 3 h at RT. Peptides were cleaved from the Rink resin with 95% TFA, which introduced an asparagine into the final cyclic peptide. The yields of the both cyclic forms varied between 10 and 15%.

Effect of the Peptides on the Enzyme Activity of KLK2

The effect of the peptides on the enzyme activity of KLK2 was studied by using the chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-pNA, Chromogenix, Milano, Italy) [11]. KLK2 (0.17 μM) was incubated with a 100-fold molar excess of synthetic peptides in 20 mM Tris-buffer, pH 8.0, containing 0.1% BSA for 30 min at RT. After addition of the substrate to a final concentration of 0.2 mM, the absorbance was monitored at 405 nm at 5–10 min intervals for 1 h on a Victor 1420 Multilabel photo- and fluorometer (PerkinElmer-Wallac, Turku, Finland).

Stability Studies

Initially, stability was studied by using modified trypsin (sequencing-grade modified trypsin V5113, Promega, Madison, USA). Four hundred microliters of each peptide (1 mg/ml) dissolved in 200 mM NH_4HCO_3 buffer (pH 8.0) was mixed with 1 μl of trypsin solution (1 mg/ml in NH_4HCO_3, pH 8.0). The peptides were incubated at +37 °C and 30 μl samples were taken every 30 min. Thirty microliters of 2% TFA and 5% ACN in water was added, and the samples were analyzed by HPLC and by mass spectrometry using ABI QSTAR XL with a MALDI interface. Open rings of the KLK2 ht1 peptide were analyzed by MSMS and the fragment ions were calculated by using Prowl (http://prowl.rockefeller.edu/). The stability of the peptides in human plasma was studied by mixing 400 μl of human male plasma and 400 μl of each peptide in PBS (1 mg/ml). The peptides were incubated at +37 °C and 100 μl aliquots were taken after 30 min and then every hour up to 4 h. In the case of the head-to-tail form, the peptide was incubated for 24 h. The peptide was separated from plasma proteins on a Microcon centrifugal filter device (Microcon YM-10, cut off 10 kDa, Millipore, Bedford, MA) by centrifugation at 14 000 rpm using an Eppendorf 5415 D centrifuge (Eppendorf, Hamburg, Germany) for 10 min. The remaining plasma proteins on the filter were washed after centrifugation with 50 μl of 01% TFA and 0.1% Tween 20 in water and centrifuged again for 10 min. The filtrates were analyzed by analytical HPLC and ABI QSTAR XL mass spectrometer using the MALDI interface.

NMR Spectroscopy

Samples were prepared for NMR by dissolving the purified and lyophilized peptides in 500 μl 10% D_2O in H_2O to a peptide concentration of 20–25 mM. The pH of the samples varied between 2 and 3. All spectra were recorded on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany) operating at 500.13 MHz for ^1H. One-dimensional experiments were recorded at different temperatures over the range 285–340 K. Two-dimensional (2D) experiments were recorded at 300 or 330 K depending on the quality and clarity of the spectra. All chemical shifts are reported with respect to the 5 mM TSP peak at 0.00 ppm. Two-dimensional experiments included COSY, TOCSY and ROESY with mixing times of 80 ms for KLK2a and 225 ms for KLK2b.

RESULTS

Inhibitory Activity of the Peptides

The inhibitory effect of the linear and cyclic forms of the KLK2a and KLK2b peptides was measured by using 0.17 μM concentration of the KLK2 enzyme and a 100-fold molar excess of each peptide. There was a clear difference in the effect of cyclization between the two KLK2-specific peptides. Most of the cyclic forms of KLK2a were totally inactive and only one KLK2a(c2) showed slight (23%) inhibition, while all cyclic KLK2b peptides were active and some of them were even more active than the original linear peptide. The most active peptide (KLK2b(c1)) inhibited 97% of the activity, while the linear peptide showed 74% inhibition. A variant with the point mutation R4K in the linear form of KLK2b showed equal activity compared to the original peptide (Table 1).

Biological Stability

The stability of linear acetylated and two cyclic forms of KLK2b was determined by using modified trypsin, which cuts peptide bonds after lysine and arginine. Complete degradation of the linear forms occurred in 30 min, producing fragments cleaved after the positively charged amino acids (Table 2). Proteolytic cleavage of the peptide with cysteine bridges produced a peptide with an additional 18 Da because of the degradation of one amide bond, the cysteine bridge remaining intact. The same addition was seen with the head-to-tail form of the KLK2 peptide (KLK2 ht1). The cleavage rate of this peptide was much slower than that of the three other forms. HPLC analysis showed that only 57% of the KLK2b ht1 peptide was cleaved by trypsin in 4 h (Figure 1(A)). According to MSMS analyses, the major cleavage site was after arginine.

To simulate the proteolytic environment *in vivo*, all four peptides were incubated in human plasma. The two linear forms as well as the cyclic form with a cysteine bridge were degraded within 30 min, whereas the head-to-tail peptide remained intact for 24 h (Figure 1(B)).

NMR Analyses

Solvent exposures of the NH groups were detected by determining their temperature coefficients. None of the investigated peptides had an amide proton showing very low $\Delta\delta/\Delta T$ values (<3 ppb/K) characteristic of strong solvent shielding [16]. No reasonable differences between the chemical shifts of $C^{\alpha}H$'s and random coil values [17] were found. Medium or strong sequential

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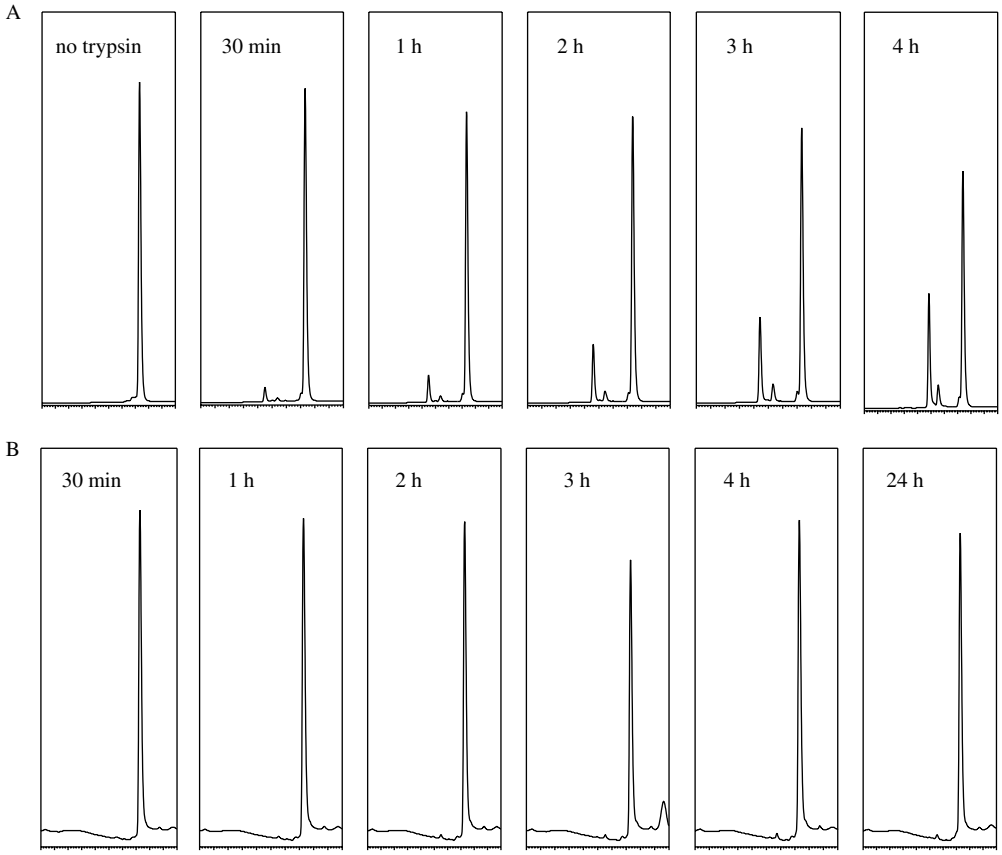

Figure 1 (A) HPLC analyses of the degradation of the head-to-tail form KLK2 ht1 with trypsin. About 57% of the peptide was cleaved after 4 h. (B) HPLC analyses of KLK2 ht1 peptide incubated with plasma. No significant degradation occurred during 24 h. Peptides and peptide fragments corresponding to the masses are shown in Table 2.

$C_\alpha H(i)–NH(i+1)$ NOEs were found between almost all residues in both peptides, indicating predominant β-conformations. Furthermore, no long-range inter-residual NOEs were detected. Some broadening of the NH peaks were detected, indicating solvent-exposed NH groups or conformational fluctuation and dynamic behavior [16,18].

DISCUSSION

Phage display is a powerful method to produce peptides with novel biological activities, which may be useful and form lead structures for the design of new diagnostic and therapeutic molecules. We have identified novel peptide inhibitors that are specific to the serine protease KLK2 by phage display [11]. Two linear synthetic analogs display specific and efficient inhibition of KLK2. Although the amino acid sequences of the two peptides are dissimilar, their inhibition of KLK2 is competitive, suggesting that they bind to the same site. Both peptides contain arginine, which cannot be replaced with alanine, suggesting its important role for binding [11]. Substrate-specificity studies indicate that KLK2 has strict preference for arginine at position P1 followed by a serine at position P′1 [19]. In spite of this, our peptides are not cleaved by KLK2 but its protease activity *in vitro* is inhibited. Replacement of arginine with lysine in the peptide KLK2b did not abolish its inhibitory effect, suggesting that inhibition is based on the positive charge that guides binding of the peptides to KLK2.

In order to study the correlation between structure and function, we synthesized a set of cyclic variants of both peptides by adding cysteines to the amino and carboxyl ends. Inhibition assays showed that essentially all the cyclic KLK2a peptides were inactive, only one of showing a slight inhibitory activity. The corresponding cyclic forms of the KLK2b peptides were active. Since the disulfide-bridged cyclic forms of KLK2b were biologically active, we further synthesized a set of cyclic peptides with the head-to-tail strategy. After cyclization, the inhibitory activity of the KLK2b peptides with various sequence lengths was similar and slightly higher than that of the corresponding linear peptide.

Structural studies of linear and cyclic peptides by NMR spectroscopy indicated the absence of major rigid conformations. Neither sequential $NH(i)–NH(i+1)$ NOEs nor low-temperature coefficients were found for acyclic forms of KLK2a. Cyclic forms of KLK2a were not investigated with NMR because of their lack

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Table 2 Four different forms of KLK2b peptides digested with trypsin for 30–240 min and analyzed by mass spectrometry. Sequences of the fragments were deduced from the masses of the fragments (MW) by mass spectrometry. A dash (–) in the sequence indicates a possible cleavage site, but the resultant peptides are still attached by cysteine bridges

	Sequence	MW
Linear		
Uncleaved	ARFKVWWG	1048.55
Fragment 1	ARFK	520.30
Fragment 2	VWWG	546.25
Fragment 3	AR	245.14
Fragment 4	FKVWWG	821.41
Acetylated linear		
Uncleaved	AcARFKVWWGG	1147.57
Fragment 1	AcARFK	562.30
Fragment 2	VWWGG	603.27
Fragment 3	AcAR	287.14
Fragment 4	FKVWWG	821.41
Cyclic with Cys-bridge		
Uncleaved	GCAARFKVWWACG	1451.470
Estimated fragments + 18 D	GCAARFK—VWWACG	1469.480
Estimated fragments + 18 D	GCAAR—FKVWWACG	1469.480
Head-to-tail cyclic		
Uncleaved KLK2b ht1	ARFKVWWN	1087.69
Linear fragment 1	VWWNARFK	1105.57
Linear fragment 2	FKVWWNAR	1105.57

of biological activity. In the case of cyclic forms of KLK2b, only weak sequential NH(i)–NH(i + 1) NOEs were found between some of the residues, and no low-temperature coefficients for amide protons were found in the cyclic and linear forms of KLK2b. A majority of the recognized NOE correlation peaks were intraresidual or sequential (i – i + 1). This suggests that there is no major conformational constrains in the KLK2b sequences. Furthermore, the chemical shifts between cyclized and acyclic forms of KLK2b were almost equal in all residues enabling structural similarity and therefore similar biological activity.

The stability of two linear and two cyclic forms of KLK2b against proteolytic digestions was studied by using trypsin and human plasma. Although the linear forms of the peptides were not degraded by KLK2, they were sensitive to trypsin digestion and they were also degraded in plasma. The fragments detected after tryptic digestion indicate that the linear forms were cleaved after arginine and lysine. An adduct of 18 Da was detected after protease treatment of the cyclic form with a cysteine bridge, indicating that the peptide was cleaved after arginine or lysine, leaving the cysteine bridge intact. The most stable form was the cyclic peptide without terminal amino acids, i.e. head-to-tail cyclic form. After a 4-h incubation with trypsin, 57% of the KLK2b ht1 peptide was still intact, and even after 24 h no fragmentation was observed in plasma. It is noteworthy that the used trypsin concentration was very high, i.e. fivefold higher than generally used for protein digestion for mass spectrometry. Head-to-tail cyclization has also been found to improve the stability of peptides against proteases of α-conotoxin, while maintaining the biological activity [20]. The cyclic form of the conotoxin was sensitive to the size of the linker between the amino and carboxy termini. Unlike our peptide, the 3D structure of the cyclic conotoxin could be determined, which indicated a rigid conformation and influence of the sequence length on the correct conformation of the peptide. Absence of a rigid conformation in both the linear and cyclic forms of the KLK2b peptide could explain why both cyclic and linear forms with variable sequence lengths displayed similar activity towards KLK2. Usually cyclization of peptides has been used to restrict conformational mobility and to enhance the affinity to the target molecule [21,22]. In the case of KLK2 inhibitory peptides, a rigid structure does not seem to be needed for biological activity or stability. Our results indicate that backbone cyclization is an efficient and simple approach to improve the resistance to proteolytic digestion also of flexible structures, and this approach, without any sequence modifications, could be useful for modification of other peptides that are used for *in vivo* studies. Although there were no defined rigid structural elements in cyclic forms, the slightly higher activity compared to the linear ones may be due to the restricted conformational space during binding. Lower internal rotational entropy may translate into higher binding affinity for the cyclic analogs.

We conclude that head-to-tail cyclization of peptide inhibitors to KLK2 dramatically improve their resistance to proteolytic digestion and enhance their inhibitory capacity. Thanks to long term stability in plasma, these peptides should be suitable for studies on the effect of KLK2 inhibition in animal models of prostate cancer.

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