

A secondary C1s interaction site on C1-inhibitor is essential for formation of a stable enzyme-inhibitor complex

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Received 11 December 1996

Abstract This paper examines the location of a secondary binding site for C1s on C1-inhibitor (C1-inh) which is required for the formation of SDS-stable C1s-C1-inh complexes. We used a synthetic peptide (residues 448–459) corresponding to the distal hinge region of C1-inh. This peptide binds to C1s and C1s preincubated with the peptide cleaves C1-inh but does not form a stable C1s-C1-inh complex. Computer modelling of C1-inh shows that residues Q452, Q453 and F455 are surface-exposed and that the secondary binding site may also include residues H291 and F292 which are conserved in serpins.

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Key words: Human; Autoantibody; Complement

1. Introduction

C1-inhibitor (C1-inh) regulates the enzymatic activities of the C1r and C1s proteases of the complement system [1] in addition to regulating activities of activated Hageman factor [2], coagulation factor XIa, tissue plasminogen activator [3] and kallikrein [4]. C1-inh belongs to the serpin family of serine protease inhibitors which includes most of the protease inhibitors found in blood as well as non-inhibitory proteins such as angiotensinogen [5,6] and ovalbumin [7–9]. Serpins inhibit proteases by forming a stable complex with them. The mechanism of complex formation is imperfectly understood, but the serpin appears to act as a pseudosubstrate: the protease cleaves the serpin at a susceptible peptide bond (R444-T445 in the case of C1-inh), but the products of cleavage do not dissociate. Instead an enzyme-product complex is formed, which is stable during analysis by SDS-PAGE. The protease must therefore have interactions with the serpin, additional to the simple recognition of the region of the bond to be cleaved. In type II acquired C1-inh deficiency, autoantibodies against C1-inh are present in the serum [10,11]. In most cases these autoantibodies cause the inhibitor to behave as a substrate, and no stable complex is formed. The patient's C1-inh is cleaved and inactivated, giving rise to symptoms similar to hereditary deficiency of C1-inh [12,13]. Recently we described six patients with type II C1-inh deficiency in whom autoantibodies were shown to recognise the epitope QQPF in the distal hinge region of C1-inh [14]. It was postulated that the autoantibodies may inhibit a secondary interaction of C1s with C1-inh which is essential for stable serpin-protease complex formation. Our data confirm this hypothesis as C1s, like anti-C1-inh autoantibodies, binds to a synthetic peptide derived from the distal hinge region of C1-inh, and although the same peptide does not inhibit cleavage of C1-inh

by C1s, it prevents the formation of a stable C1s-C1-inh complex.

2. Materials and methods

One IgG anti-C1-inh autoantibody (from patient 1 in reference [14]) was affinity-purified using C1-inh-Sepharose [13].

Three overlapping synthetic peptides (Fig. 1B) spanning the proximal hinge region (peptide 1, residues 428–440), the reactive site residue (peptide 2, residues 438–449) and the distal hinge region (peptide 3, residues 448–459) were obtained from Alta Biosciences, (University of Birmingham, UK). The anti-C1-inh autoantibody used in this study, like the other five anti-C1-inh autoantibodies studied previously, recognises the QQPF sequence (residues 452–455) in peptide 3 but also recognises a structurally similar motif LLVF (residues 446–449) in peptide 2 [14].

Activated C1s [15] and C1-inh [16] were purified by standard techniques.

Binding of C1s to solid phase synthetic peptides and C1-inh was determined by ELISA. Briefly plates were coated with peptides at 100 µg/ml or C1-inh at 5 µg/ml. C1s was used at 10 µg/ml. Bound C1s was detected using goat anti-human C1s (Inctar Ltd., Wokingham, UK) followed by the HRP-conjugated rabbit anti-goat IgG.

Effects of peptides and the anti-C1-inh autoantibody on C1s-C1-inh interactions were studied using reagents diluted in PBS containing 1% (v/v) Triton X-100. Reaction products were analysed by SDS-PAGE and Western blotting [17]. Full details are given in the figure legends.

Effects of synthetic peptides or C1-inh on C1s activity were also analysed using the chromogenic substrate propionyl L-lysyl L-arginine *p*-nitroanilide hydroacetate (AcOH-C₂H₅CO-Lys(e-Cbo)-Gly-Arg-pNA) (Immuno Ltd., Heidelberg, Germany) dissolved in 50 mM Tris-HCl, pH 8.5, containing 25 mM NaCl [13].

The secondary C1s binding site on C1-inh was modelled by computer graphics [18–20]. Graphics were created using the X-ray crystallographic database of anti-thrombin III, provided by Professor R. Carrell (University of Cambridge, UK). Residue replacements were made according to serpin sequence alignments (Fig. 4B).

3. Results and discussion

Fig. 1A shows that ¹²⁵I-labelled C1s forms an SDS-resistant complex with C1-inh. In Fig. 1C it is seen that C1s binds most effectively to peptide 2, which contains the reactive site residue. However, it also binds to peptide 3 which contains the epitope QQPF (residues 452–455) which is recognised by the anti-C1-inh autoantibodies [14]. Computer modelling shows that Q452, Q453 and F455 are exposed on the surface of the C1-inh molecule whereas P454 is concealed. In preliminary experiments using substituted peptides, we have demonstrated that the replacements of either Q452 or Q453 with E, N, D or L and replacement of F455 with L reduces binding of C1s by 10–100-fold, confirming that Q452-Q453 and F455 are critical for the binding of C1s to peptide 3. Thus it appears that these three residues, which are recognised by spontaneously occurring anti-C1-inh autoantibodies, are also recognised by C1s, possibly as part of a secondary interaction

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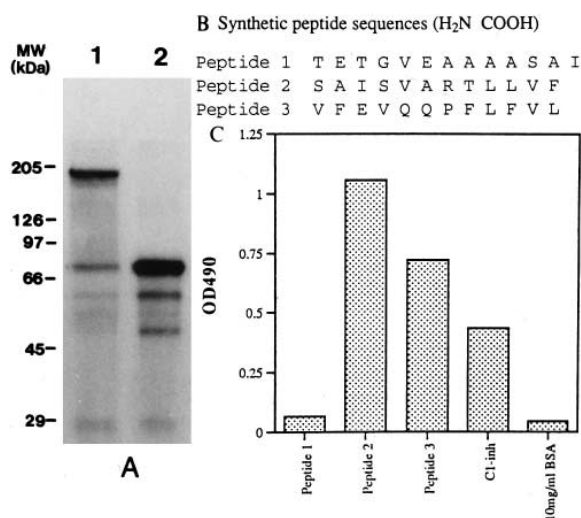


Fig. 1. Covalent binding of C1-inh to C1s, and binding of C1s to synthetic peptides. **A:** Complex formation between C1-inh and ^{125}I -labelled C1s (6 μg of each in a reaction volume of 40 μl). The samples in PBS-1% (v/v) Triton X-100 were incubated at 37°C for 2 h and then the reaction was stopped by addition of 1:1 (v/v) SDS-PAGE sample buffer (4 M urea, 125 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.8% (w/v) SDS and 0.002% (w/v) bromophenol blue). Lane 1, ^{125}I -labelled C1s and C1-inh; lane 2, ^{125}I -labelled C1s alone. The reaction products were separated in 7.5% SDS-PAGE (under non-reducing conditions) then the dried gel was exposed to X-ray film (Blue Sensitive Film Genetic Research Instrumentation Ltd, Dunmow, Essex, UK). The band seen at >190 kDa in lane 1 is the complex of C1-inh with intact C1s. The band at 87 kDa is intact C1s. Bands at 55 kDa and below are degradation products of C1s. **B:** Synthetic peptide sequences synthesised by Alta Bioscience, Birmingham University, UK. A cysteine residue was added to the N-terminus of each peptide, with the aim of forming a disulphide-linked dimer. **C:** The binding of C1s to peptides 1, 2 and 3 and C1-inh. The peptides (100 μl of 100 $\mu\text{g}/\text{ml}$) and C1-inh (100 μl of 5 $\mu\text{g}/\text{ml}$) were coated in 15 mM Na_2CO_3 and 35 mM NaHCO_3 , pH 9.6 onto wells of an ELISA plate (Falcon, Becton and Dickinson Laboratory Company, Oxnard, USA) overnight at 4°C. Non-specific binding sites were blocked by addition of 2% (w/v) BSA in PBS and incubation at 37°C for 1 h. C1s (100 μl , 10 $\mu\text{g}/\text{ml}$ in PBS) was added to each well and incubated at 37°C for a further 1 h followed by addition of goat anti-human C1s (Incstar Ltd, Wokingham, UK) (1:2000) at room temperature for 2 h. Anti-goat IgG conjugated with horseradish peroxidase (HRP) (1:4000) (Incstar Ltd) was then added and incubated at room temperature for a further 2 h. The plate was washed 3 times in PBS-0.2% (w/v) Tween 20 (Sigma Chemical Co., Poole, UK) between each step. The plate was developed using OPD (2.1% (w/v) *o*-phenylenediamine dihydrochloride (Sigma) in 22 mM sodium citrate, pH 5.0, 56 mM Na_2HPO_4 , and 5% (v/v) H_2O_2). The reaction was stopped by addition of 4 M H_2SO_4 and the absorbance was read at 490 nm. The results are averages of 5 separate experiments each of which contained 5 replicates. Although we have not determined the absolute amount, significant binding of each peptide to ELISA plates was shown by the use of three peptide-specific murine monoclonal antibodies (Tsang and Whaley, unpublished data).

site. Failure to demonstrate binding of C1s to peptide 1 was not due to poor binding of this peptide to the ELISA plates. Murine monoclonal antibodies to each of these peptides showed high levels of binding to the appropriate peptide bound to the same microtitre plate (Tsang and Whaley, unpublished data). Furthermore, the failure of peptide 1 to interfere with (a) the formation of C1s-C1-inh complexes (see below) and (b) C1s-mediated hydrolysis of a synthetic substrate (Fig. 3) support the conclusion that there is no binding site for C1s in peptide 1.

As peptides 2 and 3 (Fig. 1B) contain binding sites for C1s (Fig. 1C), both should interfere with the interaction between C1s and C1-inh. This was demonstrated by incubating each

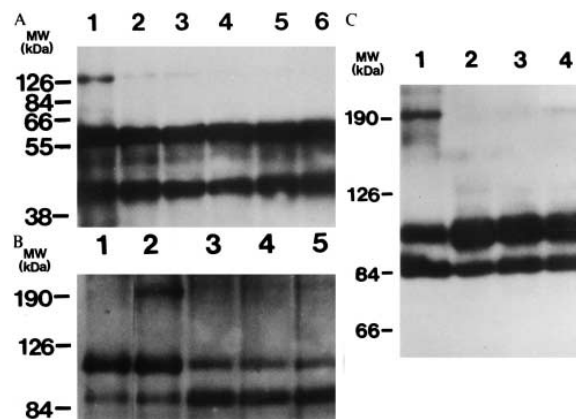


Fig. 2. Western blots of reaction products of C1s and C1-inh in the presence of the peptides. C1s and C1-inh (6.0 μg of each) were dissolved in PBS-1% Triton X-100 in a total reaction volume of 40 μl . The reactions were stopped by addition of an equal volume of SDS-PAGE sample buffer (see legend to Fig. 1A) and the products were separated in 7.5% SDS-PAGE under reducing and non-reducing conditions. The proteins were transferred to nitrocellulose membranes (Hybond-Super, Amersham International plc, Little Chalfont, UK), in 25 mM Tris/192 mM glycine buffer containing 20% (v/v) methanol and 0.1% SDS at 4°C using a current of 200 mA for 6 h. After blotting the membrane was washed in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1 mM EDTA and 0.1% (v/v) Triton X-100 (rinse buffer). Membranes were then incubated (6 h at 4°C) in blocking buffer (rinse buffer containing 2% w/v BSA (Sigma Chemical Co., Poole, UK) and 0.02% (w/v) sodium azide (Sigma)) and were incubated for 2 h at 4°C with goat anti-human C1s or goat anti-human C1-inh (both from Incstar Ltd., Wokingham, UK) diluted 1/2000 in blocking buffer, washed thrice in blocking buffer (15 min in 100 ml for each wash), incubated for 2 h at 4°C with peroxidase-labelled rabbit anti-goat IgG (Incstar) in blocking buffer, re-washed three times and finally developed using the ECL system (Amersham International plc) according to the manufacturer's instructions. The membrane was then exposed to high performance luminescence detection film (Hyperfilm ECL, Amersham International plc). **A:** Western blot of reaction products of C1s and C1-inh interaction in the presence of peptide 2 using reducing SDS-PAGE (7.5%). Blots were probed with goat anti-human C1s. Lane 1, C1s and C1-inh incubated at 37°C for 2 h; lanes 2–6, incubated with peptide 2 at molar ratios of 3:1 (lane 2), 2:1 (lane 3), 1:1 (lane 4), 1:1.6 (lane 5), 1:3 (lane 6) at 37°C for 1 h before C1-inh was added to the mixture and incubation continued at 37°C for a further 2 h. **B:** Western blot (7.5% SDS-PAGE, non-reducing conditions) of reaction products of C1s and C1-inh in the presence of peptide 3. Blots were probed with goat anti-human C1-inh. Lane 1, C1-inh alone incubated at 37°C for 2 h; lane 2, C1-inh and C1s incubated at 37°C for 2 h; lane 3, C1-inh incubated with anti-C1-inh autoantibody at a molar ratio 1:1 at 37°C for 1 h and then C1s for a further 2 h; lanes 4 and 5, C1s incubated with peptide 3 (1:3 and 1:5 molar ratios, respectively) at 37°C for 1 h and then with C1-inh for a further 2 h. Note that the 96 kDa bands were increased and the 115 kDa bands decreased in the presence of either anti-C1-inh autoantibody or peptide 3 compared with C1-inh alone (lane 1). **C:** Western blot (7.5% SDS-PAGE, non-reducing conditions) of reaction products of C1s and C1-inh in the presence of peptide 2. Lane 1, C1-inh and C1s incubated at 37°C for 2 h; lanes 2–4, C1s incubated with peptide 2 (1:5, 1:3 and 1:1 molar ratios, respectively) at 37°C for 1 h and then with C1-inh for a further 2 h. Note that the 115 kDa band in lane 1 was decreased due to the formation of C1s-C1-inh complex (>190 kDa band). However, unlike the situation with peptide 3 (B), increased cleavage of the C1-inh did not occur as shown by the relative intensities of the 115 kDa and 96 kDa bands in lanes 2–4 compared with lanes 3–5 in B.

Inhibition of C1s by synthetic peptides
(C1s activity (%))

	Concentrations	
	12 μ M	24 μ M
Peptide 1	96.35 $\pm$ 0.82	96.23 $\pm$ 1.57
Peptide 2	39.59 $\pm$ 0.75	19.54 $\pm$ 0.92
Peptide 3	76.73 $\pm$ 0.81	58.22 $\pm$ 1.23

Fig. 3. Assays of the effect of the synthetic peptides on C1s enzymic activity. The effect of the synthetic peptides on C1s enzymic activity was assayed using the chromogenic substrate, propionyl L-lysyl L-arginine *p*-nitroanilide hydroacetate (AcOH-C₂H₅CO-Lys(e-Cbo)-Gly-Arg-pNA) (Immuno Ltd., Heidelberg, Germany) which was dissolved in 50 mM Tris-HCl, pH 8.5, 25 mM NaCl. The concentration of C1s was kept constant at 8 μ M and peptides were at a final concentration of 12 μ M and 24 μ M. C1s was dissolved in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and incubated with peptides at 37°C for 20 min before addition of the chromogen to the final concentration of 10 μ M in each reaction. The absorbance changes were recorded automatically at 37°C. C1s activity (%) was calculated using $1 - [(\text{slope of C1s alone} - \text{slope of C1s and peptide}) / \text{slope of C1s alone}] \times 100$.

peptide with C1s prior to the addition of C1-inh. In the absence of peptide or in the presence of peptide 1, a stable C1s-C1-inh complex was formed as demonstrated by SDS-PAGE followed by immunoblotting, using anti-C1s as a probe. This shows that (a) peptide 1 does not bind to C1s and (b) pre-incubation of C1s for 1 h prior to the addition of C1-inh did not affect complex formation. In the presence of peptide 2 a stable C1s-C1-inh complex was not formed, and the inhibitory effect of peptide 2 was shown to be dose-dependent (Fig. 2A). An identical result was obtained when peptide 3 was substituted for peptide 2 (data not shown). When the same experiments were repeated using anti-human C1-inh as a probe for the immunoblots, the absence of the C1s-C1-inh complex in reaction mixtures containing peptides 2 and 3 was confirmed (Fig. 2B,C). However, in contrast to the peptide 2 reaction mixture which contained C1-inh with the normal M_r of 115 kDa, C1-inh in the peptide 3 reaction mixture was in the cleaved 96 kDa form (Fig. 2B), indicating that peptide 3 had 'converted' C1-inh to a substrate. The same effect was seen when the affinity-purified anti-C1-inh autoantibody was substituted for peptide 3 (Fig. 2B, lane 3). Although peptide 2 prevented the formation of the C1-inh-C1s complex, it did not 'convert' C1-inh to a substrate (Fig. 2C). The inhibitory activity of the peptides was investigated further by demonstrating their effect on the activity of C1s in cleaving synthetic substrates (Fig. 3). Peptide 2, which contains the sequence normally cleaved by C1s, inhibited complex formation between C1-inh and C1s, and is therefore likely to act as a competing (pseudo)substrate by inhibiting recognition by C1s of the R444-T445 site in C1-inh, and preventing the initial cleavage of C1-inh. Peptide 3, however, was a much less potent inhibitor of C1s enzymic activity against the chromogenic substrate (Fig. 3), but it effectively inhibited C1s-C1-inh complex formation, and, like the autoantibody, it promoted the generation of uncomplexed, cleaved C1-inh. The observations that peptide 3 (a) bound to C1s, (b) prevented C1s-C1-inh

complex formation but (c) did not inhibit the cleavage of C1-inh by C1s and (d) was not an effective inhibitor of hydrolysis of the chromogenic substrate by C1s are consistent with peptide 3 possessing a C1s binding site which prevents the binding of C1s to the residues QQ-F in the C1-inh molecule, which contribute a secondary C1s binding site.

Computer modelling of C1-inh (Fig. 4A) indicates that the residues Q452, Q453 and F455 are closely adjacent on the surface of the molecule. Interestingly Q452 is immediately adjacent to, but partially conceals, F291, which, like F455, is highly conserved amongst the serpins (Fig. 4B). H292, which is also highly conserved amongst the serpins, is also adjacent to Q452 and Q453 and is exposed on the surface of the molecule. It is possible that residues F291, F455 and H292 contribute to the secondary interaction site but the specificity is determined by Q452 and Q453. Thus our data suggest that the binding of an anti-C1-inh autoantibody to its epitope on C1-inh would, like the binding of peptide 3 to C1s, prevent the interaction of C1s with this critical secondary binding site therefore converting C1-inh to a substrate and producing acquired C1-inh deficiency. It is also possible that the conserved residues F291, H292 and F455 comprise part of a secondary enzyme binding site in all serpins with serine protease inhibitory activity but with enzyme specificity being determined by the equivalent residues to 452 and 453 in C1-inh.

Although the distal hinge region of C1-inh contains a secondary C1s binding site which is essential for the formation of a stable enzyme-inhibitor complex, residues in the proximal hinge region must also contribute to this as mutations in this region (e.g. V432E) [21] may also produce C1-inh deficiency by converting C1-inh to a substrate. However, in this case the mechanism is unlikely to be related to the binding of C1s to residues in the proximal hinge region, as C1s did not bind to peptide 1. How residues in the proximal hinge region contribute to the formation of a stable enzyme-inhibitor complex merits further investigation.

Acknowledgements: We would like to thank Miss S. Tsang for assistance with the ELISA and Dr Harvey Colten for helpful comments. This work was supported by a grant from the Wellcome Trust.

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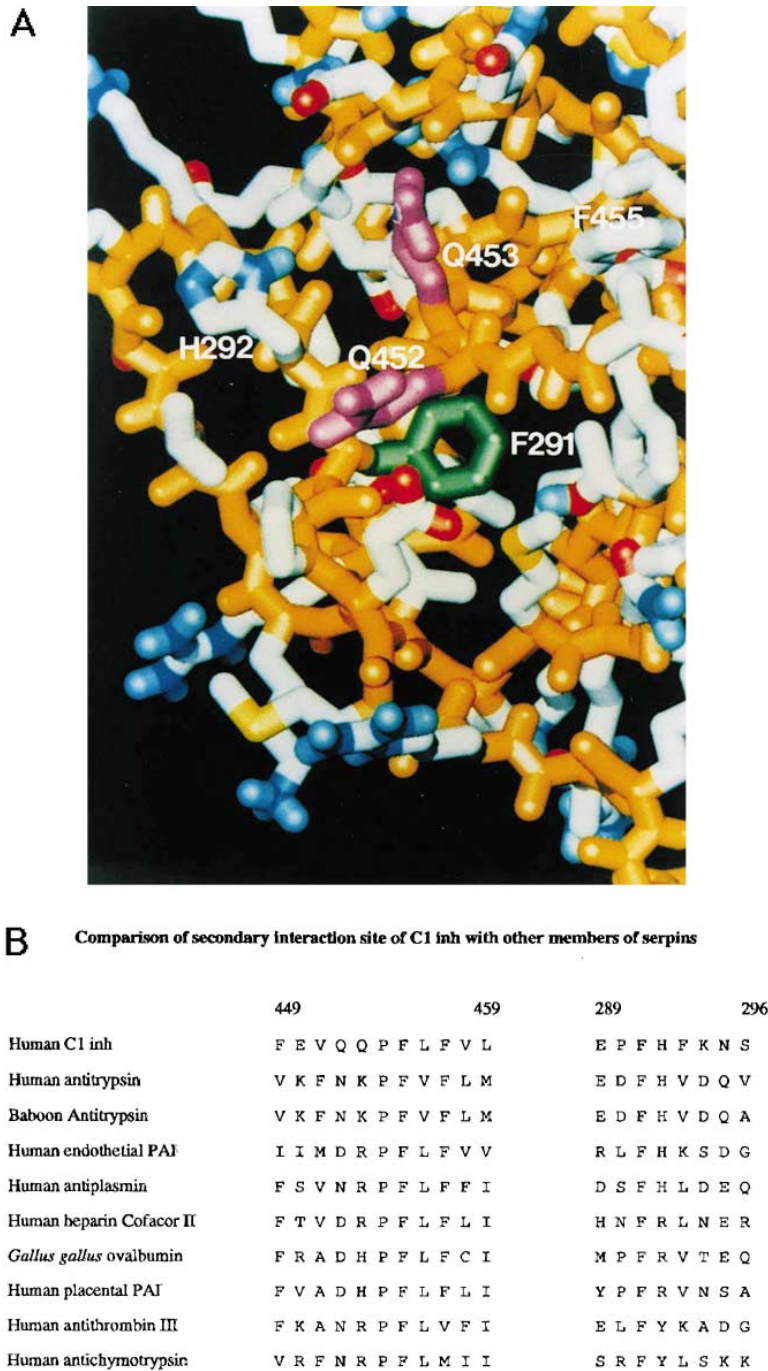


Fig. 4. Computer graphics of C1-inh showing the possible secondary interaction site and residue alignments in the interaction site. A: Computer graphics showing a local region which may play a role in forming the stable covalent complex between C1s and C1-inh. Graphics were created using the X-ray crystallographic database of antithrombin III provided by Professor R. Carrell, University of Cambridge, and residue replacements were made according to serpin sequence alignments (see B). Side-chains of Q452 and Q453, aromatic rings of F291 and F455, and imidazole ring of H292 are labelled. B: Residue alignments of serpins in the secondary interaction site. The numbers indicated are C1-inh sequence predicted from cDNA [22]. The specific residues in the secondary interaction site may be Q452 and Q453 which are located within a triangle formed by F455, F291 and H292 (see A). Human C1-inh [22]; human antitrypsin [23]; baboon antitrypsin [24]; human endothelial PAI [25]; human antiplasmin [26]; human heparin cofactor II [27]; *Gallus gallus* ovalbumin [28]; human placental PAI [29]; human antithrombin III [30]; human antichymotrypsin [31].

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