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Analysis of the contribution of the hinge region of human neutrophil collagenase (HNC, MMP-8) to stability and collagenolytic activity by alanine scanning mutagenesis

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Abstract Analysis of the hinge region of neutrophil collagenase by alanine scanning mutagenesis revealed that this sequence motif has a pronounced effect on the stability and collagenolytic activity of the active enzyme. The mutagenesis of the amino acid residues in the P_1' position of the two autoproteolytically cleaved peptide bonds (Leu^{243} and Ile^{248}) to Ala showed that the mutant enzymes were more resistant to autoproteolysis. However, these mutants were not completely stable and autoproteolysis occurred mainly at the Ala^{239}–Ile^{240} peptide bond and the half-life of the active enzyme was increased by 50%. In contrast, mutagenesis of $Pro^{247} \rightarrow Ala$ (P_1 of the minor cleavage site Pro^{247}–Ile^{248}) lead to increased susceptibility of the enzyme to autoproteolysis. However, when the other P_1 position Gly^{242} was altered to Ala no effect on stability was observed. The analysis of the ability of the mutant active enzymes to hydrolyse ^{14}C-type I collagen was assessed and our results demonstrate that the hinge sequence motif of neutrophil collagenase is important for collagenolytic activity. The alteration of the Gly^{242}–Leu–Ser–Ser–Asn–Pro–Ile–Gln–Pro^{247} sequence motif to Gly^{242}–Ala–Ala–Ala–Ala–Pro–Ala–Ala–Pro^{247} showed that the collagenolytic activity was reduced by 68.4%. In addition, mutagenesis of the downstream sequence motif Pro^{247}–Thr–Gly–Pro–Ser–Thr–Pro–Lys–Pro^{258} to Pro^{247}–Ala–Ala–Pro–Ala–Ala–Pro–Ala–Pro^{258} had an even more marked effect on the collagenolytic activity, which was reduced by 87.4%. When the Pro residues in the hinge motif (Pro^{247}, Pro^{250}, Pro^{253} and Pro^{256}) were altered to Ala the collagenolytic activity dropped to 1.5% of the value observed for wild-type enzyme.

1. Introduction

The matrix metalloproteinases (MMP[1]) form a family of at least 15 structurally related zinc metalloendopeptidases capable of degrading macromolecular components of the extracellular matrix. Connective tissue remodelling is a critical event in development, differentiation and growth and the MMPs are implicated to be involved in the regulation of these important processes. In addition, these enzymes play a considerable role in pathological processes such as tumour invasion, metastasis and arthritis which has reinforced the interest in the mechanisms of their structure and function.

The three homologous collagenases are unique members of the MMP family due to their ability to hydrolyse triple helical interstitial collagens (types I–III) at the Gly^{775}–Ile^{776} or Leu^{776} peptide bond of the corresponding α-chains. Their primary structures were elucidated by analysis of their respective cDNAs [1–4] and sequence comparison revealed that they are comprised of three domains, namely the propeptide, catalytic and C-terminal domain.

Activation of the procollagenases involves proteolytic and autoproteolytic cleavages within the propeptide domain, which effects the removal of the 'Pro–Arg–Cys–Gly–Val–Pro–Asp' sequence motif, thereby expelling the free Cys residue of the propeptide from the co-ordination sphere of the catalytic zinc ion [5–10]. The active collagenases cleave the interstitial collagens type I–III with different k_{cat}/K_M values into characteristic 3/4 and 1/4 fragments and slowly hydrolyse other substrates such as cartilage aggrecan, serpins, denatured collagen and small synthetic peptide substrates [11,12]. In addition, the active enzymes are subject to autoproteolytic fragmentation processes at different sites within the hinge region, which connects the catalytic and C-terminal domains [13–15]. The catalytic domains of the collagenases retain proteolytic activity against cartilage aggrecan, serpins, denatured collagen or small synthetic peptide substrates, but are no longer capable of cleaving triple helical collagen.

The construction of hybrid enzymes consisting of N-terminal collagenases C-terminal stromelysins and vice versa was the first tool in the determination of the structural elements of the collagenases responsible for substrate specificity, since the homologous MMPs stromelysin-1 and stromelysin-2 do not cleave triple helical interstitial collagens although stromelysin-1 does bind to collagen [16–18]. It was pointed out by Hirose et al. [17] that a 16 amino acid sequence motif within the 'hinge' region of HNC (residues 242–258) was most important for its ability to hydrolyse triple helical collagen. This group had inserted the SL hinge sequence motif into the HNC sequence and found that the mutant was no longer collagenolytic [17]. However, once this mutant was expressed in a baculovirus expression system and purified prior to analysis of its collagenolytic activity it was shown to have considerable collagenolytic activity (44% of wild type [19]). In addition, MT1-MMP has collagenolytic activity, although this enzyme has a linker peptide which is considerably longer than in the classical collagenases [20] and this suggests that the length of the linker peptide is not critical for collagenolytic activity in general. Souza and Brentani [21] had already hypothesised

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Abbreviations: FC, fibroblast collagenase; HNC, human neutrophil collagenase; MMP, matrix metalloproteinase; APMA, *p*-aminophenyl mercuric acetate; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; McaPLGLDpaAR, Mca–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–NH_2; SL, stromelysin; SL-2, stromelysin-2

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that a motif within the hinge sequence of the collagenases, 'Leu^{243}–Ser–Ser–Asn–Pro–Ile–Gln–Pro^{250}' in HNC and 'Arg^{243}–Ser–Asn–Gln–Pro–Val–Gln–Pro^{250}' in FC, was responsible for the ability of the collagenases to bind to and hydrolyse collagen. However, a chimeric mutant prepared from N-terminal FC (1–250) and C-terminal SL-2 (256–459) by Sanchez-Lopez et al. [18] which contains this motif was not collagenolytic.

Further analysis of a chimeric enzyme constructed from N-terminal HNC (1–306) and C-terminal SL, which incorporates the hinge sequence motif and blade 1 of the C-terminal β-propeller of collagenase showed 89% of the collagenolytic activity of wild-type enzyme after purification [19]. In contrast, another chimeric enzyme constructed from N-terminal HNC (1–281) and C-terminal SL, i.e. incorporating the hinge sequence and the first two β-sheets of blade 1 of the C-terminal domain of collagenase, showed only 1% collagenolytic activity of wild-type enzyme [19]. In conclusion, these results suggest that the β-sheets three and four of the C-terminal domain of collagenase are the main determinants for collagenolytic activity.

From data with other chimeras Sanchez-Lopez et al. [18] proposed that the amino acid residues encoded by exon 5, which correspond to the residues of the Zn^{2+} binding active site cleft, were also important for specificity by using a gelatin fingerprint assay.

Thus, it is not clear to date which amino acid residues within the 'hinge' region of HNC, might really be responsible for the specificity of the enzyme, if at all. We have therefore performed a more detailed analysis of the HNC hinge sequence motif in order to evaluate its role in stability and collagenolytic activity.

2. Experimental procedures

2.1. Amplification of the wild-type neutrophil collagenase (HNC) cDNA by RT-PCR

The human neutrophil cDNA was generated by RT-PCR using purified RNA from human bone marrow and the oligonucleotide primers 5′-TAT*GGATCC*ATGTTCTCCCTGAAGA-3′ and 5′-TGAT*GGATCC*TCAGCCATATCTACAGTTAGG-3′ which generated *Bam*HI sites on both ends of the cDNA in 30 rounds of amplification using 75 s denaturation at 94°C, 75 s annealing at 61°C and 120 s extension at 72°C. The RT-PCR product was cleaved with *Bam*HI and subcloned into pSVB30 and sequenced using the dideoxy method which confirmed its identity with the sequence published by Hasty et al. [3].

2.2. Construction of neutrophil collagenase hinge mutants (HNC) and expression and purification of wild-type and mutant enzymes

The neutrophil procollagenase cDNA (wtHNC) was subcloned into the *Xba*I and *Eco*RI sites of pSP64. Using pSP64 wtHNC as a template, five mutants of the hinge region of the enzyme were prepared by polymerase chain reaction using overlap extension mutagenesis [22]. An oligonucleotide that primes within the SP6 promoter of pSP64 and a second mutagenic oligonucleotide that is largely complementary to the sequence encoding residues 236-261 of HNC and defining each of the five mutations was used to generate a 900 bp DNA fragment in the presence of pSP64 wtHNC (for sequence details see Fig. 1A). A second 700 bp fragment was amplified using pSP64 wtHNC, with a oligonucleotide priming downstream of the multiple cloning site of pSP64 and a second coding mutagenic oligonucleotide complementary to the one above. The two fragments were gene cleaned, annealed and further amplified by PCR in the presence of a coding oligonucleotide priming upstream the *Bsm*I site of HNC and a non-coding vector oligonucleotide primer. The resulting 900 bp fragment, containing the desired mutation, was cleaved with *Bsm*I and *Eco*RI, purified and ligated into *Bsm*I–*Eco*RI cleaved wtHNC in pSP64. In order to construct an expression vector, the *Xba*I–*Dra*III 950 bp fragment from the pSP64 construct was ligated into *Xba*I–*Dra*III cut wtHNC in pEE12 to generate the various hinge mutants. The presence of mutagenised HNC was confirmed by dideoxysequencing of the 105 bp segment generated by PCR. The linearised pEE12 vector containing the mutagenised HNC cDNA was transfected into NSO mouse myeloma cells by electroporation. Colonies producing recombinant proteinases were selected and grown up as described [12]. High expression rates were achieved following gene amplification by methionine sulphoximine-treatment [23].

The cell conditioned medium containing the secreted enzymes was dialysed against 20 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.3, and applied to a DEAE-Sepharose fast flow column which was coupled to a S-Sepharose fast flow column (10×5 cm). The bound proteins from the S-Sepharose column were eluted using a linear gradient from 0 to 500 mM NaCl in the above buffer.

2.3. Activation of the proenzymes

Wild-type neutrophil collagenase and the subsequent hinge mutants were incubated at 37°C with 2 mM APMA prior to activity assay and SDS-PAGE. Alternatively, activation was achieved using trypsin and stromelysin as previously described [9].

2.4. Enzymatic assays and active site titrations to determine the concentration of active enzymes

Hydrolysis of the substrate McaPLGLDpaAR was monitored by measuring the increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm) as described [24]. The apparent k_{cat}/K_m values for hydrolysis of McaPLGLDpaAR were determined at substrate concentrations of 1–2 μM. It is currently not possible to determine the k_{cat} values for substrate hydrolysis since this substrate is not soluble at concentrations higher 5 μM and additionally considerable quenching of fluorescence is observed at high substrate concentrations. Collagenolytic activity was measured using ^{14}C-labelled fibrillar type I collagen at 35°C [25]. The concentrations of active wild-type and mutant neutrophil collagenase were determined by active site titrations using a standard TIMP-1 solution of known concentration and residual activity was monitored using the quenched fluorescence substrate as described above [26].

2.5. N-terminal sequencing

Fragmented wild-type and mutant neutrophil collagenases were purified by HPLC on a Vydac 218T P54 column using a linear gradient of 5–95% acetonitrile prior to automated sequencing using an Applied Biosystems 470A sequencer with on-line 190A HPLC for phenylthiohydantoin-derivative analysis.

3. Results and discussion

A number of studies have demonstrated the role of structural features of both the catalytic and the C-terminal hemopexin-like domain of the collagenases in their ability to specifically cleave the triple helix of fibrillar collagens, as has been discussed by Bode [27] and Gomis-Rüth et al. [28]. This property also depends on the correct interplay between the two domains and it has been proposed that this might be determined by both the length and the surface-guiding properties of the linker peptide [27]. In the crystal structure of pig FC [29] this peptide bulges backward before it runs loosely across the lower edge of the catalytic domain. The most significant interaction observed between the linker and the catalytic domain is a single hydrogen bond between Gln^{250} and Pro^{272} which is responsible for the relative orientation of the catalytic and C-terminal domain in the crystal structure. An additional minor interaction corresponds to a hydrogen bond between Asn^{265} and Val^{267} which creates a tight bend in the middle of the linker. Furthermore, a small kink in the linker is defined by a hydrogen bond between Pro^{275} and Val^{277} at its very end which is most likely not present in HNC due to the substitution of the Val residue by Pro (Deborah Hodges, per-

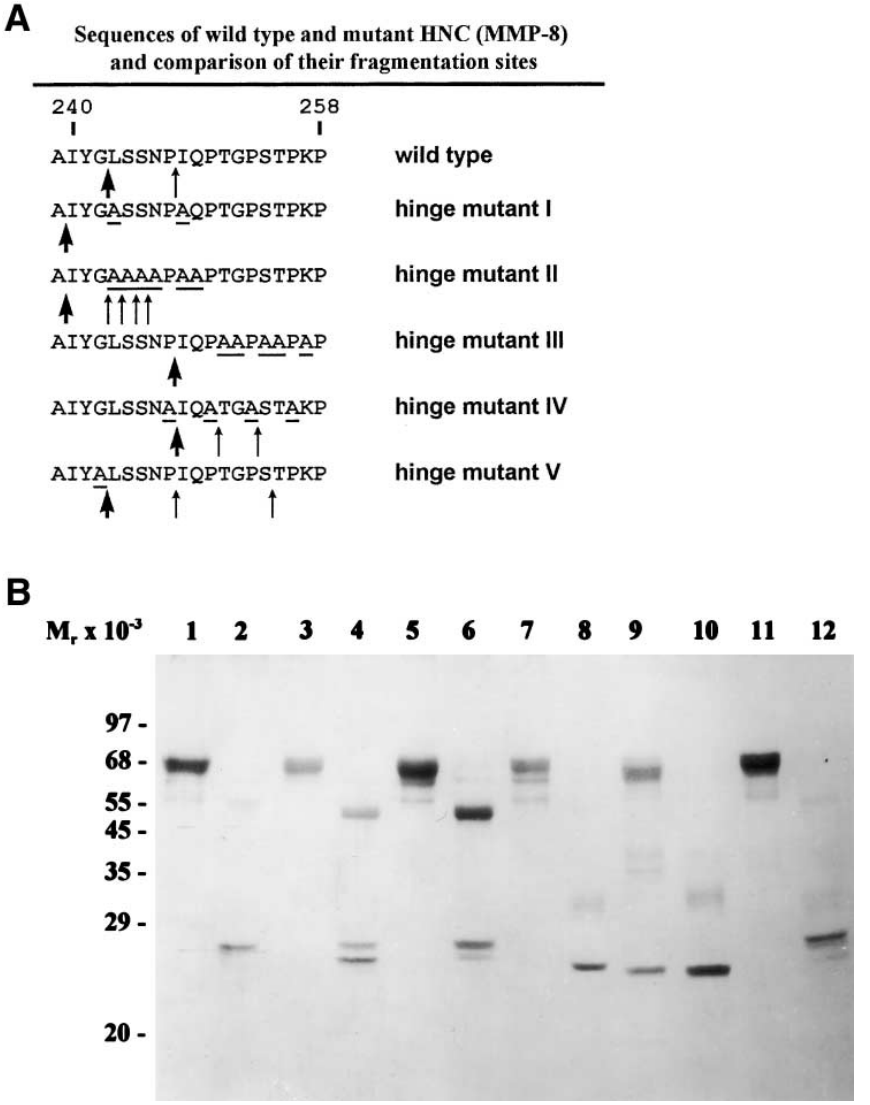

Fig. 1. A: Sequences of wild-type and mutant HNC (MMP-8) and comparison of their fragmentation following incubation of the corresponding active enzymes for 15 h at 37°C. The sequence alterations defined by each hinge mutant are shown by underlined amino acid residues in single letter code. The major fragmentation sites are indicated by bold arrows, while minor fragmentation sites are indicated by small arrows. B: Molecular mass determination of purified wild-type and mutant HNC proenzymes and demonstration of their thermostability after incubation of active wild-type and mutant enzymes for 15 h at 37°C by silver-stained SDS-PAGE. Lane 1: Wild-type HNC (proenzyme); lane 2: wild-type active HNC after 15 h at 37°C; lane 3: hinge mutant I (proenzyme); lane 4: active hinge mutant I after 15 h at 37°C; lane 5: hinge mutant II (proenzyme); lane 6: active hinge mutant II after 15 h at 37°C; lane 7: hinge mutant III (proenzyme); lane 8: active hinge mutant III after 15 h at 37°C; lane 9: hinge mutant IV (proenzyme); lane 10: active hinge mutant IV after 15 h at 37°C; lane 11: hinge mutant V (proenzyme); lane 12: active hinge mutant V after 15 h at 37°C. Molecular mass markers are indicated on the left. Please note that the hinge mutants I and II were more stable and the active enzymes (M_r 54000) is still present after incubation for 15 h at 37°C. The faint band at 32000 corresponds to the respective catalytic domain.

sonal communication). The high crystallographic B-factor observed for the hinge region indicates that this part of the molecule is very flexible and it is most likely that its conformation in solution will be quite variable. It is not quite clear to date how this motif might interact with the triple helical substrate in the interstitial collagens. In order to analyse its function we have mutagenised the hinge region of HNC and determined the effect of the mutations on enzyme stability and collagenolytic activity.

3.1. Effect of the hinge mutations on stability of active neutrophil collagenase (HNC)

The effect of the hinge region on stability of the active enzymes was assessed by SDS-PAGE following activation with APMA and incubation at 37°C (Fig. 1B). The active wild-type HNC fragmented into the catalytic and C-terminal domain essentially as previously described [13]. In contrast, the hinge mutants I and II were more stable and a considerable amount of the active enzyme (M_r 54000) was still retained after 15 h incubation at 37°C, indicating that these mutants were more resistant to autoproteolysis being more stable than wild-type HNC. In contrast, the hinge mutants III and V showed an unaltered fragmentation rate when compared to wild-type enzyme. The mutagenesis of the proline residues (Pro^{247}, Pro^{250}, Pro^{253}, Pro^{256}, mutant IV) demonstrated that these residues were important for the structural

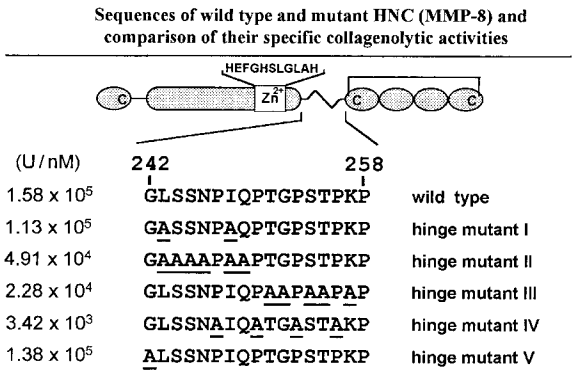

Fig. 2. Comparison of the collagenolytic activities of wild-type and mutant HNC (MMP-8) using ^{14}C-type I collagen.

integrity of the active enzyme. The final purified proenzyme preparation of mutant IV was already contaminated with the free C-terminal domain and the active enzyme fragmented at an increased rate relative to wild type (not shown). Fragmentation was prevented if wild-type or mutant forms of HNC were incubated in the presence of either triple helical type I collagen or TIMP-1 (not shown).

N-terminal amino acid sequence analysis of the C-terminal fragmentation products revealed that autoproteolysis of the wild-type enzyme and mutant V preferentially proceeded at the Gly^{242}–Leu^{243} or Ala^{242}–Leu^{243} peptide bonds with minor cleavages observed at Pro^{247}–Ile^{248}. The C-terminal fragments of the hinge mutant III and IV displayed the N-terminal sequence Ile^{248}Gln–Pro–Ala–Ala or Ile^{248}Gln–Ala–Thr–Gly, while mutant I and II were hydrolysed further upstream leading to the generation of the N-terminal sequences Ile^{240}Tyr–Gly–Ala for both mutants (Fig. 1A). Thus mutagenesis of the hinge sequence motif led to the alteration of the autoproteolytically hydrolysed peptide bonds and changed the rate of hinge autolysis of the corresponding active enzymes.

The evaluation of the stability of the HNC hinge mutants established that the alteration of the two autoproteolytic cleavage sites in their P_1' position to Ala results in increased stability. Similar mutagenesis in fibroblast collagenase has been demonstrated recently and the conversion of Ile^{251} → Ser and/or Ile^{241} → Leu leads to resistance to autoproteolysis when the active enzymes were stored at 4°C [30,31]. In contrast, mutagenesis of the P_1 position of the peptide bonds cleaved by autoproteolysis has no effect on stability (mutant V), or leads to increased autoproteolysis (mutant IV).

3.2. *Effect of the hinge mutations on the specific collagenolytic activity of neutrophil collagenase (HNC) for ^{14}C-type I collagen*

Proenzymes were activated by treatment with APMA at 37°C for 2 h, a time interval not sufficient for hinge autolysis, followed by active site titration versus a standard TIMP-1 solution in order to quantitate the functional enzyme concentrations. The specific activities values for collagen cleavage of active wild-type and mutant HNC were then determined using ^{14}C-type I collagen in a fibrillar assay and the results are summarised in Fig. 2. Mutants II, III and IV have significantly reduced specific collagenolytic activities, with mutant IV being the most pronounced. Hinge autolysis does not occur in the presence of triple helical collagen which was present during the determination of activity and the employment of short-term activity assays (3 h) also reduced the risk of hinge autolysis of active enzymes. Thus it is unlikely that the reduction in the specific collagenolytic activity values was due to the altered stability of the corresponding enzymes, and additionally, mutants with increased or unaltered stability showed reduced specific collagenolytic activities when compared to wild-type HNC. The k_{cat}/K_M values of the mutants versus a synthetic peptide substrate (McaPLGLDpaAR) were unaltered (not shown) thus indicating that the catalytic domains of all mutants were correctly folded and fully functional. Thus the alteration of the hinge sequence motif caused significantly reduced the specific activity for triple helical substrate hydrolysis in three out of our five mutants. The mutations of the proline residues (Pro^{247}, Pro^{250}, Pro^{253}, Pro^{256}) had the most effect, reducing the collagenolytic activity to 1.5% of wild-type enzyme. It can be predicted that Pro^{253} might be important for anchoring the linker to the catalytic domain as discussed above for the homologous pig FC and mutagenesis of this residue to Ala would disrupt the anchoring hydrogen bond which is responsible for the relative orientation of the catalytic and C-terminal domain during crystal packing. However, a recent modelling analysis for the interaction of collagenase with triple helical collagen revealed that the collagenase hinge sequence motif adopts a collagen-like conformation [32], which means that the conformation of the linker after binding to collagen is different from the conformation in the crystal structure. It was hypothesised that upon binding to the triple helical substrate the enzyme is able to disturb the quaternary organisation of the triple helix near the collagenase susceptible site by forming a 'Pro zipper' which leads to unwinding and allows proteolytic cleavage. Our results obtained with mutant IV substantiate this concept, but the Pro residues are not the sole determinants of the collagenolytic activity (see below).

The alteration of 'Leu^{243}–Ser–Ser–Asn–Pro–Ile–Gln–Pro^{250}' to Ala–Ala–Ala–Ala–Pro–Ala–Ala–Pro (mutant II) lead to a decrease to 31.6% supporting an earlier hypothesis of Souza and Brentani [21] that this motif mediates binding and collagenolysis using the concept of sense-antisense peptide interactions. However, the downstream Pro^{250}–Thr–Gly–Pro–Ser–Thr–Pro–Lys^{257} sequence motif was shown to be more important since alteration of the above motif to Pro^{250}–Ala–Ala–Pro–Ala–Ala–Pro–Ala^{257} (mutant III) leads to a reduction in the specific collagenolytic activity to 12.6% of wild-type enzyme. It can be assumed that the alterations in mutant III will have no pronounced effect on the overall structural organisation of the active enzyme, since all the residues responsible for hydrogen bonds with the catalytic domain or within the linker are still in place.

It is not quite clear how the linker of HNC interacts with the triple helical substrate, especially in conjunction with the β-sheets three and four in blade 1 of the C-terminal β-propeller, which mediate specificity as well [19]. This clearly awaits an X-ray crystallographic approach in the presence of a triple helical substrate. However, sequence alignments of the linker sequences of all known collagenolytic MMPs shows that these enzymes all have the four Pro residues in similar positions which suggests that they are vital for function.

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