Identification and Expression of the cDNA-encoding Human Mesotrypsin(ogen), an Isoform of Trypsin with Inhibitor Resistance

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The cDNA encoding a novel isoform of human trypsin-ogen was identified. The isoelectric points of the proenzyme and active forms calculated from the deduced amino acid sequence are consistent with those of mesotrypsin(ogen), known to be an inhibitor-resistant trypsin isoform. The cDNA attached with a bacterial signal peptide sequence was expressed in Escherichia coli. The recombinant proenzyme purified from periplasm showed enterokinase-dependent activation similar to a major isoform of human trypsinogen. The enzyme was far less inhibited by trypsin inhibitors such as soybean trypsin inhibitor, aprotinin, or pancreatic secretory trypsin inhibitor than the control trypsin. A gel filtration assay showed that the enzyme and aprotinin did not form a stable complex. It is noteworthy that the amino acid at position 198, which is in close vicinity to the active Ser, is Arg while those of other major trypsins are all Gly. It is concluded that the cloned cDNA encodes human mesotrypsinogen, a unique isoform of trypsinogen with inhibitor resistance.

Pancreatic trypsin is a key enzyme, which leads to activation of a number of pancreatic digestive proenzymes including chymotrypsinogen, procarboxypeptidases, proelastases, and pro-phospholipase A2 as well as trypsinogens themselves. Three different trypsinogens, which show unique isoelectric points (pl), have been described in human pancreatic juice using two-dimensional isoelectric focusing/SDS-PAGE. These trypsinogens have been designated as trypsinogen 1 (pl = 4.9), 2 (pl = 5.7), and 3 (pl = 6.2); the isoforms having been numbered from anode to cathode in accordance with the IUPAC-IUB Commission on biochemical nomenclature of multiple forms of enzymes (1).

Trypsinogen 1 and 3 represent 23.1 and 16.0%, respectively, of total pancreatic secretory proteins in humans and play a major role in the proteolytic digestion (1). Trypsinogen 2, alternatively called mesotrypsinogen (based on its intermediate pl relative to the other two major trypsinogens) accounts for only a trace amount (<0.5%) and displays a specific characteristic best demonstrated when challenged by naturally occurring trypsin inhibitors such as soybean trypsin inhibitor or aprotinin; mesotrypsin is far less inhibited by these inhibitors than are other isoforms of trypsin (1, 2).

Researchers have found that levels of mesotrypsinogen are increased in chronic alcoholics and decreased in patients with pancreatitis (3). These findings raise the question of whether inhibitor-resistant activity of mesotrypsin has a role in the pathogenesis of pancreatic diseases (4). However, the limited availability of mesotrypsinogen has made determination of its biological properties and physiological significance difficult.

Two major trypsinogen cDNAs, TRYI and TRYII, which correspond to trypsinogen 3 (pl = 6.2) and 1 (pl = 4.9), respectively, based on estimated pl values, have been identified (5). Another human pancreatic trypsinogen cDNA, trypsinogen III, has also been described (6) which has been proposed to correspond to trypsinogen 2. However, at present, little is known of trypsinogen III, and the estimated pl based on the deduced amino acid sequence (pl = 5.94 for trypsinogen form; pl = 7.0 for active trypsin form) is different from those experimentally obtained in native mesotrypsinogen (pl = 5.7 for trypsinogen form; pl = 6.4 for active trypsin form) (1, 2).

Here we describe the cDNA encoding a novel isoform of human trypsinogen. The deduced amino acid sequence displays a pl that is in accordance with the biochemical data, and furthermore, characterization of the heterologously expressed product shows that the cDNA encodes the human trypsin isoform, which resists trypsin inhibitors, suggesting that it could be a human trypsinogen 2/mesotrypsin gene.

EXPERIMENTAL PROCEDURES

General Recombinant DNA Methods—DNA modification enzymes and restriction enzymes were purchased from standard commercial sources and used according to the manufacturers’ instructions. PCR was performed as described (7) except that Taq polymerase was used with Pfu polymerase (1:1 unit ratio) to increase the fidelity of the template amplification. The recombinant plasmids constructed as described below were confirmed by a combination of restriction enzyme mapping and DNA sequencing. The oligonucleotides used are as follows: F1, 5'-GATGACAAAGCTTGGGGAATAGCTA-3' ; F2, 5'-GGGATCCGCTGGAATCCCGCGGGGACG-3'.

Vector Construction—Expression of mesotrypsinogen in bacteria was performed by ligating the cDNA-coding sequences for mesotrypsinogen to the DNA sequence encoding a bacterial signal peptide at the HindIII site in pFlag-1 (IBI). The cloning region of mesotrypsinogen with HindIII sites at both ends was generated by using PCR with synthetic oligonucleotide primers, F1 and F2, from a λ clone carrying the mesotrypsinogen cDNA. The PCR-derived mesotrypsinogen cDNA used in the expression vector was sequenced, and its identity with the mesotrypsinogen cDNA sequence derived from λ clones was confirmed. The vector is hereafter referred to as pFlag-meso. In this vector, the native signal peptide of premesotrypsinogen is replaced with the signal peptide of OmpA to ensure efficient translocation of the expressed protein into the periplasmic space of bacteria. The probability that the protein would fold with correct disulfide formation was also expected to be increased in the oxidizing environment of the periplasm. The use of vector allows for regulated expression of mesotrypsinogen by adding IPTG. An

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‡ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; BAPNA, benzoyl arginine p-nitroanilide; SBTI, soybean trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor; TLCK, Nα,Nα-tosyl-L-lysine chloromethyl ketone; FOY-305, N,N,N,N-tetramethylcarbamoylmethyl-4-(4-guanidinobenzoyloxy)phenylacetate methane sulfone; PSTI, pancreatic secretory trypsin inhibitor.
expression vector was also constructed in the same manner (with a cDNA-encoding human trypsinogen 1 (anionic trypsinogcn, pl = 4.9)) and utilized as a control. This vector is referred to as pPlag-T1.

Purification—Escherichia coli strain JM109 was transformed with pFlag-meso or pFlag-T1 and grown to log phase. IPTG was added to a final concentration of 0.5 mM, and the cultures were further incubated in the presence of 5 mM reducing glutathione, which is known to accelerate expression and correct folding of secretory proteins in E. coli (8). IPTG-induced expression was analyzed with SDS-PAGE. A major induced band (about 20% of total protein) was shown to have a molecular size corresponding to trypsinogen (24 kDa). Cells were collected by centrifugation at 6000 × g for 20 min, washed with a Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, and then treated with lysozyme (0.1 mg/ml) in the same buffer at 4 °C for 30 min. Periplasmic proteins were obtained as supernatant fractions after centrifugation at 10,000 × g for 20 min. Expressed trypsinogens were further purified with differential sedimentation with ammonium sulfate (9), and the resulting material was further purified by high pressure liquid chromatography with a TSK3000 gel filtration column. Elution was monitored at 280 nm and then by SDS-PAGE. The fractions containing the 24-kDa band were pooled and stored at −20 °C until use in the presence of 0.5 mM HCl to prevent the autoactivation of zymogens.

Enzyme Assay—The recombinant mesotrypsinogen or trypsinogen 1 (0.1 mg/ml) was activated with enterokinase (1 μg/ml) (New England Biolabs) at 37 °C for 40 min, and then benzoylarginine p-nitroanilide (BApNA, Wako Pure Chemicals, 2 mM) was added as a substrate according to the method described (10). The reaction mixtures were incubated at 37 °C for 15 min, and then the reactions were stopped by addition of acetic acid (20%). p-Nitroanilides liberated by tryptic activities were measured with a spectrophotometer (Beckman DU-650) at 410 nm. Under the experimental conditions, linearity between increments of p-nitroanilide and incubation time was maintained up to 40 min. Thus the reaction was routinely incubated for 15 min, and then trypsin activity was calculated as the rate of A410/min unless specified.

Degradation of Macromolecule by Trypsin—Proteolytic activities of trypsins were examined using azocasein (Sigma) as substrate according to the method described by Hofsten and Renhammar (11). Hydrolysis of azocasein was monitored in increments at 5.7 for trypsin.

Trypsin Inhibitory Assay—-The recombinant mesotrypsinogen or trypsinogen 1 (0.1 mg/ml) was activated with enterokinase (1 μg/ml) as described above, and then various proteinaceous or synthetic trypsin inhibitors were added at given concentrations as shown in the figures. The reaction mixtures were incubated at 22 °C for 15 min to allow inhibitor/enzyme interaction, after which BApNA was added. The reaction was carried out at 37 °C for 15 min and then the reaction was routinely incubated for 15 min, and then trypsin activity was calculated as the rate of A410/min unless specified.

Structure of Mesotrypsin cDNA—The open reading frame of the cDNA encodes a protein of 247 amino acids (from 741 nucleotides), exhibiting a high, although not identical, similarity to the previously reported human trypsins as shown in Fig. 2. The deduced amino acid sequence contains the major features characteristic of trypsins; a negatively charged (cationic) or TRYII (trypsinogen 1, anionic) DNA sequences. Of the remaining 20% a category comprising 10 clones shared the same pattern, but this pattern was different from those of the known trypsin cDNAs. We selected these clones for further analysis. Five individual cDNA inserts in λ DNAs from this category were subcloned into Bluescript and then the DNA sequences were analyzed. All five clones showed identical DNA sequences as shown in Fig. 1.
DNA of Human Mesotrypsin

**Expression of Mesotrypsin cDNA in E. coli Periplasm—-E. coli** transformed with pFlag-meso carrying mesotrypsinogen cDNA produced mesotrypsinogen protein at about 20% of total protein upon IPTG induction (Fig. 3). A large portion (>90%) of the expressed protein remained insoluble in the bacteria even after lyset with 1% Triton X-100. A small portion (about 10%) of the expressed mesotrypsinogen was obtained as soluble protein in the periplasm and a signal peptide of OmpA (its predicted size is 2046 Da; 21 amino acids) was released resulting in the proenzyme (mesotrypsinogen) form. The apparent low efficiency of translocation may be due to saturation of *E. coli* translation machinery with high levels of exogenous protein expression.

Further purification was needed to obtain stable mesotrypsinogen, which can be used for enzymatic studies. Activation of trypsinogen to trypsin was observed in some preparations without addition of enterokinase. An affinity chromatography procedure using anti-Flag monoclonal antibody M1 (IBI) was not successful in purifying mesotrypsinogen. Antibody M1 is designed to bind Flag, an N-terminal activation peptide of trypsinogen (DDDDD), when it is expressed as a tag with a foreign protein. However, our results suggest that antibody M1 is not able to access and thus bind the Flag peptide when it is expressed as a portion of the authentic structure of trypsinogen. Chemical bonds that are supposed to be required for Flag peptide/antibody interaction would be already occupied by intramolecular interactions within trypsinogen. A series of differential sedimentations with ammonium sulfate and a gel filtration were employed to obtain purified mesotrypsinogen.

The recombinant mesotrypsinogen showed weak cross-reactivity with anti-anionic and cationic trypsinogen antibodies (provided from Dr. Scheele) indicating that mesotrypsinogen shares structural homology to other major trypsinogens (data not shown).

**Characterization of Recombinant Mesotrypsinogen—** Recombinant mesotrypsinogen showed enterokinase-dependent activation in a time-related manner, which was very similar to that shown by recombinant trypsinogen 1 (anionic human trypsinogen) (Fig. 4). In the absence of enterokinase neither mesotrypsinogen nor trypsinogen 1 were activated (Fig. 4). A control preparation similarly purified from *E. coli* periplasm transformed with vector alone did not show any significant trypsin activity in the presence or absence of enterokinase (data not shown) indicating efficient separation of endogenous *E. coli* proteases by the purification. Activated mesotrypsin was completely inactivated by 5 mM diisopropyl fluorophosphate, which is sufficient to inactivate ordinary serine proteases, confirming that mesotrypsin is a serine protease. The proteolytic activity of mesotrypsin to azocasein was the same for those of human trypsin 1 and bovine trypsin (Fig. 5).

When recombinant mesotrypsin was mixed with various concentrations of protein inhibitors, it showed significant resistance to those inhibitors. When equal molar amounts of SBTI (*M* = 24,000, 0.1 mg/ml) was added to the recombinant trypsin 1 (prepared as a control using the same procedure) more than 95% of the activity was inhibited while mesotrypsin was hardly affected (Fig. 6A). Although increasing the amount of inhibitor had a dose-dependent effect on inhibition, about 30% of trypsin activity remained even with a 1000-fold molar excess of SBTI. A marked difference was obtained when mesotrypsin or trypsin 1 was mixed with human PSTI (*M* = 6,000), which is a phys-

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**TABLE I**

Summary of human pancreatic trypsinogen isoforms

<table>
<thead>
<tr>
<th>Cloned cDNAa</th>
<th>Geneb</th>
<th>Productc</th>
<th>Deduced pld</th>
<th>pl observed with IEEe</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>Not mapped</td>
<td>Trypsinogen 2/mesotrypsinogen</td>
<td>5.68</td>
<td>5.7</td>
</tr>
<tr>
<td>TRYI (5)</td>
<td>T4</td>
<td>Trypsinogen 3</td>
<td>6.08</td>
<td>6.2</td>
</tr>
<tr>
<td>TRYII (5)</td>
<td>T8</td>
<td>Trypsinogen 1</td>
<td>4.36</td>
<td>4.9</td>
</tr>
<tr>
<td>Trypsinogen III (6)</td>
<td>Not mapped</td>
<td>Not known</td>
<td>5.94</td>
<td>7.00</td>
</tr>
</tbody>
</table>

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a Names of the cDNAs have been based on each reference shown in parentheses. Note that the numbers do not correspond to either genes or products.
b Two cDNAs have been assigned to correspond to two loci, which have been found within a cluster of eight trypsinogen genes (three seem functional) in the beta T cell receptor region of the human genome (see Ref. 19).
c Trypsinogen 1, 2, and 3 (protein products of cDNAs) are numbered from anode to cathode in accordance with the IUPAC-IUB Commission on biochemical nomenclature of multiple forms of enzymes (see Ref. 1).
d Deduced pl values are calculated by the authors based on amino acid sequences deduced from published cDNAs using software DNASIS (27).
e Data are cited from publications (1, 2) where denatured conditions were used in isoelectric focusing (IEF), which are supposed to fit better to deduced pl values (27).
iological inhibitor coexisting with trypsinogen in the secretory granule in the pancreas and may serve to prevent premature activation of trypsins (12). With a 4-fold molar excess of human PSTI (0.1 mg/ml) trypsin 1 was 100% inhibited while almost full activity of mesotrypsin remained (Fig. 6B). In the presence of a 40-fold excess of human PSTI (1 mg/ml), about 80% of the activity of mesotrypsin survived. Similar results were obtained using purified rat PSTI (data not shown). These profiles were also observed in the presence of BPTI (aprotinin), another type of protein inhibitor purified from bovine pancreatic tissue (Fig. 6C). On the other hand, mesotrypsin was sensitive to low molecular weight, synthesized serine protease inhibitors including phenylmethylsulfonyl fluoride (Fig. 6D), TLCK (Fig. 6E), and FOY-305 (Fig. 6F). The sensitivity of mesotrypsin to these inhibitors was similar to that shown by human trypsin 1. For each inhibitor, mesotrypsin was almost 100% inhibited at a concentration great enough to completely inhibit trypsin 1. Such characteristics are consistent with the results found in native mesotrypsin (2). In the case of FOY-305, however, mesotrypsin showed slightly higher resistance when intermediate concentrations of the inhibitor were used (Fig. 6F).

**DISCUSSION**

It is known that human trypsin, when compared with bovine trypsin, is less inhibited by naturally occurring trypsin inhibitors such as chicken ovomucoid (13). The occurrence of two forms (anionic and cationic) of trypsinogen has been reported by a number of researchers (3, 14–17). The cationic form of human trypsin, which represents the major part of trypsin activity of the whole pancreatic juice, is shown to be less inhibited by ovomucoid and soybean trypsin inhibitor than the anionic trypsin (18, 19). Both human trypsins are completely inhibited by aprotinin or human PSTI, a physiological inhibitor co-localized with trypsins in the zymogen granules, at a stoichiometric enzyme-to-inhibitor ratio of one to one (18). More recently, a third minor trypsinogen in human pancreatic juice, named trypsinogen 2 (1) or mesotrypsinogen (2), has been reported (1, 2) that shows almost no inhibition by either naturally occurring trypsin inhibitors or human PSTI. (Thus, ani-
onic and cationic trypsinogens are referred to as trypsinogen 1 and 3 according to their pI values as summarized in Table I.)

In this study, after extensive screening of a human pancreatic cDNA library, we identified and characterized a cDNA-encoding mesotrypsinogen. The identification was based on the following points: 1) deduced amino acid sequence shows a unique isoform containing the major features characteristic of trypsinogens, 2) the calculated isoelectric point is consistent with that obtained from native mesotrypsinogen, and 3) recombinant protein expressed from the cDNA shows resistance against protein inhibitors, which has been observed in native mesotrypsin.

It has been predicted, based on Southern analysis, that the human genome carries at least ten trypsinogen genes (5). Recently, a large scale DNA sequencing of the human beta T receptor locus has revealed that eight trypsinogen genes (denoted as T1 through T8) are intercalated in the locus (20); among them, three (T4, T6 and T8) are apparently functional while the rest are pseudo or relic trypsinogen genes. Data base analysis reveals that T4 corresponds to TRYI and T8 to TRYIII (see Table I), two major trypsinogens. However, T6 does not correspond to any of the reported trypsinogen cDNAs. We examined whether mesotrypsinogen cDNA corresponds to T6 or any other trypsinogens found in this locus but found no match.

Analysis of the mesotrypsinogen cDNA in the data bases reveals that there are several trypsinogen-related cDNAs from the pancreas and brain, which show close similarities to the mesotrypsinogen sequence described here. Relatively high similarity between the mesotrypsinogen and trypsinogen III (6) genes might suggest these are allelic divergences in the human genome. However, we thought that this possibility is unlikely because differences in sequences (Thr167-Gln168, Tyr175, Cys196...)

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**Fig. 6. Inhibition profiles of mesotrypsin and trypsin 1 with various trypsin inhibitors.** Recombinant mesotrypsinogen (●) or trypsinogen 1 (○) (0.1 mg/ml, Mr = 24,000) was activated with enterokinase (1 µg/ml) as described and then incubated with one of the given trypsin inhibitors at various concentrations as indicated at 22 °C for 15 min. Residual trypsin activity was assayed with BApNA and expressed as the percentage of activity without the inhibitor. A, SBTI, Mr = 24,000; B, human PSTI, Mr = 6,000; C, BPTI/aprotinin, Mr = 6,000; D, phenylmethylsulfonyl fluoride (PMSF); E, TLCK; F, FOY-305. Data are expressed as the mean of three experiments, all of which had variations of less than 5%.

**Fig. 7. Interaction profiles of mesotrypsin or trypsin 1 with BPTI.** Recombinant mesotrypsin or trypsin 1 (0.1 mg/ml) was incubated with BPTI/aprotinin (250 µg/ml) and then the mixture (about equal molar ratio of enzyme/inhibitor) was loaded onto a Sephadex G-100 gel chromatography column. Elution profiles were monitored with a UV spectrophotometer at 280 nm. Upper, mesotrypsin (○) or BPTI (●) were loaded separately and the monitored profiles were superimposed. Peaks for mesotrypsin (about 24 kDa) and BPTI (about 6 kDa) are indicated with arrows. Trypsin 1 (about 24 kDa) was eluted with a similar profile (data not shown). Middle, a mixture of trypsin 1 with BPTI was loaded and the elution profile was monitored. Note that a single peak appeared at 30 kDa indicating that the two molecules form a complex. Lower, a mixture of mesotrypsin with BPTI was loaded and the elution profile was monitored. Note the two peaks indicating that the molecules do not interact.
Gln<sup>197</sup> versus Arg<sup>197</sup>, Glu<sup>168</sup>, Cys<sup>175</sup>, Trp<sup>196</sup>, Lys<sup>197</sup>) include major substitutions of charged amino acids in the opposite direction and replacement of conserved Cys, which are quite unusual for regular allelic divergences, and partly because our intensive search for the trypsingen III sequence in the screened clones failed. This suggests that trypsingen III is a very minor component, or else it is highly up-regulated upon stimulation, similar to the expression of rat trypsingen isoform P23 (21, 22). Products of trypsingen III cDNA and the T6 gene remain to be further investigated.

Another mesotrypsin-like sequence found in the data bases is D23456, which has been identified in the human brain using a PCR technique (23, 24). The D23456 and our mesotrypsingen sequences have about 200 amino acids in common at their C-terminals but the amino acid sequences at the N-terminals are quite different; the brain sequence lacks any signal sequence characteristic of pancreatic trypsinges. It was suggested that tissue specific alternative splicing might generate the brain-type (cystosolic) and the pancreas-type (secretory) trypsinges (23). Such a hypothesis is quite attractive; however, how a trypsingen lacking a signal peptide forms correct disulfide bonds to generate an enzymatically active structure in the cystosolic environment that is reductive should be further investigated. Moreover, the alternative (secretory) form expressed in the pancreas was not detected with PCR (23). We did not find a cystosolic form of trypsingen even after intensive screening of the pancreatic cdna library. A reason for the failure of previous studies could be partly explained by the fact that they designed the PCR primer to detect a secretory partner of the brain-type trypsingen in the pancreas based on trypsingen III (6), the nucleotide sequence of which differs by more than 3% from the sequence of mesotrypsingen cdna described here. Mesotrypsingen in the pancreas could be a better candidate for an alternative spliced form of a brain-type trypsingen gene if the splicing model is correct.

Although elucidation of the molecular basis of the inhibitor resistance of mesotrypsin will require site-directed mutagenesis and x-ray crystallography, both of which are currently underway in our laboratory, it is noteworthy to point out that amino acid substitutions are unique in mesotrypsin when compared with other major isoforms of trypsingen that are inhibitor sensitive, in particular at and around the reactive center that interacts with inhibitors. Between mesotrypsin and the other two major human trypsinges, the catalytic triad of His<sup>63</sup>, Asp<sup>107</sup>, and Ser<sup>200</sup> as well as an obligatory Asp at position 194 are all conserved. However, at amino acid 198 of mesotrypsingen an Arg residue takes the place of a Gly residue. The Gly residue is normally found in the active center of the known rat and human sequences and participates in the interactions with trypsingen inhibitors such as BPTI (aprotinin) (25). Substitution by a positively charged amino acid at Gly<sup>198</sup> position might interfere with the trypsingen/trypsingen inhibitor interaction because a site in the trypsingen inhibitor that interacts with trypsingen also contains Arg. In a large number of Kunitz- or Kazal-type trypsingen inhibitors the consesus sequence has been found to be Arg/Lys-Ile (26). By utilizing x-ray crystallography data of rat trypsingen and BPTI complex (24) and superimposing the replacement of Gly by Arg at position 198 using the software Quanta (Molecular Simulations), the Van der Waals radius of both Arg<sup>198</sup> in mesotrypsin and Arg<sup>17</sup> in BPTI was found to overlay at any possible rotation. This suggests that two Arg residues are mutually exclusive in the complex. In fact two molecules could not bind tightly to each other. The model partly explains why mesotrypsin and BPTI did not form a stable complex in the gel filtration assay shown in Fig. 7.

The fact that mesotrypsin has a strong inhibitor resistance suggests several physiologically relevant functions. First, mesotrypsin may serve as the inhibitor-resistant trypsingen for effective digestion when certain diets rich in naturally occurring trypsingen inhibitors such as SBTI are ingested. Second, if levels of mesotrypsingen are increased with a certain stimulus or by pathogenic conditions the inhibitor-resistant trypsingen may cause potentially deleterious effects on autoactivation within pancreatic tissues in particular in the case of pancreatitis; autoactivated mesotrypsin can further activate trypsingen even in the presence of PSTI or other endogenous trypsingen inhibitors. The work presented in this paper provides essential information for future studies designed to reveal the physiological significance of mesotrypsin and its underlying molecular basis.

REFERENCES

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