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Epitope Mapping of Region 11-70 of Ovalbumin (*Gal d I*) Using Five Synthetic Peptides

Key Words

Allergenicity
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Synthetic peptide

Abstract

Five successively located peptides, in region 11-70 of the major allergen of ovalbumin (OA) *Gal d I* (11-19, 20-33, 34-46, 47-55, 56-70), were obtained by manual solid-phase peptide synthesis. These peptides together with the previously reported OA region 1-10 comprise a segment of 70 amino acid residues located at the N-terminal of ovalbumin. The crude peptides were purified by gel filtration and reversed-phase high-performance liquid chromatographies and their sequences were verified. Polyclonal antibodies against the peptides conjugated to carrier protein (BSA) were raised in rabbits. Rocket line immunoelectrophoresis showed that four peptides (20-33, 34-46, 47-55 and 56-70), could deflect OA-line immunoprecipitates. The peptide's affinity to rabbit polyclonal Ig was examined by quantitative precipitation inhibition and the results suggested that an epitope was encompassed in segments 34-55 and 47-55. Allergenicity was tested by inhibition of specific IgE binding of ovalbumin, using several sera and a serum pool from 16 egg-allergic patients. The results showed that the allergenicity was distributed over the whole region. These findings suggested that: (a) the region 11-70 of OA seemed not to encompass continuous epitopes; (b) the antigenicity of this region was convincing for peptides 34-46 and 47-55; (c) the allergenicity, though dependent on the patient serum used, was distributed over the whole of region 11-70; (d) peptide 11-19, although weak antigenically was capable of specific IgE inhibition; (e) human and rabbit polyclonal antibodies did not show analogous affinities to the present peptides.

Introduction

Egg is an essential ingredient in human and animal food, and is difficult to eliminate from the diet [1]. Adverse reactions to egg are well recognised due to the intimate relation between food ingestion and the reaction elicited. Egg allergy is more frequent in young children than

in adults [2]. The allergenic proteins of hen egg white extract have been characterised [3]. The allergenic structure of ovalbumin OA (*Gal d I*) was briefly described [4] and an epitope analysis using a panel of mouse monoclonal antibodies against the native OA was reported [5]. The complete amino acid (AA) sequence of OA has been established [6] and the secondary structure corresponding

Fig. 1. The covalent structure of the five peptides in the region 11–70 of ovalbumin. The sequences were deduced from the known primary structure of the molecule. No internal homology was found between these peptides. Both the molecular weight (M_r) of the peptides and the crude recoveries (Rec) are shown. The peptide selection was based on assuring optimal hydrophobicity, mass and availability of Boc-amino acid resins used in solid-phase synthesis.

Residue No.	Covalent structure	M_r	Rec
11–19	C F D V F K E L K.	1109	43.5%
20–33	V H H A N E N I F Y C P I A.	1612	32.6%
34–46	I M S A L A M V Y L G A K.	1350	24.0%
47–55	D S T R T Q I N K.	1087	48.4%
56–70	V V R F D K L P G F G D S I E.	1660	91.4%

to the initiation region of OA mRNA has been determined [7]. Furthermore, a preliminary X-ray analysis of two crystals of the molecule has been elucidated [8,9]. Recombinant OA was further synthesised and secreted by *Escherichia coli* [10, 11]. Although the biological activity of the molecule is not understood, it was suggested to be mainly a structural protein and substrate for the elastase-like enzyme group [12].

Knowledge on the allergenic/antigenic structure of allergens is mandatory for establishing modern therapeutic methods for allergy and for regulation of IgE response. In addition, OA is universally used in a large number of publications and its structures elucidation using various antibody isotypes seemed mandatory [11, 13–16].

The allergenic structures of many other allergens have been elucidated [17–19]. The number of IgE-binding epitopes varies in different allergens. Although the minimal requirements of an allergenic peptide were demonstrated to be 4 amino acids, a molecular size of 12–15 amino acids may be necessary for antibody binding which may be linked to a requirement for helical conformation [17, 20–22].

In previous studies the activities of two OA peptides (1–10 and 323–339) were studied [23, 24]. For 323–339, a correlation between peptide Ia interaction and MHC restriction were demonstrated and found responsible for 25–35% of BALBc T cell response to intact OA [25]. Peptide 323–339 was concluded to encompass one B cell epitope [24].

The present communication reports the antigenicity and allergenicity of the region 11–70 of the molecule. Five successive peptides were examined and assigned the reactivities of this segment using rabbit antibodies and specific IgE from egg-allergic patients. The selection of the five peptides was favored on the basis of the optimal hydrophobicity, mass and the availabilities of the C-terminal Boc-amino acid resin, necessary for solid-phase synthesis. The chosen peptides together with the previously report-

ed 1–10 [23], comprise the 70 amino acid residues covering the N-terminal of region OA.

Materials and Methods

Hen Egg White

Extract of hen egg white proteins (8.43 g/l protein) and OA (*Gal d I*) were prepared and antibodies against both the crude extract and purified OA were raised in rabbits as described previously [26].

The Synthesis and Purification of the Peptides

Solid-phase peptide synthesis (SPPS) of the five peptides of region 11–70, namely 11–19, 20–33, 34–46, 47–55 and 56–70 with the covalent structures illustrated in figure 1, were prepared by manual methods. The synthetic preparations were cleaved from the resin by HBr and trifluoroacetic acid (TFA) [27–29].

The purification of the peptides by gel filtration chromatography (GFC) was done using Biogel P2 column (dimensions 61 × 1 cm), 200–400 mesh resin obtained from Biorad, Richmond, Calif., USA. The void volume peak (V_o) contained the peptide in question. High-performance liquid chromatography (HPLC) was performed using an ion-exchange column, Bio Siol TSK DEAE 2-SW (dimension 250 × 4.6 mm; Biorad) with the following solvent system at pH 6.2: (A) 20 mmol/l Tris, and (B) 20 mmol/l Tris 500 mmol/l NaCl. A linear gradient of 100% A:0% B to 100% B:0% A was run in 14 min. The last solvent was continued for the same time, and then back to the initial composition (100% A:0% B). Aliquots of 100 µg peptide were originally run and the absorbance was monitored at 220 nm [24]. The initial concentration of the peptides (2 µM) was usually used in 6-fold serial dilutions for examining their activity.

Judging the Peptide Structure

This was done by AA analysis on a Biotronic LC 7000 analyser (Wissenschaftliche Geräte, Munich, FRG). Aliquots of 50 µl of the peptides were drawn from the reaction bottle after coupling the amino acids at different stages of synthesis and after the termination and cleavage. The samples were hydrolyzed in vacuum by 6 M HCl and then analyzed [30].

Later, a 477A Applied Biosystem Automatic Sequence Analyzer was provided to our laboratory and the sequence of the five peptides were further confirmed by their entire AA sequences [27].

Protein-Peptide Conjugate

Coupling of the peptides to bovine serum albumin (BSA) was performed using 100 μ M of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), 10 μ M peptide and 500 nM BSA. The peptide conjugates were lyophilized and purified on the same GFC column to provide preparations with no free AA or EDC [31].

Immunization

Ten adult rabbits were immunized, two with each of the five peptide conjugates by 4 dorsal intradermal injections, with 10-day intervals. An emulsion of 2 mg/ml of the five peptides conjugated to BSA in 0.5 ml of 0.9% NaCl and 0.5 ml of complete Freund's adjuvant (CFA) was injected [32].

Antisera were titrated against the respective antigens (BSA and OA) and the optimal dilutions (10-, 100- and 1,000-fold) were used in quantitative precipitation inhibition (QPI) tests. Rabbit antisera were used with no dilution in rocket line immunoelectrophoresis (RLIE) experiments.

Immunoelectrophoretic Techniques

RLIE was performed using the five peptides, OA and egg white extract as controls. The experimental details of RLIE were described in previous publications [28, 33, 34]. The concentrations of the peptide conjugates used were 400 μ g (20 μ l of 20 mg/ml) and 15 μ g (15 μ l of 1 mg/ml) of the controls including EDC. Antisera (400 μ l) were incorporated in the antibody gel and the OA and BSA lines were obtained by incorporating 15 μ g of the protein in 1 ml of the secondary gels as in the legend to figure 2.

Quantitative Precipitation Inhibition

The QPI test was performed on a Laser Nephelometer (Behring Institute, Marburg, FRG), wavelength 840 nm. Saline/15% Tween-20 solution was used for tenfold dilution of the antibody. Twofold series of dilutions of OA (150 nM) and the peptides (10 μ M) were used for the precipitation inhibition tests. Fifty microliters of the peptide-BSA conjugate and 200 μ l of the diluted antiserum were incubated for 1 h at room temperature prior to the addition of 50 μ l of OA. Birch pollen major allergen (*Bet v I*) at a concentration of 2 mg/ml and an unrelated peptide (*Bet v I* 23–38) were used as control [27]. The turbidity signals (volts) were directly correlated to the quantity of the Ag/Ab complex. Experimental details for quantifying proteins by QPI were described in Schliep and Flegenhaver [35].

Human Sera

Specific IgE antibodies against egg white extract were obtained from 16 patients with clinical histories of food allergies including egg or egg and fish, with moderately high total and egg-specific serum IgE (radio-allergosorbent test, RAST, class 2–4). Informed consent was obtained from the patients before samples were donated. None of the patients had been treated with systemic corticosteroids or undergone immunotherapy. A pool was prepared from equal aliquots of sera (No. 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 and 50). Three of these sera were selected due to their high total IgE and RAST class (No. 20, 29, 33). Pool of 3 healthy individuals' sera was also included in the control.

Specific IgE Analysis

RAST inhibition was performed using CNBr-activated paper discs (0.25 g) coated with OA (10 mg in 10 ml of 100 mmol/l phosphate-buffered saline, pH 7.4) and commercially available RAST egg

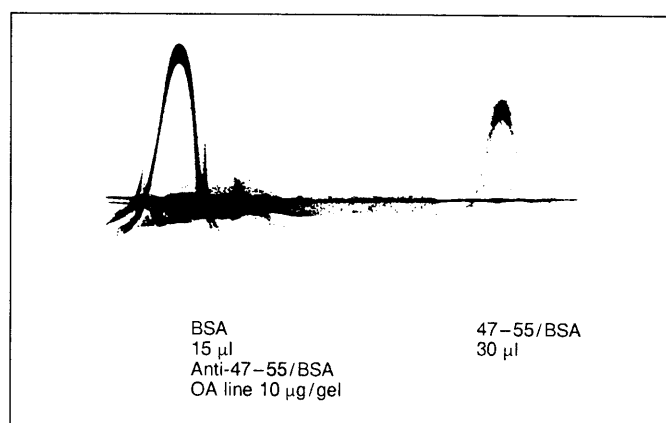


Fig. 2. RLIE run using 400 μ l rabbit anti-BSA 47–55 in the antibody gel (upper), 10 μ l of BSA and 20 μ l of the peptide conjugate in the first and second wells, respectively, in the middle gel. Birch pollen allergen and peptide 11–19 were used at identical concentrations as negative control in a parallel run [not shown]. Both BSA and the BSA 47–55 conjugate did not deflect the OA line but showed a clear direct rocket of BSA. The OA line was done by incorporating 15 μ g OA in the lowest line gel. Experimental details are further described in the literature [28, 33, 34].

discs (f1; Pharmacia, Uppsala, Sweden) [36]. Briefly, triplicates of 50 μ l serum and 100 μ l of peptide aliquots and controls (*Bet v I* and OA) were incubated for 3 h under gentle mixing. Paper discs were added, followed by 3 h incubation. After washing, 50 μ l of ^{125} I-anti-Fc_ε was added.

Labelling was performed overnight and the discs were washed and counted in a γ -scintillation counter. The mean % inhibition was calculated relative to the noninhibited control [37].

Results

The covalent structures, molecular weights (M_r) and recoveries (Rec) of the five synthetic peptides (11–19, 20–33, 34–46, 47–55 and 56–70) are depicted in figure 1. Their lengths varied from 9 to 15 residues and no AA homologies were present.

The peptide synthesis was done by the SPPS technique [29] using well-established strategies, protecting groups and procedure [28, 38]. Peptides 20–33 and 34–46 were obtained at relatively low yields (32.6 and 24%, respectively) (fig. 1). The purification of the peptides by GFC showed two peaks of which the V_0 peak was run on an HPLC ion-exchange column. Those were further resolved into apparently homogenous symmetrical peaks with the following retention times (Rt): 11–19 (Rt 2.42); 20–33 (Rt 1.44); 47–55 (Rt 1.41), 34–46 (Rt 1.64) and 56–70 (Rt 1.59). Deletion peptides or contaminants were resolved at different Rt and discarded.

Table 1. Amino acid composition of GFC-purified OA peptides

Amino acid	Peptide R/M									
	11-19		20-23		34-36		47-55		56-70	
	T	E	T	E	T	E	T	E	T	E
Asp	1	0.96	2	1.60	-	-	2	1.70	2	2.12
Thr	-	-	-	-	-	-	2	1.60	-	-
Ser	-	-	-	-	1	0.95	1	0.92	1	0.90
Glu	1	0.80	1	0.85	-	-	1	1.05	1	-
Pro	-	-	1	0.77	-	-	-	-	1	0.83
Gly	-	-	-	-	1	0.80	-	-	1	2.16
Ala	-	-	2	2.00	3	3.30	-	-	-	-
Cys	1	0.83	1	0.70	-	-	-	-	-	-
Val	1	0.77	1	0.77	1	0.70	-	-	2	2.20
Met	-	-	-	-	2	1.60	-	-	-	-
Ile	-	-	2	1.85	1	0.60	1	0.99	1	1.03
Leu	1	1.44	-	-	2	2.00	-	-	1	1.0
Tyr	-	-	1	1.15	1	0.80	-	-	-	-
Phe	2	1.40	1	1.24	-	-	-	-	2	2.10
Lys	2	2.00	-	-	1	1.80	1	1.20	1	1.02
His	-	-	2	1.69	-	-	-	-	-	-
Arg	-	-	-	-	-	-	1	1.50	1	0.72
Total	9		14		13		9		15	

R/M = Residue per mol; T = theoretical value; E = experimental value.

The covalent structures of the peptides were confirmed retrospectively by their automatic AA sequence analyses.

The AA compositions of the peptides showed that their syntheses proceeded linearly. Table 1 shows the AA compositions of the GFC-purified peptide chains. The covalent structures of the five peptides were reconfirmed by automatic Edman AA sequence analyses as mentioned in 'Materials and Methods'.

The nonconjugated peptides were not immunogenic in rabbits.

The antigenicities of the BSA/peptides could be detected by RLIE and 8 sera gave clear immune responses for BSA [results not shown]. Rocket lines were found for peptides/BSA: 20-33; 34-46; 47-55 and 56-70 upon using antisera from rabbits immunized with BSA conjugates of 20-33, 34-46, 47-55 and 56-70, respectively. Some cross-reactivities were shown for OA 47-55 giving line deflection with two antisera: BSA 20-33 and 56-70, respectively

Table 2. Antigenicity

Antigen OA/BSA	Antibody OA/BSA	OA line deflection		BSA line deflection	
		peptide	BSA	peptide	BSA
11-19	11-19	-	+	-	+
	34-46	-	(+)	-	+
	20-33	(+)	+	n.d.	n.d.
20-33	11-19	-	-	-	-
	47-55	-	-	-	-
	34-46	+	+	n.d.	n.d.
34-46	20-33	-	-	-	-
	47-55	-	(+)	-	(+)
	47-55	+	+	n.d.	n.d.
47-55	20-33	+	+	-	+
	56-70	+	+	-	+
56-70	56-70	(+)	+	-	+
	34-46	(+)	+	-	+

(+) and (-) qualitative analyses indicating positive and negative generation of deflection in the antigenic lines. (+) = Uncertain non-specific deflection; n.d. = not done.

A summary of the rocket line immunoelectrophoresis of OA peptide in region 11-70 using rabbit polyclonal antibodies raised against BSA/peptide conjugates. The antigenic sites were suggested to be located regions 34-46 and 47-55. Peptides 20-33 and 56-70 have none/weak activity under these experimental conditions. Some cross-reactivity was shown for OA BSA 47-55 against anti-BSA 20-33, 47-55 and 56-70 conjugates. Peptide in either form was used at a concentration of 20 mg/ml and a sample of 30 µl as applied to the well. BSA, OA and EDC were used at 1 mg/ml and 15 µl was applied in the wells.

(table 2). BSA conjugates of peptides 11-19, 20-33 and 56-70 did not show specific deflections. Furthermore, no antigenicity was found for the nonconjugated forms. Only BSA 34-46 and 47-55 gave clear rockets in the antibody gel area (one run for 47-55 is illustrated in fig. 2). QPI analysis showed that the antigenicity was covering the N-terminal segment encompassing residues 20-33, 47-55 and 56-70. The crude preparations showed high inhibitions at concentrations >300 nM (fig. 3a-c).

The allergenic activity was investigated by specific IgE inhibition using three individual sera and a serum pool. The mean inhibitions % using serum 29 are shown in table 3. Peptide 11-19 was capable of inhibition of binding IgE from sera 29, 33 and serum pool. Both the crude and GFC-purified peptide preparations showed similar IgE inhibition. Peptide 20-33 was shown to be inactive.

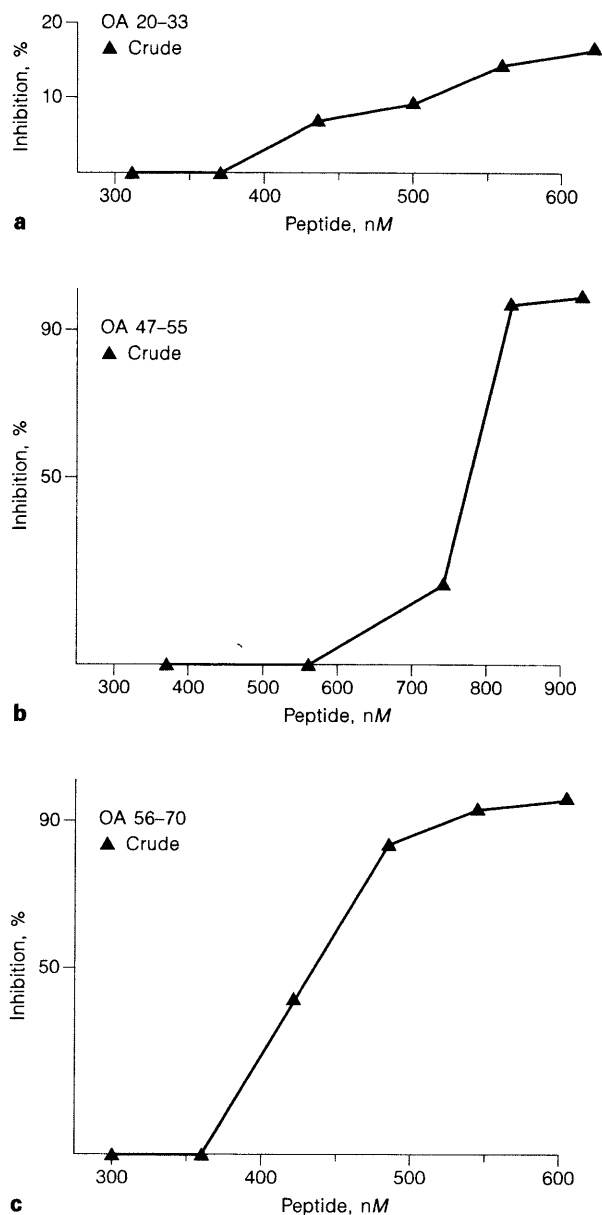


Fig. 3. Quantitative precipitation inhibition diagrams of the peptides in region 11-70 of ovalbumin at relatively high concentrations of OA 20-33 (a), 47-55 (b) and 56-70 (c).

The activity of 34-46 was extremely weak particularly with serum 29 (<7%). Similar low inhibitions for peptide 47-55 were also found. Peptide 56-70 was moderately active using serum 20, 29 and pool I (maximum inhibition 16.9%).

Table 3. Specific IgE inhibition

Concentration at peptide/mean value in % IgE inhibition							
nM	11-19	nM	34-46	nM	47-55	nM	56-70
1,800	25	1,480	-	1,180	8.3	1,200	16.9
900	13	740	4.5	590	0.68	600	10.6
450	4	370	6.7	290	0.34	300	5.9
222	6	185	2.8	145	0.17	150	8.0
111	2	92	2.0	72	0.08	-	-
OA (1 mg/ml)		36	91.4	91	81.7		
Bet v I (2 mg/ml)		4.9	0.2	3.1	7.4		

- = Not determined. RAST inhibition values were not corrected to the nearest figure.

The five synthetic peptides were tested using inhibition of specific IgE of patients allergic to egg white. It was shown that except for OA 20-33 [results not shown] the activity was distributed in the whole region examined.

Table 4. Qualitative overview of the allergenicity and antigenicity of region 11-70 of OA

Region	Ab affinity	
	Human specific IgE	polyclonal rabbit IgG
11-19	moderate	none
20-33	low	low/moderate
34-46	low/moderate	moderate
47-55	low/moderate	high
57-70	moderate	low/none

The low and moderate affinities suggest that the region studied comprises fragments of conformational epitope(s).

Discussion

Earlier studies from our laboratory described the synthesis of two OA epitopes 1-10 and 323-339 [23, 24]. In the present paper, five consecutively located peptides were synthesized and their activities were examined [39, 40]. Due to the ambiguity regarding epitope prediction in hydrophobic regions, the synthesis of a whole N-terminal segment was initiated at residue 11 and terminated at residue 70 [22, 41, 42].

The allergenic structure of the rest of OA chain (region 71-385) was elucidated using different strategies for peptide selection, synthesis, sequence and purification. Region 71-385 will therefore be reported separately [40, 43].

The HBr/TFA cleavage of the peptides from the solid-phase resin did not seem optimal for all the peptides, occasionally resulting in low yields. Despite the low yields of 20–33 and 34–46 (fig. 1), these were available in relatively large excess for all the tests performed.

All the peptides were easily purified by GFC followed by ion-exchange HPLC. The V_0 peak of the GFC, was further resolved on the HPLC column into homogeneous symmetrical peaks.

The linearity of the sequence was confirmed by: (a) AA composition (table 1) and (b) AA sequence analysis. Some minor deviation of the AA concentrations from the theoretical values did not appear to impel structural irregularities.

The immunogenicity and antigenicity of the peptides were investigated. The nonconjugated peptides were not immunogenic in rabbits. The animals were immunized by the peptides/BSA conjugates, despite some concern about structural changes that might be induced by conjugation.

Peptides 11–19, 20–33 and 56–70 conjugates did not show confident deflections. Briefly, RLIE runs illustrated that only BSA/34–46 and 47–55 gave specific immunoprecipitates (fig. 2).

QPI analysis showed that the antigenicity was covering the region encompassed by 20–33, 47–55 and 56–70. The crude preparations showed high inhibitions at concentrations > 300 nM (fig. 3a–c).

The nonconjugated peptides gave no immune responses in the 8 animals used. RLIE showed that peptides/BSA conjugates of 20–33, 34–46, 47–55 and 56–70 could give rocket lines when using Abs against BSA 20–33, 34–46, 47–55 and 56–70 conjugates, respectively (table 2).

The allergenic activity was investigated by specific IgE inhibition using three individual sera and a pool con-

sisting of 13 sera. The mean average % inhibitions are shown in table 3.

Peptide 11–19, although antigenically weak, was capable of inhibition of binding IgE from sera 29, 33 and serum pool which could be attributed to different individual avidities of sera used. Considering the variation coefficients of RIA method used, the activity of 34–46 was extremely weak particularly with serum 29 ($< 7\%$). Similar low inhibitions for peptide 47–55 were also found. Peptide 56–70 was moderately active using serum 20, 29 and pool I (maximum inhibition 16.9%).

In conclusion, only two peptides, 11–19 and 56–70 showed inhibition by the panel of sera used (table 3). Serum 29 and 33 gave approximately 90% inhibition with control OA while negative with *Bet v* I. Serum 20 showed affinity for egg allergens other than OA (only 36% inhibition for the intact OA).

These findings suggested that: (a) region 11–70 of the OA molecule seemed to encompass conformational rather than continuous motifs; (b) the antigenicity was stronger for peptides at the C-terminal part of segment 11–70; (c) the allergenicity was not located on a distinct peptide but distributed over region 33–70; (d) peptide 11–19, although antigenically weak, was capable of inhibition of binding IgE; (e) rabbit antibodies and human IgE against the intact OA did not always have analogous affinities to the peptides.

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