Visualization of clustered IgE epitopes on α-lactalbumin

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Background: α-Lactalbumin (α-La) is a major cow's milk (CM) allergen responsible for allergic reactions in infants.
Objective: We performed molecular, structural, and immunologic characterization of α-La.
Methods: Recombinant α-lactalbumin (rα-La) was expressed in *Escherichia coli*, purified to homogeneity, and characterized by means of mass spectrometry and circular dichroism, and its allergenic activity was studied by using microarray technology, as well as in a basophil histamine release assay. IgE epitope mapping was performed with synthetic peptides.
Results: According to circular dichroism analysis, rα-La represented a folded protein with a high thermal stability and refolding capacity. rα-La reacted with IgE antibodies from 57.6% of patients with CM allergy (n = 66) and induced the strongest basophil degranulation with sera from patients with CM allergy who had exhibited gastrointestinal symptoms or severe systemic reactions on CM exposure. rα-La contained sequential and conformational IgE epitopes. Superposition of IgE-reactive peptides onto the 3-dimensional structure of α-La revealed a close vicinity of the N- and C-terminal peptides within a surface-exposed patch.
Conclusions: rα-La can be used for the diagnosis of patients with severe allergic reactions to CM and serves as a paradigmatic tool for the development of therapeutic strategies for CM allergy. (J Allergy Clin Immunol 2010;125:1279-85.)

Key words: *Allergen, food allergy, milk allergy, recombinant allergen, IgE epitope*

Cow's milk (CM) is the first component introduced into the diet, and it is the most common cause of food allergy in children younger than 5 years, affecting about 2% to 3% of infants and young children in developed countries.[1]

Several studies have identified αS1-casein as a major CM allergen that induces strong immediate, as well as delayed-type, allergic reactions.[2-4] Although αS1-casein represents a class I food allergen, it was found to contain both conformational and sequential IgE epitopes.[4] β-Lactoglobulin represents another important CM allergen that is recognized by approximately 50% of patients with CM allergy and for which IgE and T-cell epitopes have been studied.[5-7] However, for α-lactalbumin (α-La), a widely varying prevalence of IgE recognition ranging from 6% to 100% has been reported in the literature.[2,3,8-10] α-La plays an important role in the biosynthesis of lactose through the interaction with lactose synthase.[11] It is expressed exclusively during lactation in the mammary gland and accounts for 20% of bovine whey, the remaining 80% being mostly β-lactoglobulin.[12]

We report the isolation of a cDNA coding for α-La, the expression of the recombinant allergen in *Escherichia coli*, and its purification to homogeneity. Recombinant α-lactalbumin (rα-La) was characterized regarding its fold by means of circular dichroism (CD), and a mapping of both sequential and conformational IgE epitopes was performed to localize the major IgE epitopes on the 3-dimensional structure of the allergen. By using IgE microarray technology and the transfer of serum IgE from patients with CM allergy with defined clinical symptoms to rat basophils transfected with the human FcεRI, the IgE reactivity and allergenic activity of rα-La were determined and related to clinical manifestations in patients.

METHODS

Isolation of cDNA and expression of rα-La

The cDNA coding for mature α-La without N-terminal signal sequence and with a C-terminal hexahistidine tag was obtained from a mammary gland cDNA library by means of PCR amplification with the Pfu DNA polymerase system (Fermentas Life Sciences, Vilnius, Lithuania) and the following oligonucleotides (MWG Biotech, Ebersberg, Germany), as previously

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Abbreviations used
α-La: α-Lactalbumin
CD: Circular dichroism
CM: Cow's milk
EGTA: Ethyleneglycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid
HSA: Human serum albumin
IPTG: Isopropyl-β-D-thiogalactopyranoside
nα-La: Natural α-lactalbumin
NIH: National Institutes of Health
PBST: PBS with 0.5% vol/vol Tween 20
rα-La: Recombinant α-lactalbumin
RBL: Rat basophil leukemia

described[4]: α-La 5′, 5′-GCGGATCCACATATGGAACAGTTAACAAAATG TGAG-3′ (*Nde*I underlined); α-La 3′, 5′–CGGAATTCCTGCAGAACTCA *GTGATGATGATGATGATG*CAACTTCTCACAGAGCCACTGATCCAGC–3′(*Eco*RI underlined; His tag-encoding DNA in italics).

The sequence of α-La from cow (National Institutes of Health [NIH] accession no. NP_776803), as determined by us, and the protein sequences from α-La of other mammalian species that had been obtained from the NIH database (http://www.ncbi.nlm.nih.gov) were aligned manually in Fig 1, *A*, for maximal fit. Potential glycosylation sites were predicted with a NetNGlyc 1.0 Server-Program (http://www.cbs.dtu.dk/services/NetNGlyc/).

rα-La was expressed in the *E coli* strain BL21 Codon Plus (DE3)-RIPL (Stratagene, La Jolla, Calif) and purified with Ni-NTA resin affinity columns (Qiagen, Hilden, Germany).

The protein was blotted onto nitrocellulose[13] and incubated with an anti-hexahistidine antibody (Histidin-Tag; Dianova, Hamburg, Germany) 1:1,000 diluted in PBS with 0.5% vol/vol Tween 20 (PBST) overnight and detected with iodine 125–labeled sheep anti-mouse IgG antibody (GE Healthcare, Little Chalfont Buckinghamshire, United Kingdom) diluted 1:1,000 in PBST to confirm the identity of his-tagged rα-La.

Molecular characterization of rα-La

Laser desorption mass spectra were obtained in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, United Kingdom; piCHEM R&D, Graz, Austria).

CD spectra were performed on a Jasco J-810 spectropolarimeter (JASCO Corp, Tokyo, Japan) fitted with a Jasco PTC-423S/L Peltier-type temperature control system. All measurements were performed in 1 mmol/L Tris (pH 8.5) or water. For certain experiments, 10 mmol/L ethyleneglycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) was added to the recombinant protein before CD analysis.

The secondary structure content of rα-La was calculated by using the secondary structure estimation program CDSSTR.[14]

Patients and biological materials

Sera were obtained from patients with CM allergy who had a positive case history of CM allergy, a positive skin prick test reaction to CM, and/or specific IgE to CM extract, as measured with the ImmunoCAP System (Phadia, Uppsala, Sweden). The group of patients with CM allergy is described in the Methods section and Table E1 of this article's Online Repository at www.jacionline.org.

Pasteurized CM containing 3.5% fat was bought at a local market (NÖM, Baden, Austria, batch 22 550 2:00) and purified natural α-La (nα-La) was purchased from Sigma-Aldrich (Vienna, Austria).

IgE reactivity testing and calcium depletion experiments

For more information, see the Methods section and Fig E2 of this article's Online Repository.

Synthesis of α-La–derived peptides and determination of surface-exposed amino acids in the peptide sequences

α-La–derived peptides, as displayed in Table E2 in this article's Online Repository at www.jacionline.org and Fig 1, *A*, were synthesized by using the Fmoc (9 fluorenylmethoxy carbonyl) strategy with (2-/1H-Benzotriazol-1-yl)1,1,3,3, tetramethyluronium hexafluorophosphate activation (0.1 mmol small-scale cycles) on an Applied Biosystems peptide synthesizer Model 433A (Foster City, Calif) and purified as previously reported.[15]

The coordinates of the known bovine α-La structure were retrieved from the Protein Data Bank (PDB-1F6S) to determine the extent of surface exposure of the amino acid residues in the peptides in the complete α-La.[16] The solvent-accessible surface areas were calculated with the program AREAIMOL[17,18] by rolling a probe sphere of 1.4 Å radius over the Van der Waals surface of the protein. The solvent-accessible surface is specified by the center of the probe sphere. In this way the solvent-accessible surface of the complete α-La, as well as the individual residues, were determined.

Rat basophil leukemia assays

For the assessment of the allergenic activity of rα-La and nα-La, rat basophil leukemia (RBL) cell mediator release assays were performed, as described previously.[4,19]

RESULTS

Expression in *E coli*, purification, and biochemical and structural characterization of rα-La

We obtained an α-La cDNA from which an amino acid sequence could be deduced that was identical to the α-La protein sequence deposited in the NIH database (http://www.ncbi.nlm.nih.gov, accession no. NP_776803). Fig 1, *A*, shows the alignment of the deduced α-La amino acid sequence with α-La amino acid sequences from different mammalian species. α-La contains an N-terminal leader sequence that is cleaved from the mature protein (Fig 1, *A*). A prediction of N-glycosylation sites revealed the presence of potential N-glycosylation sites in the α-La sequence (Fig 1, *A*, underlined: 45NDS, 71NIC). The 8 cysteine residues that form disulfide bridges are conserved throughout the species and have been boxed (blue) in Fig 1, *A*. Also, the domain forming the calcium-binding loop, residues 79K-88D, is highly conserved among the different species (Fig 1, *A*, yellow box). The amino acids responsible for the binding of Ca^{2+} have been printed in italics. They are identical in all but 2 sequences. In the rat and murine sequences, the aspartic acid shows a conservative exchange to a glutamic acid. Overall, there were strong similarities between the α-La amino acid sequences from cows to human subjects and rodents, ranging at approximately 75% identity.

The α-La cDNA coding for the mature protein and a C-terminal hexahistidine tag was expressed in *E coli*, and approximately 6 mg/L culture of the recombinant allergen was purified by means of nickel affinity chromatography to homogeneity (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org and Fig E1, *B*). The immunoblot shows the reactivity of this protein with an anti-hexahistidine antibody, and matrix-assisted laser desorption/ionization time-of-flight analysis of purified rα-La resulted in a mass peak of 15.1 kd, which corresponds to the molecular weight calculated from the sequence (ie, 15.14 kd, including the methionine and the C-terminal hexahistidine tag; see Fig E1, *B* and *C*).

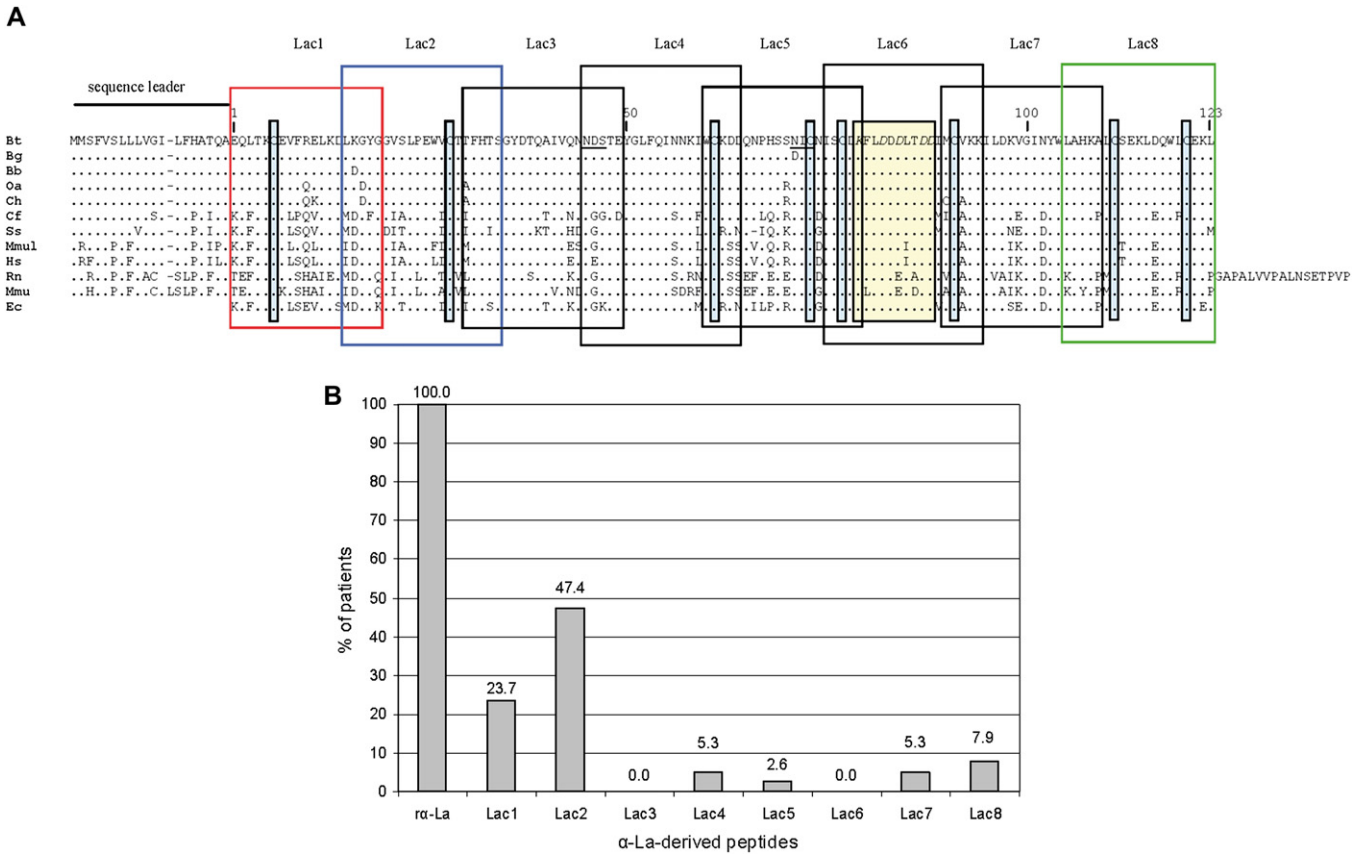

FIG 1. Alignment of α-La amino acid sequences from different species. The 8 overlapping synthetic peptides (Lac1-Lac8) are boxed. Cysteine residues are in blue, and residues that form the calcium-binding loop are in a yellow box. *Bb*, water buffalo (ABG78269); *Bg*, yak (AAF06793); *Bt*, cow (NP_776803); *Cf*, dog (BAA95930); *Ch*, goat (CAA28797); *Ec*, horse (LAHO); *Hs*, human (BAC06860); *Mmu*, mouse (AAA37208); *Mmul*, rhesus monkey (XP_001102116); *Oa*, sheep (CAA29665); *Rn*, rat (CAA25150); *Ss*, pig (AAA31060). *Dots* represent identical amino acids, and *dashes* represent gaps. The 2 potential N-glycosylation sites are underlined **(A)**. The prevalence *(y-axis)* of IgE recognition of 8 α-La–derived peptides tested with sera from those 38 patients with IgE reactivity to rα-La by means of microarray analysis is also shown **(B)**.

The far-UV CD spectrum of purified rα-La was recorded at 25°C (see Fig E1, *D*). It showed minima at 208 nm and 222 nm and a maximum at 193 nm, which are typical for α-helical structures. The CD spectrum of nα-La possessed a more pronounced minimum at 222 nm (see Fig E1, *E*). The CD spectrum of rα-La recorded at 95°C was shifted to the left (ie, lower wavelengths), indicating an increase in denatured protein, although with the protein remaining folded. The CD spectrum recorded after cooling to 25°C was almost identical to the spectrum obtained before heating, which suggests that the protein has almost completely folded back to its original conformation.

It has been observed that calcium-chelating agents, such as EGTA, can destabilize the structure of calcium-binding food allergens.[20] However, when we added 10 mmol/L EGTA to rα-La, the CD spectra at 25°C and 95°C were very similar to those recorded without EGTA (data not shown).

Secondary structure analysis performed with the program CDSSTR using the reference database 7 showed that rα-La consists of 8% α-helices, 32% β-sheets, 19% β-strands, and 40% random coils. The normalized root mean SD value of 0.022 confirmed a good fit between the calculated and experimentally derived spectra.

SDS-PAGE analysis demonstrates that rα-La forms oligomers, whereas the natural protein appears only as a monomer (see the Methods section and Fig E1, *F*, in this article's Online Repository). The latter was confirmed by means of gel filtration analysis showing the presence of oligomers in the rα-La preparation, whereas nα-La eluted only as monomer (see the Methods section and Fig E1, *G*, in this article's Online Repository).

rα-La is a major CM allergen according to IgE reactivity

In first experiments the specific IgE reactivity of rα-La was demonstrated by means of immunoblot analysis with serum from a patient with CM allergy. Serum IgE from patient A2 reacted specifically with rα-La, whereas a control serum from a nonallergic subject did not react (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org).

Next, we tested whether the binding of patients' IgE might be affected by depletion of protein-bound Ca^{2+} with EGTA. Depletion of Ca^{2+} from rα-La led to a reduction of the IgE reactivity with sera from 2 of 3 rα-La–reactive patients (see Fig E2, *B*).

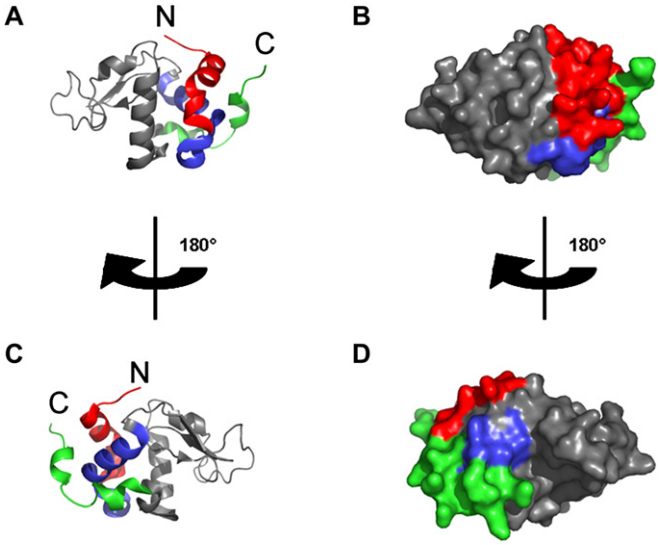

FIG 2. Ribbon (**A** and **C**) and molecular surface (**B** and **D**) presentations of α-La. The N- and C-terminus are indicated in Fig 2, *A* and *C*. Peptides Lac1, Lac2, and Lac8 are colored in red, blue, and green, respectively.

We then investigated whether rα-La contains the IgE epitopes of nα-La by using ELISA competition analysis. When tested with sera from 4 patients with allergy to α-La, we found that rα-La inhibited the binding to nα-La to the same extent as nα-La (see Fig E2, *C*).

From most of the patients with CM allergy, only small amounts of serum were available. We therefore determined the prevalence of IgE recognition for rα-La by using microarray technology. We found that 57.6% of the patients with CM allergy (n = 66) who showed IgE reactivity to CM reacted with rα-La (ie, 38 patients), and 75.8% reacted with nα-La (ie, 50 patients).

Identification of IgE epitopes of α-La

To identify IgE-reactive epitopes of α-La, we synthesized 8 peptides spanning the α-La sequence (see Table E2). The 8 peptides had a length of 19 to 20 amino acids and overlapped with each other in 5 amino acids. The 8 overlapping peptides were tested by using microarray analysis with sera from the 38 patients with IgE reactivity to rα-La (Fig 1, *B*). We found that 9 patients (ie, 23.7%) reacted to Lac1, 18 patients (ie, 47.4%) reacted to Lac2, 2 patients (ie, 5.3%) reacted to Lac4, 1 patient (ie, 2.6%) reacted to Lac5, 2 patients (ie, 5.3%) reacted to Lac7, and 3 patients (ie, 7.9%) reacted to Lac8 (Fig 1, *B*). In total, 22 of the 38 patients with IgE reactivity to rα-La reacted to at least 1 α-La–derived synthetic peptide.

Interestingly, when we tested sera from patients without IgE reactivity to complete α-La, we found 5 patients who reacted to α-La–derived peptides (ie, Lac1, Lac2, Lac4, and Lac8).

Fig 2 shows the position of the most frequently recognized peptides, Lac1 (red), Lac2 (blue), and Lac8 (green), in the ribbon presentation (Fig 2, *A* and *C*) and in the molecular surface presentation (Fig 2, *B* and *D*). Although peptides Lac1 and Lac2 are part of the N-terminal portion of α-La and peptide Lac8 is located at the C-terminal end of α-La, all 3 peptides appear in close vicinity on the surface of α-La and seem to define an IgE-reactive patch on the protein. Peptides Lac1 and Lac8 contain amino acids that are exposed on the surface of α-La and comprise a high percentage of the α-La surface (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org), whereas the other peptides, in particular Lac4, contain less surface-exposed amino acids. The surface calculations of the peptides (see Fig E3, *B*) show that Lac1 and Lac8 occupy 24.4% and 25.7% of the total α-La surface, respectively.

rα-La induces specific basophil degranulation

RBL cells loaded with serum IgE from patients with CM allergy (n = 59) were stimulated with CM, rα-La, and nα-La to assess the allergenic activity of the purified rα-La. Of the 59 tested patients, 78% had shown IgE reactivity to nα-La, and 59.3% exhibited IgE reactivity to rα-La. However, when tested for allergenic activity, basophil degranulation was observed for 11.9% with nα-La and for 23.7% with rα-La. For those patients whose sera induced basophil degranulation with both allergen preparations, similar magnitudes of degranulation were observed, ranging from 6.6% to 45% (median, 12.22%) for nα-La and 8.5% to 53.6% (median, 15.62%) for rα-La. The allergenic activity of nα-La and rα-La was specific because no degranulation was observed when cells were loaded with sera from non-allergic subjects (n = 10, see Fig E4 in this article's Online Repository at www.jacionline.org).

Association of CM-induced symptoms with IgE reactivity and basophil degranulation

Fig 3 shows the intensities of IgE reactivity and allergenic activity to CM, rα-La, and α-La–derived peptides for patients with CM allergy mounting IgE responses against rα-La. The patients had been grouped according to the type and magnitude of their symptoms. Patients were grouped into those without clinically relevant reactions (n = 2), those with oral allergy syndrome (n = 1), those with gastrointestinal symptoms (n = 3), those with gastrointestinal symptoms and other symptoms (urticaria, atopic dermatitis, eczema, asthma, and rhinoconjunctivitis; n = 5), those with skin symptoms (n = 5), those with skin and respiratory symptoms (n = 5); and those who had severe anaphylactic reactions (n = 7) on CM exposure.

We found a tendency for rα-La–specific IgE levels to be highest in the patients who had experienced systemic reactions on CM exposure followed by those patients with gastrointestinal and other symptoms or with skin and respiratory symptoms (Fig 3). IgE reactivities to α-La–derived synthetic peptides were found in each of the patient groups, except for the patients who had only skin and respiratory symptoms to CM (Fig 3).

The strongest induction of basophil release with rα-La was observed for sera from those patients who had experienced systemic reactions to CM and to a lesser degree for those with gastrointestinal and other symptoms, whereas almost no relevant basophil degranulation was found for patients without reactions or with only skin reactions to CM (Fig 3).

DISCUSSION

In this study we isolated a cDNA coding for the CM allergen α-La and characterized the recombinant allergen. Bovine α-La represents a calcium-binding protein containing a single calcium-binding domain and exhibits high sequence homology with α-La from several species, including human subjects. The high degree of sequence homology of α-La might explain the cross-reactivity

Symptoms	Patient	Age	IgE reactivity									Mediator release	
			CM	ra-La	Lac1	Lac2	Lac3	Lac4	Lac5	Lac6	Lac7	Lac8	CM	ra-La
No reaction	C1	16y	5998.5	817.3	-2.3	115.0	-6.0	10.0	-5.0	6.0	0.7	25.0	6.40	-1.00
	C38	2y	3959.3	406.0	58.3	145.0	92.0	111.3	3.0	7.0	6.3	12.3	5.96	1.09
OAS	A5	42y	270.7	153.0	245.0	83.7	22.0	15.7	1.3	-8.7	-9.3	4.3	3.32	-0.07
GI only	C6	10y	16071.8	2631.7	76.7	253.7	-135.7	-95.7	-71.7	-94.3	-38.3	63.7	20.15	12.29
	C44	7mo	1849.0	350.3	34.3	14.7	-7.3	19.3	-9.7	11.0	0.0	-16.3	nd	nd
	C50	3mo	11928.8	1515.7	44.7	44.7	3.7	11.0	6.7	4.7	9.3	14.0	8.83	2.75
GI + others	C3	13y	30760.3	2524.3	-155.0	264.7	-124.7	-111.3	6.7	-3.0	-5.7	2.7	41.51	3.50
	C4	13y	4326.7	1845.0	40.0	66.7	1.3	31.3	25.0	19.3	10.3	20.0	1.92	1.26
	C31	3y	48117.3	7277.3	372.0	1655.0	-392.7	-516.3	89.0	93.0	154.7	114.3	32.22	5.12
	C34	2y	35437.7	599.7	-267.0	1687.7	1.3	19.3	-6.0	32.7	19.7	111.7	55.70	9.87
	C43	9mo	2522.8	1244.3	23.0	35.0	-7.7	17.7	-6.3	3.7	2.0	6.7	16.03	13.10
Skin only	C33	2y	628.7	162.3	9.3	12.0	5.7	27.0	8.3	5.3	0.3	-6.3	-2.41	4.59
	C45	6mo	4345.5	549.0	15.0	26.7	-5.3	5.0	-8.0	2.0	-7.3	0.0	nd	nd
	C49	3mo	7792.3	790.3	79.7	175.7	8.3	45.7	-5.0	27.7	49.0	16.3	1.90	2.24
	C51	3mo	2129.0	469.7	478.3	383.7	-3.0	36.7	7.7	7.7	5.7	18.0	7.25	0.02
	C54	3mo	2444.3	181.7	32.3	35.0	-3.7	144.7	2.3	6.0	5.3	15.0	nd	nd
Skin + respiratory	C5	11y	24790.3	623.7	-209.7	13.3	-97.7	2.0	55.0	-24.3	-2.0	-6.7	6.97	-0.15
	C8	8y	13870.3	1838.0	-91.7	75.7	11.0	34.0	32.3	29.3	-3.3	62.7	7.20	0.39
	C9	7y	14460.3	152.7	41.3	53.7	19.3	57.7	47.7	39.0	38.0	38.0	16.10	-2.01
	C35	2y	5250.7	977.3	10.7	46.3	5.3	12.3	6.7	3.0	-12.0	0.7	2.11	0.73
	C40	1y	449.2	170.0	71.7	66.3	3.3	45.3	23.0	22.0	15.3	41.3	4.01	4.28
Systemic reactions	A2	64y	8535.7	10206.0	2377.7	2263.0	85.3	3834.7	-84.0	16.7	47.3	1080.3	64.00	53.57
	A8	22y	64927.8	16351.7	-233.0	732.3	25.7	-173.0	76.3	10.0	240.7	149.7	13.25	8.47
	C17	4y	46582.5	9896.7	-409.7	465.0	-97.0	-135.7	206.7	-73.0	-13.7	3.7	36.61	27.56
	C20	4y	23448.3	2271.3	967.3	175.0	-11.7	19.0	-18.0	14.0	12.3	9.0	8.75	6.71
	C27	3y	17137.0	2873.3	-8.0	277.0	-5.0	65.0	25.0	34.3	17.0	73.7	36.79	14.59
	C28	3y	3780.7	125.3	39.7	155.7	-2.3	16.3	5.3	15.0	34.7	15.0	14.05	1.63
	C42	9mo	42028.0	8449.3	32.0	127.0	-14.7	12.0	-1.3	8.0	2.3	25.7	19.37	16.65

Microarray (FI)
<125
125-350
350-4850
>4850

Mediator release
0 - 4.9%
≥5%

FIG 3. Association of CM-induced symptoms with IgE reactivity and basophil degranulation. Displayed are fluorescence intensities of IgE reactivities after subtraction of HSA values. Positive values are highlighted. For the mediator release, CM and rα-La were tested. Values greater than 5% of release were considered as positive and highlighted in gray. *nd*, Not done.

of IgE antibodies from patients with CM allergy, even including the human protein.[21,22] IgE cross-reactivities have also been reported for caseins from different mammalian species in human subjects allergic to CM.[23] We expressed α-La in *E coli* and demonstrated that the recombinant allergen represents a folded protein that exhibited a remarkable thermal stability. The high stability of the allergen might be explained by the presence of protein-bound calcium, which is also a feature of other calcium-binding allergens, including, in addition to respiratory allergens, the major fish allergen parvalbumin.[20] α-La and fish parvalbumin preserve their allergenic activity, even after boiling. For α-La, it has been shown that the allergen can interact with low-molecular-weight organic compounds, including phospholipids, which might also protect the allergen from digestion.[24] Accordingly, α-La represents a class I food allergen that can sensitize through the gastrointestinal tract.[25] The prevalences of IgE recognition reported for α-La show considerable variability. Wal et al[2] found IgE reactivity to α-La in 51% of patients with CM allergy (n = 92), whereas other authors reported recognition frequencies ranging from 6% to 100%.[3,8-10] Goldman et al[26] described a positive reaction to α-La in oral challenge tests in 53% of patients with CM allergy (n = 34).

We found that 57.6% recognized the recombinant protein and 75.8% recognized the natural protein. α-La thus represents a major CM allergen, although it does not bind all of the CM-specific IgE, which can be explained by the fact that patients with CM allergy often react to several different CM allergens.

However, when rα-La was compared with nα-La regarding allergenic activity, we found that the recombinant protein more often (ie, 23.7%) induced degranulation of basophils than nα-La (ie, 11.9%). This result was unexpected because nα-La had reacted more frequently with serum IgE than rα-La but might be explained by the presence of certain IgE epitopes on nα-La that exhibit low or no allergenic activity, as has been reported for hapten-like structures[27] or carbohydrate epitopes.[28,29] We therefore searched for the presence of carbohydrate epitopes on nα-La. Although IgE reactivity of certain sera to nα-La was sensitive to periodate treatment (data not shown), matrix-assisted laser desorption/ionization time-of-flight analysis demonstrated that the molecular mass of nα-La is 14,165 d, which corresponds well to the calculated mass of the protein without methionine (ie, 14,186 d) and the mass reported for nonglycosylated nα-La (ie, 14,178 d).[30] We therefore think that carbohydrate epitopes did not contribute to the differences in IgE reactivity between nα-La and rα-La. The analysis of nα-La and rα-La by means of SDS-PAGE under reducing and nonreducing conditions (see Fig E1, *F*), as well as gel filtration experiments (see Fig E1, *G*), revealed that nα-La contained only monomers, whereas rα-La contained also oligomers. This finding might explain the different folding patterns of the natural and recombinant proteins when analyzed by means of CD (see Fig E1, *D* and *E*) and the differences in IgE reactivity and allergenic activity.

We found that patients who had experienced systemic reactions on CM exposure contained higher rα-La–specific IgE antibody levels compared with those seen in patients who had experienced milder symptoms, which supports earlier observations that patients with more severe symptoms tend to have higher CM-specific IgE levels.[31-33] However, it seems impossible to unambiguously identify patients with systemic reactions to CM only on the basis of IgE reactivity. In this context several recent studies performed with highly purified allergen molecules have shown that IgE reactivity and allergenic activity do not always correlate.[34,35] Using the model of RBL cells allowed us to identify α-La-reactive patients with severe systemic reactions and those with gastrointestinal symptoms. Application of rα-La in RBL assays might therefore contribute to the improvement of *in vitro* diagnostic methods for the identification of patients with severe systemic allergic reactions to CM.

Two approaches were pursued to define IgE epitopes of α-La. First we studied whether α-La might contain conformational IgE epitopes by means of depletion of protein-bound calcium, which is similar to what has been done for other calcium-binding allergens.[20,36] We could indeed demonstrate that the apo-form (ie, calcium-depleted form) of rα-La showed a reduced IgE-binding capacity that might be explained by an alteration of the protein conformation caused by calcium depletion.[37] The existence of conformational epitopes in class I food allergens is quite unexpected but was lately described also for other class I food allergens, namely Cyp c 1,[20] Ara h 2,[38] and αS1-casein.[4] The presence of conformational IgE epitopes on certain stable food allergens might be explained by sensitization to intact undigested allergen through the gastrointestinal tract and possibly through the respiratory tract or skin.

In addition to conformational epitopes, we identified sequential epitopes in α-La by using 8 synthetic overlapping peptides spanning the α-La sequence. Interestingly, despite different localization within the α-La sequence, 2 N-terminal (ie, Lac1 [1E-G19] and Lac2 [15L-S34]) peptides and a C- terminal peptide (ie, Lac8 [105L-L123]) defined an IgE epitope–containing patch on the surface of α-La.

Our IgE epitope mapping data are in good agreement with other studies. Jarvinen et al[7] described 4 IgE epitopes ranging from amino acid 1E-K16, 13K-W26, 47S-K58, and 93K-N102 in the native bovine α-La. The study of Maynard et al[39] showed that sequence 17G-K58 and large tryptic peptides sharing this sequence were most strongly and frequently recognized. Adams et al[40] showed that the synthetic peptide ranging from 5K-A18 contains an IgE-binding epitope. In addition to the IgE-reactive peptides Lac1, Lac2, and Lac8, which formed the major IgE-reactive cluster, we found that patients exhibited IgE reactivity to Lac4 (45N-D64), Lac5 (60W-K79), and Lac7 (90M-A109) as well.

A similar clustering of IgE epitopes as described for Lac1, Lac 2, and Lac8 has been found for several other respiratory and food allergens, such as Bet v 2,[41] Phl p 5,[42] Phl p 1,[43] Phl p 2,[44] and Bos d 5,[45] and might be important for efficient cross-linking of IgE antibodies on effector cells and hence determination of the degree of allergenic activity of an allergen.

The knowledge of IgE epitopes might be of great relevance for the rational design of allergy vaccines because it allows one to construct hypoallergenic allergen derivatives[46] or peptide vaccines.[15,47,48] Allergy vaccines based on hypoallergenic allergen derivatives of respiratory allergens have already been tested in promising immunotherapy trials in human subjects and are currently being developed for important food allergens.[49,50]

The rα-La defined by us represents an important CM allergen that can be used for the diagnosis of patients with severe CM allergy. Based on the IgE epitope mapping data, it might be possible to develop new preventive and therapeutic strategies for CM allergy.

Clinical implications: rα-La can be used for the diagnosis and treatment of patients with severe allergic reactions to CM.

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METHODS

Patients

The group of patients with CM allergy consisted of persons from Austria (n = 9), France (n = 1), Germany (n = 33), Greece (n = 2), Italy (n = 2), and Spain (n = 19). Table E1 shows the available demographic, clinical, and serologic data for the adults (age, 22-70 years) and children (age, 1 month to 16 years) with IgE reactivity to rα-La. For the anonymous analysis of serum IgE antibodies, the permission of the Ethical Committee of the Medical University of Vienna was obtained.

IgE reactivity testing

For immunoblot analysis, 1 μg per gel slot of rα-La was separated by means of SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany).[E1,E2] For testing the IgE reactivity to calcium-depleted rα-La, aliquots of 1 μg of rα-La were dot blotted onto nitrocellulose membranes (Schleicher & Schuell). The nitrocellulose strips were blocked with PBST and exposed to sera from patients with CM allergy diluted 1:10 or 1:20 in PBST overnight at 4°C, and for calcium depletion experiments, incubation was performed with sera in the presence of 5 mmol/L EGTA (Sigma-Aldrich) or 0.5 mmol/L $CaCl_2$ (Merck, Darmstadt, Germany). Bound IgE antibodies were detected with iodine 125–labeled anti-human IgE antibodies (IBL, Hamburg, Germany) diluted 1:15 in PBST and visualized by means of autoradiography with Kodak XOMAT films with intensifying screens (Kodak, Heidelberg, Germany) at −80°C.

For measurement of IgE reactivities to microarrayed milk components, whole CM extract, rα-La, nα-La, α-La–derived peptides, and, for control purposes, HSA were spotted onto a capillary-flow membrane attached to an ordinary microscope glass slide, as previously described,[E3,E4] and incubated with 30 μL of patients' sera. rα-La, nα-La, and α-La–derived peptides were spotted in 3 spot replicates after each other in the flow direction, with 1 HSA spot in front of the first spot of each component. CM extract was spotted later in the flow in 6 spot replicates across the flow direction, with 1 HSA spot spotted in front of each CM spot. Bound IgE antibodies were detected with a fluorophore-conjugated anti-IgE antibody at a wavelength of 670 nm. All values were corrected for their proximate HSA values. Values exceeding 125 fluorescence intensities, the highest values obtained with sera from nonallergic subjects, were considered positive.

To compare the IgE-binding capacity of rα-La with nα-La, 5 μg of nα-La/mL in sodium carbonate buffer (pH 9.6) was coated onto ELISA plates (Nunc Maxisorb, Roskilde, Denmark) overnight at 4°C. Patients' sera were diluted 1:10 in Tris-buffered saline containing 0.5% vol/vol Tween 20 and preincubated overnight at 4°C with 10 μg/mL nα-La or rα-La or, for control purposes, with Tris-buffered saline containing 0.5% vol/vol Tween 20 (not inhibited), and the following steps were performed, as previously described.[E3]

Analysis of nα-La and rα-La by means of SDS-PAGE under reducing and nonreducing conditions and gel filtration

nα-La and rα-La were loaded under reducing (buffer containing 5% vol/vol 2-mercaptoethanol) and non-reducing conditions (buffer without 2-mercaptoethanol) onto SDS-PAGE gel and evaluated by means of Coomassie brilliant blue staining of the gels (Bio-Rad, Richmond, Calif).

A Superdex 200 5/150 GL column (GE Healthcare) was used for size exclusion experiments at a flow rate of 0.3 mL/min. The column was calibrated with a gel filtration standard (Bio-Rad). The column was equilibrated with 10 mmol/L Tris-HCl (pH 8.5) and 150 mmol/L NaCl, and later 20 μL of recombinant protein (1.3 mg/mL) and 5 μL of natural protein (10 mg/mL) were loaded. The eluted proteins were detected by means of absorbance at a wavelength of 280 nm, and results are presented in arbitrary units.

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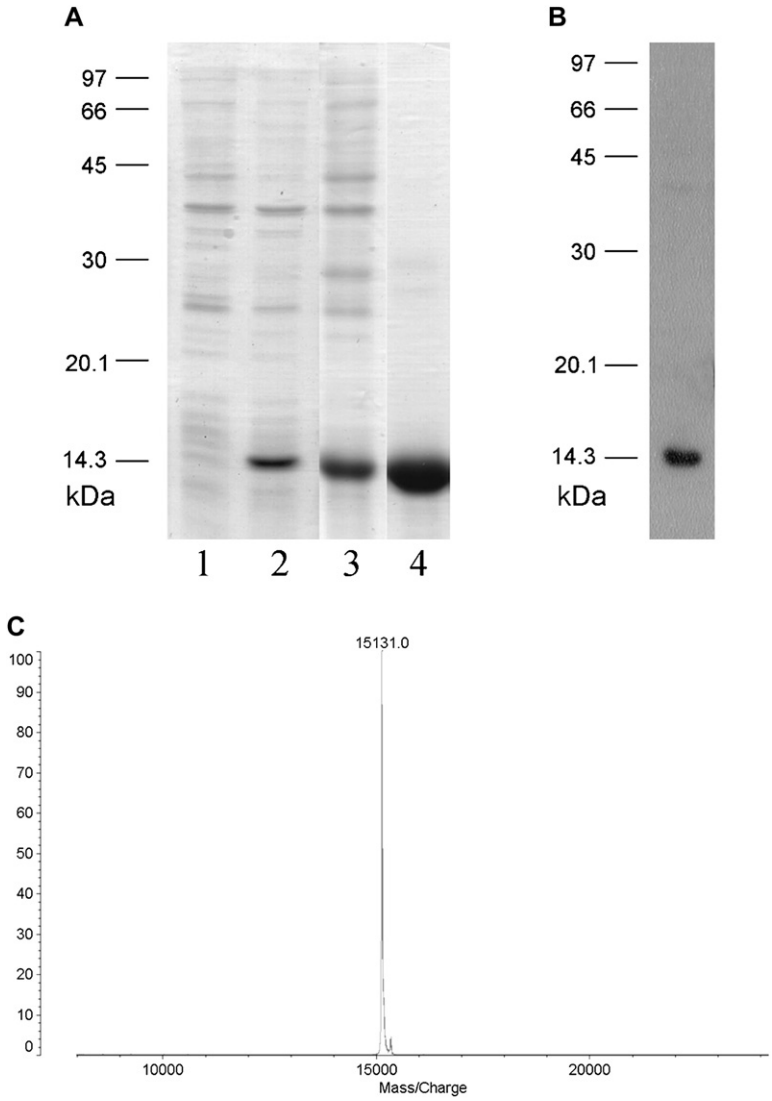
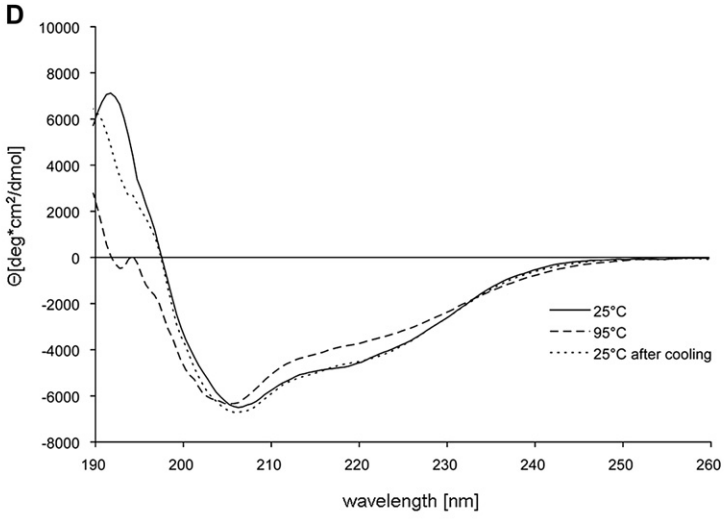


FIG E1. **A**, Expression and purification of rα-La. Coomassie brilliant blue–stained SDS-PAGE of a bacterial extract (*1*, without IPTG induction; *2*, after IPTG induction) and urea extraction of rα-La before *(3)* and after *(4)* purification through Ni-NTA agarose are shown. **B**, Western blot of rα-La incubated with an anti-his tag antibody. Molecular masses (in kilodaltons) are indicated on the left side. **C**, Mass spectrometric analysis of purified rα-La. The mass/charge ratio is shown on the *x-axis*, and the signal intensity is displayed on the *y-axis* as the percentage of the most intensive signal obtained in the investigated mass range. **D**, Structural characterization and thermal unfolding of rα-La. The figure shows the far-UV CD analysis of purified rα-La in 2 mmol/L Tris (pH 8.5). The mean residue ellipticities (Θ, *y-axis*) recorded at 25°C *(continuous line)*, 95°C *(dashed line)*, and 25°C after cooling *(dotted line)* at given wavelengths are shown *(x-axis)*. **E**, CD analysis of nα-La recorded at 25°C. **F**, SDS-PAGE analysis of nα-La *(lanes n)* and rα-La *(lanes r)* under reducing and non-reducing conditions. Molecular masses (in kilodaltons) are shown. **G**, Gel filtration performed with nα-La and rα-La. nα-La elutes as a single monomeric peak *(1)*. rα-La elutes as a monomeric peak *(2)* with a broad shoulder containing oligomeric species *(3)*. Apparent molecular masses were calculated by using linear regression of logarithm of molecular weight versus elution volumes *(x-axis)* derived from UV measurements at 280 nm (given in arbitrary units, *y-axis*).



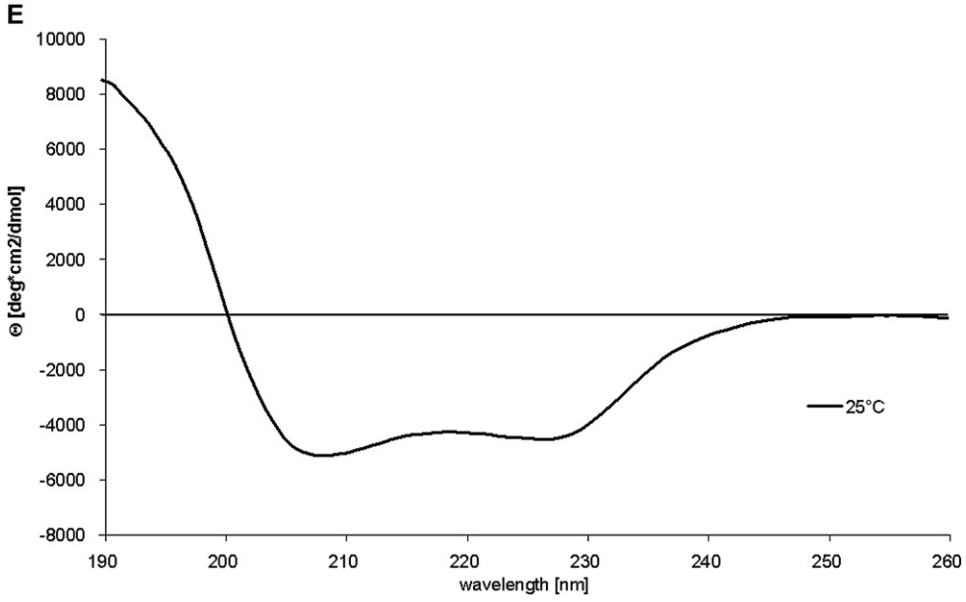
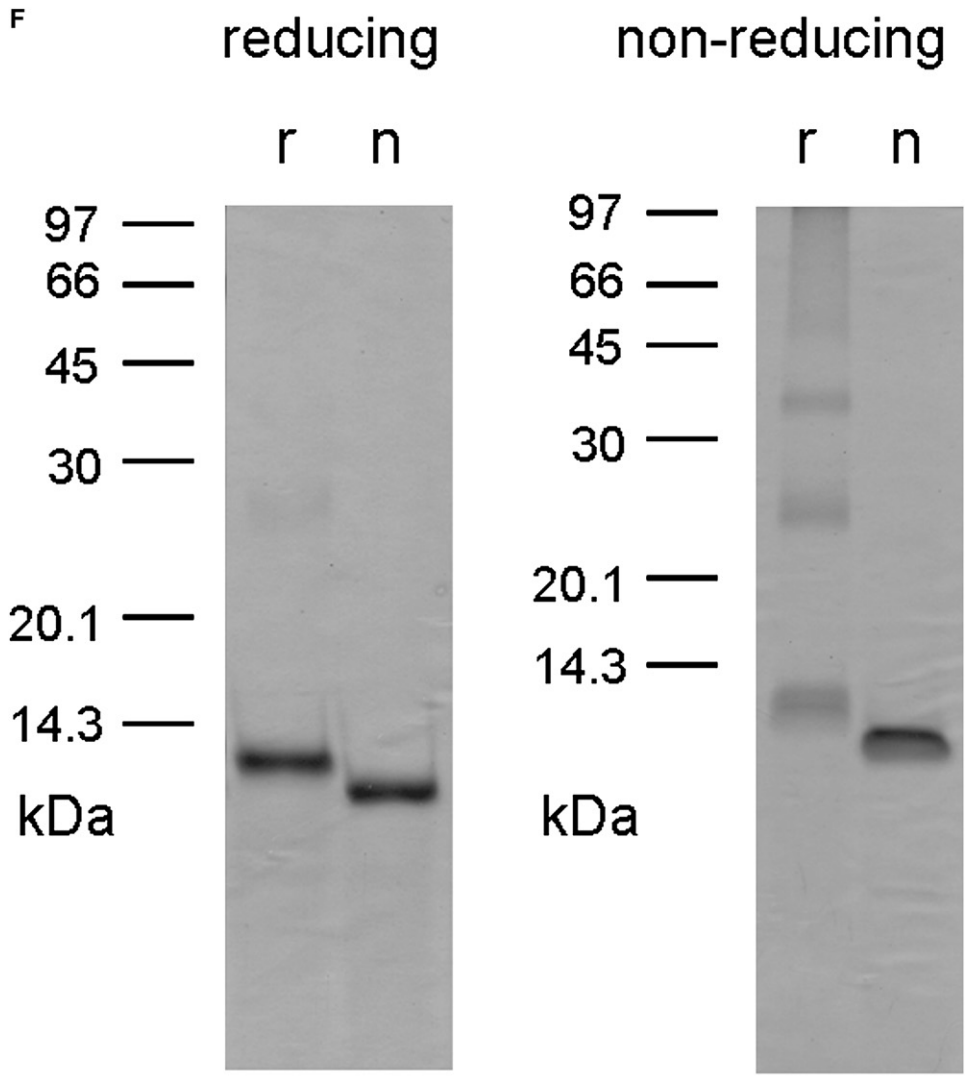


FIG E1. *(Continued).*



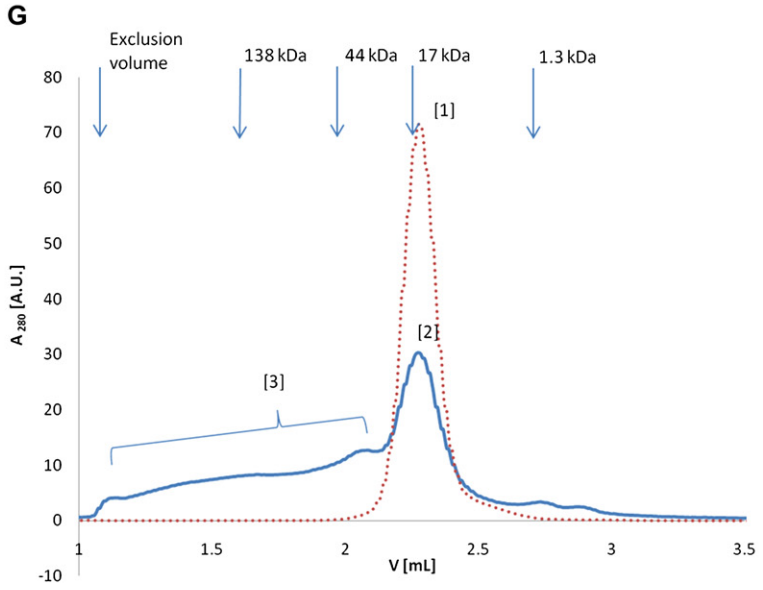


FIG E1. *(Continued).*

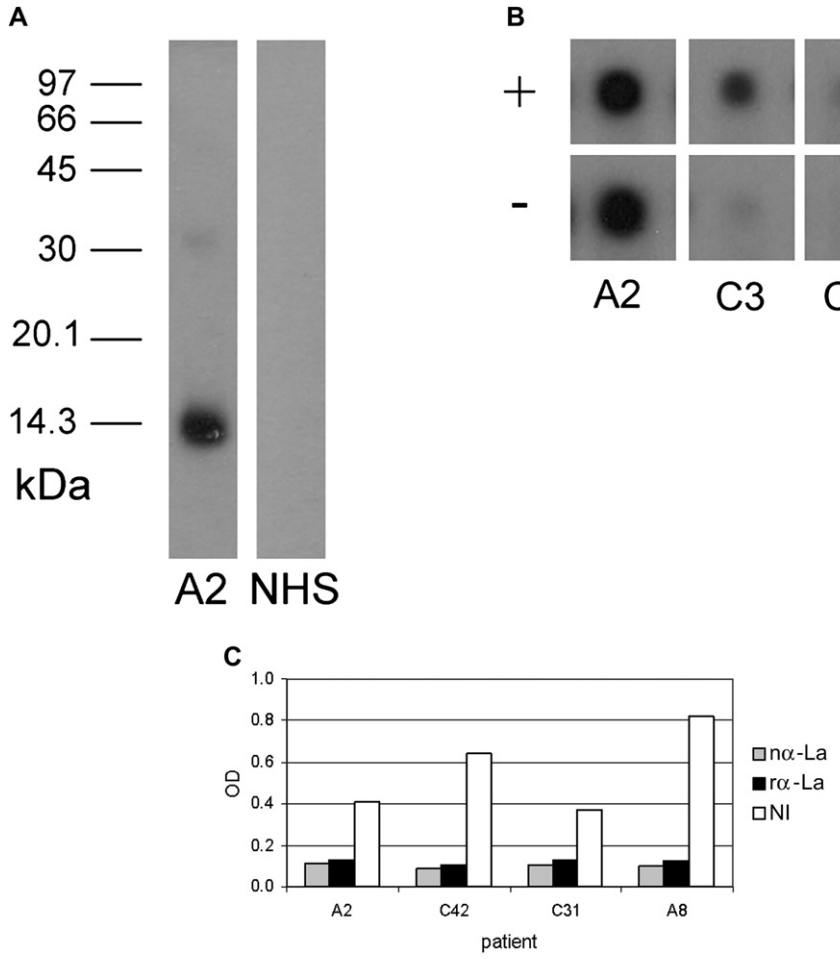


FIG E2. **A**, IgE reactivity of blotted rα-La with sera from a patient with CM allergy (patient A2) and from a non-allergic person *(NHS)*. **B**, Nitrocellulose-dotted rα-La was exposed to sera from 3 patients with CM allergy (patients A2, C3, and C34) and from a non-allergic subject *(NHS)* in the presence *(+)* or absence *(−)* of calcium. Bound IgE antibodies were detected with iodine 125–labeled anti-IgE antibodies and visualized by means of autoradiography. **C**, IgE reactivity to nα-La after preincubation of sera from 4 patients (patients A2, C42, C31, and A8) with nα-La or rα-La or with buffer (no inhibitor *[NI]*) is shown. IgE reactivity to ELISA plate–bound nα-La corresponds to optical density values *(y-axis)*.

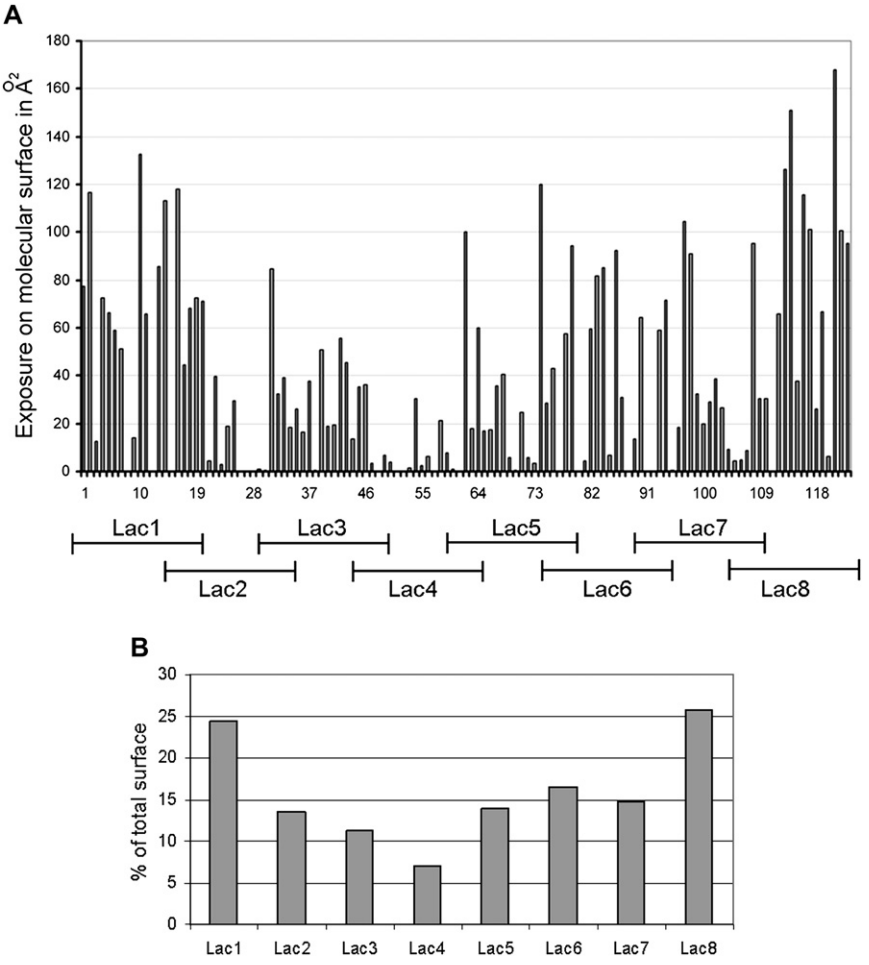


FIG E3. Contribution of amino acids (1-123) to the molecular surface in Å² **(A)** and percentages of total surface represented by peptides Lac1 to Lac8 **(B)**.

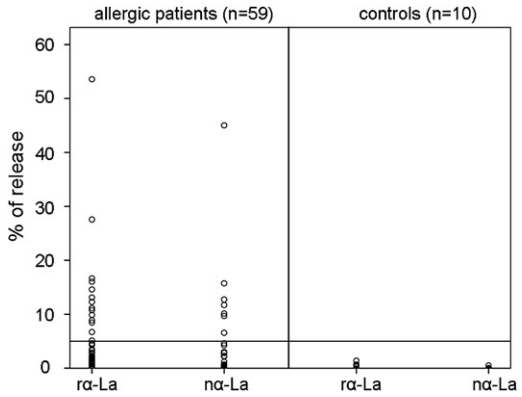


FIG E4. Comparison of basophil degranulation induced by nα-La and rα-La. The percentages of mediator release *(y-axis)* are displayed for allergic patients and non-allergic control subjects. The cutoff level at 5% is indicated with a *horizontal line*.

TABLE E1. Demographic, clinical, and serologic characterization of rα-La–positive adults (A2-A8; age, 22-64 years) and children (C1-C57; age, 3 months to 16 years)

Patient no.	Age	Sex	Country	Milk-related symptoms	Other allergies	Total IgE (kU/L)	CM (RAST class)
A2	64 y	F	F	U, Sys, GI	No	ND	6
A3	61 y	f	A	NK	Cat, WF, T, K, P	355	4
A5	42 y	f	A	OAS	Mite, cat	148	3
A8	22 y	f	A	Sys	HE, nuts, pets, PO	3,350	6
C1	16 y	f	G	NR	HE	433	3
C3	13 y	m	I	U, E, V, AS	PO, HE	909	6
C4	13 y	m	G	RC, V	HE	866	3
C5	11 y	f	I	U, AE, AS	*Candida*	ND	4
C6	10 y	f	G	Ap	ND	1,432	4
C8	8 y	m	G	E, U, AS	HE	2,200	4
C9	7 y	m	G	U, R, AS	HE	399	4
C11	6 y	NK	G	NK	NK	246	3
C17	4 y	f	G	Sys	NK	974	6
C19	4 y	m	G	NK	NK	1,894	3
C20	4 y	f	G	Sys	HE	489	5
C21	4 y	f	G	NK	NK	116	3
C24	3 y	f	G	NK	NK	125	3
C25	3 y	m	G	NK	NK	26	3
C27	3 y	m	G	Sys	NK	325	4
C28	3 y	f	G	Sys	NK	125	3
C29	3 y	f	G	NK	NK	201	3
C31	3 y	NK	Gr	U, GI	Beef, fish, Alt	2,000	5
C33	2 y	f	S	U (face)	HE	ND	3
C34	2 y	f	S	U, AD, V	HE, fish	ND	5
C35	2 y	m	A	AD, OB	Mite	134	3
C36	2 y	f	G	NK	NK	83.4	4
C37	2 y	m	G	NK	NK	193	3
C38	2 y	m	G	NR	NK	117	3
C40	1 y	f	A	AD, OB	HE, soja, nuts	217	3
C42	9 mo	NK	Gr	U, Sys	HE, W	1,066	6
C43	9 mo	f	S	U, GI	No	351	3
C44	7 mo	m	S	GI	No	32	3
C45	6 mo	m	S	U (face), AD	No	133	3
C49	3 mo	f	S	U	NK	1,292	3
C50	3 mo	f	S	GI	No	29	3
C51	3 mo	m	S	U, AE	No	51	3
C54	3 mo	f	S	U (face), AD	No	50	3
C57	NK	NK	G	NK	NK	1,129	5

A, Austria; *AD*, atopic dermatitis; *AE*, angioedema; *Alt*, *Alternaria*; *Ap*, abdominal pain; *AS*, asthma; *E*, eczema; *f*, female; *F*, France; *G*, Germany; *GI*, gastrointestinal symptoms; *Gr*, Greece; *HE*, hen's egg; *I*, Italy; *K*, kiwi; *m*, male; *ND*, not done; *NK*, not known; *NR*, no reaction; *OAS*, oral allergy syndrome; *OB*, obstructive bronchitis; *P*, pork; *PO*, pollen; *R*, redness; *RC*, rhinoconjunctivitis; *S*, Spain; *Sys*, systemic reaction; *T*, tomato; *U*, urticaria; *V*, vomiting; *W*, wheat; *WF*, wheat flour.

TABLE E2. Synthetic α-La-derived peptides

Peptide	Sequence	Length	pI	MW (d)
Lac1	EQLTKCEVFRELKDLKGYG	19 aa	6.3	2,256.60
Lac2	LKGYGGVSLPEWVCTTFHTS	20 aa	6.7	2,182.48
Lac3	TFHTSGYDTQAIVQNNDSTE	20 aa	4.3	2,228.27
Lac4	NDSTEYGLFQINNKIWCKDD	20 aa	4.4	2,403.60
Lac5	WCKDDQNPHSSNICNISCDK	20 aa	5.3	2,307.51
Lac6	ISCDKFLDDDLTDDIMCVKK	20 aa	4.1	2,317.67
Lac7	MCVKKILDKVGINYWLAHKA	20 aa	9.5	2,330.88
Lac8	LAHKALCSEKLDQWLCEKL	19 aa	6.7	2,228.65

aa, Amino acids; *Lac1-Lac8*, α-La–derived peptides 1 to 8; *MW*, molecular weight; *pI*, isoelectric point.