

## Original article

Identification of amino acids critical for IgE-binding to sequential epitopes of bovine  $\kappa$ -casein and the similarity of these epitopes to the corresponding human  $\kappa$ -casein sequence

**Background:** The delineation of allergenic (i.e. IgE-binding) epitopes in cow's milk proteins and the amino acids (AAs) critical for IgE-binding is necessary to understand better the structural properties of an allergen and to develop more efficacious immunotherapeutic reagents. Furthermore, this information may enable us to understand better cross-sensitivity between different allergens.

**Methods:** Eleven peptides, 10–14 AAs in length, representing the IgE-binding epitopes of  $\kappa$ -casein were synthesized on a derivatized cellulose membrane with single AA substitutions at each position. Membranes were incubated with pooled sera from 15 milk-allergic patients and individual sera from 10 of the patients included in the pool.

**Results:** For 10/11 allergenic peptides, one to five different single AA substitutions resulted in elimination of IgE-binding of pooled patient sera. Overall at least one mutated peptide could be found for these 10 IgE-binding sites that resulted in a reduction of IgE-binding in at least 80% of the patients who recognized the native protein. Furthermore, the IgE-binding region at AA104–112 on bovine  $\kappa$ -casein showed a high degree of similarity with the human  $\kappa$ -casein, respectively, including the AAs critical for IgE-binding.

**Conclusion:** This finding suggests that critical AAs should be assessed with both pooled and individual patient sera to account for the B-cell epitope heterogeneity between patients, with cow's milk allergy. In addition, we identified two potentially cross-reactive peptides between bovine and human caseins of unknown clinical relevance.

**N. Han\*, K. M. Järvinen\*, R. R. Cocco, P. J. Busse, H. A. Sampson, K. Beyer**

Division of Pediatric Allergy & Immunology, Jaffe Institute for Food Allergy, The Mount Sinai School of Medicine, New York, NY, USA

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Kirsi M. Jarvinen, MD, PhD  
Department of Pediatrics  
The Mount Sinai School of Medicine  
PO Box 1198  
One Gustave L. Levy Place  
New York  
NY 10029-6574  
USA

\*Authors should be considered as first author.

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In industrialized countries, cow's milk is typically one of the first foreign proteins ingested by an infant. Cow's milk allergy (CMA) is a common disease in early childhood affecting up to 2.5% of children (1). Resolution of CMA occurs in about 80% of affected children, but it remains a significant problem in a subgroup of older children, adolescents and adults. To date, the only treatment for CMA is avoidance of milk protein, which is very difficult because of its ubiquitous use in the western food supply. Therefore, immunotherapeutic reagents are being developed for foods using 'engineered' recombinant proteins that possess decreased allergenicity, but retain their capability of activating milk-specific T cells (2). The IgE-binding regions of all major cow's milk allergens (3–6) and the amino acids (AAs) critical for IgE-binding to epitopes of  $\alpha_{s1}$ -casein have been delineated (7).

Previous reports on mutational analysis of other food allergens, such as the peanut allergens Ara h1, Ara h2 and

Ara h3, showed that single AA substitutions within each IgE-binding epitope led to elimination or marked reduction of IgE-binding by antibodies within pooled sera from peanut-allergic patients (8–10). Similar findings were reported with Jug r 1, an English walnut major allergen (11) and with  $\omega$ -5 gliadin (12). However, studies of the major shrimp allergen, Pen a1, found that more than two AA substitutions were generally required to abolish IgE-binding to this protein (13, 14). In our previous studies of  $\alpha_{s1}$ -casein, we found that a single or double AA substitution in each allergenic epitope eliminated IgE-binding by pooled sera derived from children with CMA. However, when sera from individual patients were tested against these mutated epitopes, IgE-binding was not necessarily abolished and a heterogeneous pattern of critical AAs was observed between different patients (7).

Milk contains caseins and whey proteins, accounting for 80% and 20% of total protein, respectively (15).  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -caseins as well as the whey proteins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin are the most important allergens. Characteristically, the IgE-binding regions of

Abbreviations: AA, amino acid; CMA, cow's milk allergy; OD, optical density; PBS, phosphate buffer saline.

these milk proteins are multiple and spread throughout the protein (3–6, 16). Caseins have common structural characteristics, with no rigid tertiary structure but a random coil conformation that is stabilized by hydrophobic interactions (16). Caseins are largely heat-resistant, but are susceptible to hydrolysis by dietary enzymes. Based on these characteristics, it may be inferred that the major IgE-binding epitopes in caseins are mainly sequential.  $\kappa$ -casein, a low molecular weight allergen with 169 AAs, comprises 10% of total cow's milk protein. Most milk-allergic patients have a strong humoral and cellular response to this protein (17). We previously identified 11 immunodominant IgE-binding regions in  $\kappa$ -casein (4).

Sensitization to multiple cow's milk proteins is seen in 74% of patients with CMA (16). It has been hypothesized that this may be caused by a cross-sensitization to common epitopes that might exist among caseins. In addition,  $\kappa$ -casein, as well as  $\alpha$ -lactalbumin and  $\beta$ -casein, are present in human milk and share about 50%, 78% and 50% sequence homology with their bovine counterpart. This has led to the suggestion that cross-reactivity between conserved regions of bovine and human caseins exists (18).

In the present study, we identified the AAs critical for IgE-binding to  $\kappa$ -casein and compared the patterns of critical AA recognition between individual patients. In addition, we compared the degree of sequence homology and presence of critical AAs for IgE-binding between bovine and human  $\kappa$ -casein.

## Material and methods

### Patient population

Sera from 15 patients with documented IgE-mediated cow's milk hypersensitivity (median age at the time of collection of sera was 8.5 years; range 2–18 years) were used to identify the AAs essential for IgE-binding to  $\kappa$ -casein. Milk-specific IgE antibodies ranged from 25 to >100 kU/l (median >100 kU/l) as measured by the CAP System FEIA™ (Phadia Diagnostics, Uppsala, Sweden). Based on the clinical history of the children, eight had atopic dermatitis, three had gastrointestinal symptoms (vomiting and abdominal pain) and four presented with both. Three children also wheezed following milk ingestion. In eight children, diagnosis was confirmed by double-blind, placebo-controlled food challenges and in three by an open challenge. Four children had a history of anaphylaxis to an isolated ingestion of milk protein and therefore were not challenged. Informed consent was obtained from the patients and their parents and the study was approved by the Mount Sinai School of Medicine Institutional Review Board.

### Peptide synthesis

To determine the AAs essential for IgE-binding, we synthesized 11 native peptides, 10–14 AAs in length, representing IgE-binding regions previously identified (4): epitopes no. 1: AA11–24, no. 2: AA17–26, no. 3: 23–36, no. 4: AA29–42, no. 5: AA53–66, no. 6: AA67–78, no. 7: AA97–108, no. 8: AA101–114, no. 9: AA111–124, no. 10: AA137–148 and no. 11: AA153–166. In addition,

mutated peptides with single AA substitutions at each residue position were synthesized. An alanine was substituted for each AA residue in turn, or when alanine was present in the native peptide, glycine was used for the substitution. These peptides were then probed with pooled sera and 10 randomly selected sera from individuals comprising the serum pool to determine the effect of individual AAs on binding of milk-specific IgE in individual subjects.

Individual peptides were synthesized on a cellulose membrane (SPOTs™ membrane; Sigma Genosys, Woodlands, TX, USA) containing free hydroxyl groups using Fmoc-AAs according to the manufacturer's instructions. Cycles of coupling, blocking and de-protection were repeated until the peptides of the desired length were synthesized. Peptide synthesis reactions were monitored by bromophenol blue colour reactions during certain steps of the synthesis. Membranes containing synthesized peptides were either probed immediately with serum IgE or stored at –20°C until needed.

### IgE-binding assays

Membranes containing  $\kappa$ -casein peptides were blocked overnight at room temperature with 1.5% human serum albumin in phosphate buffer saline (PBS) containing 0.01% Tween 20 and 2% human serum from a nonallergic individual. The serum pool (comprised sera from 15 milk allergic patients) and sera from 10 individual patients (whose sera were used to generate the pool) were diluted 1 : 50 and incubated with the membrane-bound peptides overnight in a cold room (4°C). Serum from a nonallergic donor was used to check for nonspecific binding. Following overnight incubation, membranes were washed three times with PBS-Tween and then incubated for 3 h with biotin-labelled goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Membranes were again washed with PBS-T and then peroxidase streptavidin conjugate (Kirkegaard & Perry Laboratories) was added. The membranes were developed with a chemiluminescent-avidin-horseradish peroxidase detection system (Amersham, Arlington Heights, IL, USA), and exposed on X-ray film.

After developing the X-ray film, the optical density (OD) of each individual peptide spot was measured by reflection densitometry. The OD of each peptide spot was recorded as the difference between the OD of the peptide spot and background. The OD of the native and its mutated variants were compared to determine the per cent inhibition of IgE-binding resulting from the substitution of each AA residue.

### Comparison of sequences bovine $\kappa$ -casein and their human counterpart

Using Vector NTI Software (Invitrogen, Carlsbad, CA, USA), InforMax sequence homologies between bovine and human  $\kappa$ -caseins were compared considering the known IgE-binding regions in bovine  $\kappa$ -casein and their AAs critical for IgE-binding.

## Results

### Mutational analysis of $\kappa$ -casein

Eleven native peptides representing the IgE-binding regions of  $\kappa$ -casein and their mutated homologues with AA substitutions at each position were used to identify critical AAs for IgE-binding. Peptides were probed with pooled sera and 10 individual patients sera, randomly

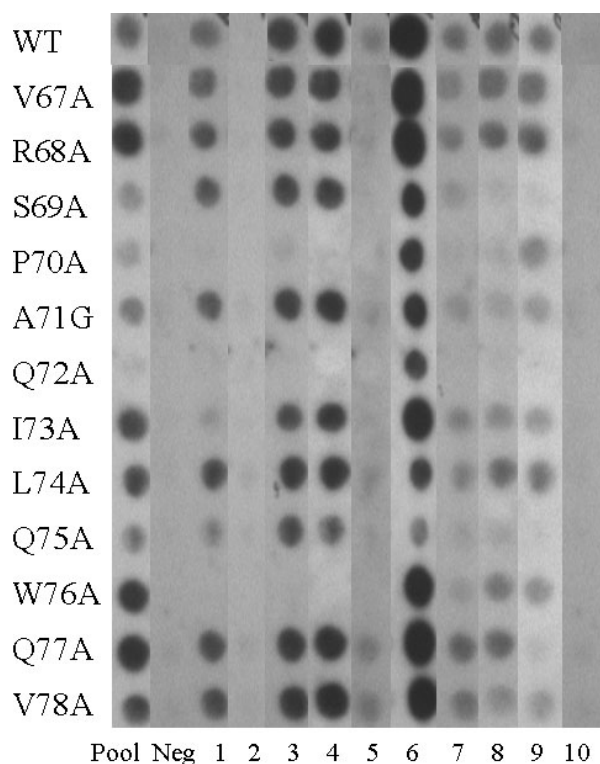


**Figure 1.** An example of the SPOTs membrane showing one of the synthesized wild type (WT) peptides (AA67–78) of  $\kappa$ -casein and the corresponding mutated peptides with single amino acid (AA) substitutions. Shown is the IgE-labelling with pooled sera from 15 children and 10 individual patients from the pool with cow's milk allergy.

selected from the patients used to generate the pool. Figure 1 shows an example of IgE-binding to the epitope no. 6 (AA 67–78). For this epitope, individual patients showed a distinct pattern of critical AAs. For example, a change of AA at position Q72A resulted in loss of binding in all patients except patient no. 6 whereas a change in AA at position W76A resulted in loss of binding only in patients no. 2, 3, 4 and 5, but not 6, 8 and 9. Patient no. 6 did not show a significant decrease of IgE-binding to any of the synthesized peptides.

Figure 2 illustrates IgE-binding to all the native and mutated peptides by pooled and individual patient's serum. Only the mutated peptides that resulted in a loss or a marked decrease ( $\leq 25\%$  of IgE-binding remaining when compared with the wild type peptide or  $OD \leq 0.25$ ) in IgE-binding in the pooled sera and sera from the majority of individual patients are depicted. For epitope no. 1, pooled sera showed a loss of binding to four mutated peptides, whereas only peptide F17A (shown in bold italics) showed a loss of binding by serum antibodies from all individual patients who recognized the native peptide. Three other mutations, F18A in epitope no. 2, Y35A in epitope no. 4 and N160A in epitope no. 11, resulted in a loss of binding in all patients. In contrast, in

the remaining 7 epitopes, no single AA substitution at the same residue position resulted in a loss of IgE-binding by all individual patients' sera. However, in most patients at least one mutation at a different position resulted in decreased binding by patient IgE antibodies. Previous studies on  $\alpha_{s1}$ -casein have shown that double or triple substitutions of these individual AA ultimately resulted in abrogation of IgE-binding in all patients. Interestingly, some patients (patient no. 2 with AA137–148 or no. 4 in AA53–66), show a significant increase in IgE-binding after the introduction of peptide mutations compared with the wild type. This phenomenon has been previously reported (6, 14), and may be caused by a particular change in the AA sequence transforming the peptide into a more allergenic epitope for certain individuals. In total, 13 AAs that are critical for IgE-binding have been identified in  $\kappa$ -casein. Those substitutions that gave the best overall loss/decrease of binding by pooled sera and individual patient's serum for each epitope are shown in bold italics in Fig. 2. Five (38%) of these 13 AAs are hydrophobic. Tyrosine and phenylalanine were the most common critical AAs (23% and 23%, respectively). The greatest decrease in binding occurred with point mutations in the centre of the IgE-binding region.

Most of the single AA substitutions resulted in no marked decrease (i.e.  $\geq 25\%$  of IgE-binding remaining when compared with the wild type peptide or  $OD \geq 0.25$ ) in IgE-binding by the majority of the individual patients' sera. These peptides are shown in Table 1 and represent the rest of the mutated peptides that are not shown in Fig. 2. As shown, most of the mutated peptides resulted in no change in IgE-binding by the majority of the patients, although in some individuals either abrogation, decreased or even increased IgE-binding was seen, when compared with the IgE-binding to the wild type peptide.

#### Comparison of IgE-binding epitopes and critical AAs of bovine milk proteins and human milk caseins

About 50% of the AA sequence of bovine  $\kappa$ -casein is similar to its human counterpart. Therefore, the degree of homology between IgE-binding regions on this allergen and the corresponding areas on its human counterpart were investigated based upon the AAs identified as critical for IgE-binding in the present study. Two areas were identified that possessed a high degree of homology between the human and the bovine protein (Fig. 3). The first area, AA104–112 in bovine  $\kappa$ -casein, which is a part of the larger IgE-binding area, is comprised of nine core AAs and includes identical AAs in the critical positions for IgE-binding. The second IgE-binding area, AAs 111–124, which is also a part of a larger IgE-binding area, has 11/14 AA residues identical to the human peptide. In contrast to the first epitope described, the critical AAs for IgE-binding by the majority of patients is glutamic acid (E), which differs from the human protein.

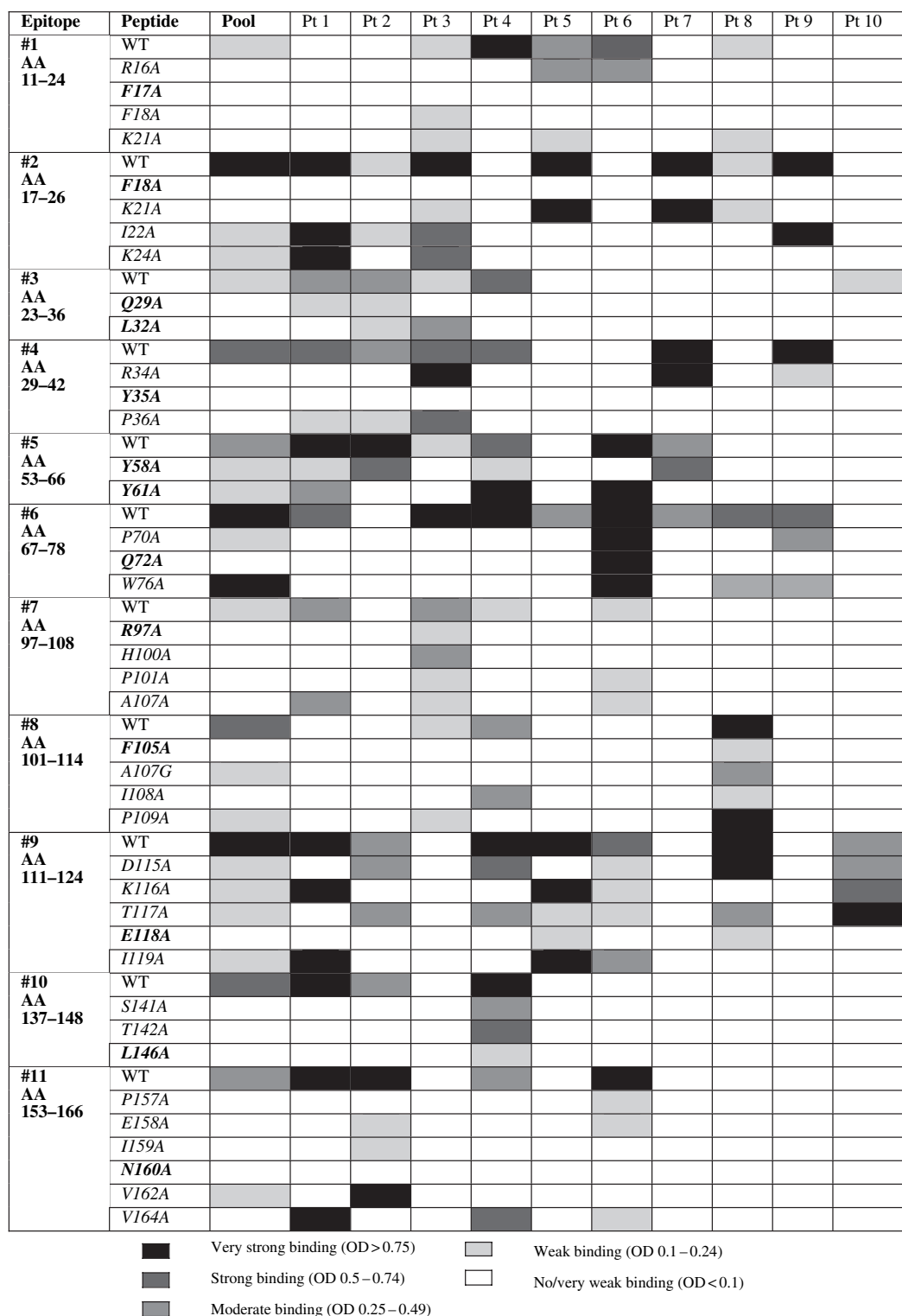


Figure 2. Shown is the IgE-binding to the wild type (WT) and mutated peptides of  $\kappa$ -casein by pooled and individual patient's sera. Only the mutated peptides that resulted in a loss or a marked decrease ( $\leq 25\%$  of the IgE-binding remaining when compared with wild type peptide or OD  $\leq 0.25$ ) in IgE-binding in the pooled sera and sera from the majority of individual patients are presented. The binding of IgE antibodies to each peptide was measured by optical densitometry (OD) and is expressed as a colour-coded system based on the strength of binding. The AA substitutions shown in bold italics are highlighting the critical amino acid (AA) for IgE-binding that gave the best overall loss/decrease of binding by pooled sera and individual patients for each epitope.

Table 1. The mutated peptides of  $\kappa$ -casein that resulted in no significant decrease (i.e.  $\geq 25\%$  of IgE-binding remaining when compared with the wild type peptide, or OD  $\geq 0.25$ ) by the majority of the patients

| Epitope no. | Peptide | No change | Abrog | Decr | Incr | Epitope no. | Peptide | No change | Abrog | Decr | Incr |
|-------------|---------|-----------|-------|------|------|-------------|---------|-----------|-------|------|------|
| <b>1</b>    | C11A    | 6         |       |      | 4    | <b>6</b>    | V67A    | 9         | 1     |      |      |
|             | E12A    | 8         |       | 1    | 1    |             | R68A    | 8         |       | 1    | 1    |
|             | K13A    | 7         |       | 3    |      |             | S69A    | 5         | 3     | 1    | 1    |
|             | D14A    | 5         |       | 1    | 4    |             | A71G    | 5         |       | 4    | 1    |
|             | E15A    | 7         |       |      | 3    |             | I73A    | 6         | 1     | 3    |      |
|             | S19A    | 9         |       |      | 1    |             | L74A    | 8         |       | 1    | 1    |
|             | D20A    | 8         |       | 1    | 1    |             | Q75A    | 4         | 2     | 4    |      |
|             | I22A    | 6         |       | 1    | 3    |             | Q77A    | 7         | 1     |      | 2    |
|             | A23G    | 6         |       | 2    | 2    |             | V78A    | 7         |       | 2    | 1    |
|             | K24A    | 7         | 3     |      |      | <b>7</b>    | H98A    | 7         | 1     | 1    | 1    |
| <b>2</b>    | F17A    | 8         | 2     |      |      |             | P99A    | 8         |       | 1    | 1    |
|             | S19A    | 7         | 2     | 1    |      |             | H102A   | 6         | 1     |      | 3    |
|             | D20A    | 5         | 1     | 2    | 2    |             | L103A   | 6         | 1     |      | 3    |
|             | A23G    | 7         |       | 3    |      |             | S104A   | 7         |       | 1    | 2    |
|             | Y25A    | 4         | 1     | 4    | 1    |             | F105A   | 8         | 2     |      |      |
|             | I26A    | 9         |       | 1    |      |             | M106A   | 10        |       |      |      |
|             | A23G    | 7         |       | 1    | 2    |             | I108A   | 7         |       | 2    | 1    |
|             | K24A    | 9         |       | 1    |      | <b>8</b>    | P101A   | 9         |       | 1    |      |
|             | Y25A    | 7         |       | 2    | 1    |             | H102A   | 9         |       | 1    |      |
|             | I26A    | 5         |       |      | 5    |             | L103A   | 8         |       | 1    | 1    |
| <b>3</b>    | P27A    | 6         | 2     | 1    | 1    |             | S104A   | 9         |       | 1    |      |
|             | I28A    | 6         | 2     | 2    |      |             | M106A   | 9         |       |      | 1    |
|             | Y30A    | 6         | 1     | 3    |      |             | P110A   | 8         |       | 1    | 1    |
|             | V31A    | 6         | 1     | 3    |      |             | K111A   | 7         | 1     | 1    | 1    |
|             | S33A    | 6         | 1     | 2    | 1    |             | K112A   | 8         | 1     |      | 1    |
|             | R34A    | 7         | 1     | 1    | 1    |             | N113A   | 6         |       |      | 4    |
|             | Y35A    | 7         |       | 2    | 1    |             | Q114A   | 9         |       |      | 1    |
|             | P36A    | 6         |       | 1    | 3    | <b>9</b>    | K111A   | 8         |       |      | 2    |
|             | Q29A    | 5         |       | 4    | 1    |             | K112A   | 7         |       | 1    | 2    |
| <b>4</b>    | Y30A    | 7         |       | 2    | 1    |             | N113A   | 8         |       |      | 2    |
|             | V31A    | 6         |       | 2    | 2    |             | Q114A   | 7         |       | 2    | 1    |
|             | L32A    | 8         |       |      | 2    |             | P120A   | 5         | 1     | 4    |      |
|             | S33A    | 7         |       | 2    | 1    |             | T121A   | 5         | 2     | 3    |      |
|             | S37A    | 5         |       | 4    | 1    |             | I122A   | 5         | 4     | 1    |      |
|             | Y38A    | 5         | 3     | 3    |      |             | N123A   | 6         | 2     | 2    |      |
|             | G39A    | 4         | 2     | 3    | 2    |             | T124A   | 5         | 1     | 4    |      |
|             | L40A    | 5         |       | 4    | 1    | <b>10</b>   | E137A   | 8         | 1     | 1    |      |
|             | N41A    | 5         | 2     | 1    | 2    |             | A138G   | 8         | 1     | 1    |      |
|             | Y42A    | 5         |       | 3    | 2    |             | V139A   | 8         |       | 2    |      |
| <b>5</b>    | N53A    | 9         |       |      | 1    |             | E140A   | 8         | 2     |      |      |
|             | Q54A    | 7         |       | 1    | 2    |             | V143A   | 9         |       | 1    |      |
|             | F55A    | 4         | 1     | 3    | 2    |             | A144G   | 8         | 1     |      | 1    |
|             | L56A    | 5         | 2     | 3    |      |             | T145A   | 8         |       | 2    |      |
|             | P57A    | 6         | 2     | 1    | 1    |             | E147A   | 9         |       | 1    |      |
|             | P59A    | 7         | 2     |      | 1    |             | D148A   | 8         |       | 2    |      |
|             | Y60A    | 5         | 3     | 2    |      | <b>11</b>   | I153A   | 10        |       |      |      |
|             | A62G    | 9         |       | 1    |      |             | E154A   | 9         |       |      | 1    |
|             | K63A    | 5         | 3     | 1    | 1    |             | S155A   | 10        |       |      |      |
|             | P64A    | 7         |       | 2    | 1    |             | P156A   | 9         |       |      | 1    |
|             | A65G    | 7         |       | 1    | 2    |             | Q163A   | 8         |       | 1    | 1    |
|             | A66G    | 5         |       | 2    | 3    |             | T165A   | 7         |       | 2    | 1    |
|             |         |           |       |      |      |             | S166A   | 6         |       | 4    |      |

Total number of patients (10), who showed no change, abrogation (Abrog) of binding ( $\leq 25\%$ ), decrease (Decr; 25–75%) or increase (incr;  $\geq 125\%$ ) of IgE-binding when compared with the wild type peptide.

## Discussion

In the present study, critical AAs necessary for IgE-binding in bovine  $\kappa$ -casein were identified using pooled

and individual patient sera. This information is necessary to engineer recombinant proteins with decreased allergenicity for use in immunotherapeutic reagents for reversing CMA. Furthermore, knowledge of the AA

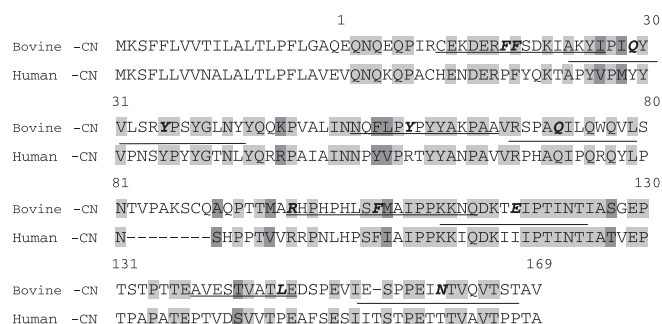


**Figure 3.** Shown are amino acid (AA) sequences of precursors of bovine and human  $\kappa$ -caseins. Identical AAs are shadowed with light grey and similar ones with dark grey. IgE-binding peptides of bovine  $\kappa$ -casein are indicated by a solid line, with the AA critical for IgE binding by the majority of sensitized patients shown in bold italics.

positions that are not as critical for IgE-binding may help in developing strategies to silence epitopes on other allergens. Interestingly, a heterogeneous pattern of critical AAs was found between individual patients that was not apparent when screening with pooled sera. This could present a significant problem for future engineering of proteins if only pooled sera are utilized when identifying the critical AAs for IgE antibody binding. ‘Engineered’ modified peptides identified with pooled sera may retain significant IgE-binding capacity in some individual patients, and may therefore not be suitable for immunotherapy for all patients. However, Li et al. (19) reported that treatment of peanut-allergic mice with modified peanut proteins, identified by mutational analysis using pooled sera from peanut-allergic individuals, effectively desensitized the mice against peanut-induced anaphylaxis with no adverse effects. Nevertheless, our results with milk proteins suggest that for future immunotherapeutic interventions with modified recombinant proteins, critical AAs should be determined with individual patient sera to account for the B-cell epitope heterogeneity found among patients with CMA.

The location and specific AAs critical for IgE-binding in  $\kappa$ -casein appeared consistent with our previous findings on  $\alpha_{s1}$ -casein (7) and those in a study of tropomyosin (14). According to these reports, substitutions of AAs in the central portion of the epitope were most likely to induce a loss of antibody binding. Moreover, hydrophobic residues comprised the most common type of critical AA. Sequential epitopes are commonly found in hydrophobic portions of the protein and are inaccessible to antibodies in the native conformation. However they may become accessible after denaturation and hydrolysis of the protein in the digestive tract (16). Some of these sequential epitopes have been associated with persistence of CMA (20, 21). For  $\kappa$ -casein, the presence of specific IgE antibodies against three epitopes (AA 13–22, 115–124

and 155–164) has been found only in patients with long lasting (beyond 7 years of age) CMA. For immunotherapeutic purposes aimed at treating patients with potentially life-long CMA, engineering of these epitopes appears to be critical. For all these epitopes, single point mutations resulted in a loss or significant decrease in IgE-binding by the pooled sera and all 10 patients' individual sera. The epitopes for which similar decreases of binding were not achieved by single AA substitutions were associated with persistence of CMA.

One limitation of this study is that it uses a method, which considers each peptide separately outside its chemical environment and outside any interaction with the whole protein molecule and with other proteins/constituents of whole milk. It also does not consider the effect that post-translational modifications may have on the IgE-binding capacity. Experiments with the whole protein would add valuable information to confirm the power of particular mutations in reducing IgE-binding to the whole molecule. Nonetheless, it has been shown that the treatment of peanut-allergic mice with modified peanut proteins, identified by mutational analysis of IgE-binding peptides (but not the whole protein) effectively desensitized mice against peanut-induced anaphylaxis (19). This suggests that such experiments with the whole protein may not be necessary to prove the *in vivo* efficacy of desensitization with mutated peptides.

A recent report described the first case of IgE-mediated allergy to endogenous human milk protein (22). A young father showed contact urticaria following exposure to human milk, but not cow's milk, and the presence of specific IgE antibodies to human, but not bovine  $\alpha$ -lactalbumin. Since approximately 50% of the AA sequence of bovine  $\kappa$ -casein is similar to its human counterpart, we were interested in evaluating the potential cross-reactivity given the high degree of identity between known IgE epitopes of bovine  $\kappa$ -casein and human  $\kappa$ -casein sequence. Interestingly, two IgE-binding regions in bovine  $\kappa$ -casein were identified that shared high degrees of similarity with their human counterpart (AA104–112 and AA111–124). Most importantly, the AAs critical for IgE-binding in the first one of these regions were identical. These findings imply that IgE antibodies specific to these bovine IgE-binding epitopes could therefore cross-react with the corresponding human casein, but it is not known whether the degree of similarity is sufficient to cross-link IgE on effector cells leading to IgE-dependent activation. Studies are ongoing to further address the *in vitro* cross-reactivity of bovine whey and casein fractions and their human counterparts. Similarly, the clinical importance of this finding remains to be determined. It can only be speculated at this point whether these epitopes play a role in some CMA, exclusively breast-fed infants who continue having symptoms even when their mothers are no longer ingesting cow's milk products. In some cases these babies do not

show resolution of symptoms until breast-feeding is discontinued (23). Previously, Bernard et al. (18) have suggested that a conserved region at AA127–141 in bovine  $\beta$ -casein and AA 118–132 in human  $\beta$ -casein might be responsible for cross-reactivity.

In conclusion, the identification of AAs necessary for IgE-binding to bovine  $\kappa$ -casein, a major CMA, will enable us to engineer modified proteins with decreased IgE-binding capacity. However, the observed heterogeneous pattern of critical AAs for IgE-binding to  $\kappa$ -casein needs to be taken into account when using this modified protein in immunotherapeutic reagents. In addition, we

identified two cross-reactive peptides between bovine and human caseins, the clinical relevance of which remains to be determined.

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