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Structural Basis of IgE Binding to α- and γ-Gliadins: Contribution of Disulfide Bonds and Repetitive and Nonrepetitive Domains

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Supporting Information

ABSTRACT: Wheat products cause IgE-mediated allergies. The present study aimed to decipher the molecular basis of α- and γ-gliadin allergenicity. Gliadins and their domains, the repetitive N-terminal and the nonrepetitive C-terminal domains, were cloned and expressed in *Escherichia coli*. Their secondary structures and their IgE binding capacity were compared with those of natural proteins before and after reduction/alkylation. Allergenicity was evaluated with sera from patients who had a wheat food allergy or baker's asthma. The secondary structures of natural and recombinant proteins were slightly different. Compared with natural gliadins, recombinant proteins retained IgE binding but with reduced reactivity. Reduction/alkylation decreased IgE binding for both natural and recombinant gliadins. Although more continuous epitopes were identified in the N-terminal domains of α- and γ-gliadins, both the N-terminal and C-terminal domains contributed to IgE binding. As for other members of the prolamin superfamily, disulfide bonds appear to be of high importance for IgE binding.

KEYWORDS: *IgE epitopes, recombinant wheat allergens, synchrotron radiation circular dichroism, wheat allergy, wheat gliadins*

INTRODUCTION

The prolamin superfamily comprises the largest number of allergenic plant proteins. It includes proteins such as the sulfur-rich prolamins, the seed storage 2S albumins, the nonspecific lipid transfer proteins (LTP), and the cereal α-amylase/trypsin inhibitors (ATI) that are involved in food or respiratory allergies.[1−3] Prolamin superfamily members are grouped on the basis of a conserved skeleton of eight cysteine residues that are connected by intramolecular disulfide bonds. In LTPs, 2S albumins, and ATIs, these disulfide bonds contribute significantly to the stabilization of a compact, α-helix-rich 3D structure that is resistant to thermal and enzymatic denaturation and plays an essential role in the allergenic potential of these proteins.[4−6] The sulfur-rich prolamins, α-gliadins, γ-gliadins, and low molecular weight (LMW) glutenin subunits, differ from the other members of the prolamin superfamily by their organization into two structural domains: the C-terminal (Ct-) domain, which includes the cysteine skeleton, and the N-terminal (Nt-) domain, which contains repeating motifs that are rich in glutamine and proline residues. To date, no 3D structure of prolamins has been determined. Sulfur-rich prolamins are considered to be extended molecules,[7] although their Ct-domain is assumed to be more compact than the Nt-domain due to the disulfide bonds. Studies of the secondary structures of these proteins showed that α- and γ-gliadins contain approximately 30% or more α-helical structures.[3,8,9] Tatham et al. found that the Ct-domain obtained through the enzymatic hydrolysis of a γ-gliadin was rich in α-helices but that its repetitive domain contained poly-L-proline II and β-turn structures.[10]

Many α- and γ-gliadin peptides have been identified as T-cell epitopes in celiac disease, with most of them being from the repetitive domain.[11] With respect to wheat allergies, much attention has been paid to ω5-gliadins because of their role in wheat-dependent exercise-induced anaphylaxis.[12−14] However, α- and γ-gliadins have also been identified as IgE binding proteins in several studies.[15−19] A minor role of α-gliadin was also reported in bakers with occupational asthma.[20,21] IgE binding epitopes of gliadins were primarily found in the repetitive domains of these proteins.[4,22−24] This different antigenicity of the two gliadin structural domains may be related to their asymmetric organization. The more structured Ct-domain may mostly contain discontinuous epitopes.[4,22]

Wheat gliadins present a very large intra- and intercultivar polymorphism; thus, each gliadin subgroup contains many homologous proteins.[25,26] Purifying individual natural gliadins is thus very difficult. To overcome these problems, recombinant

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proteins provide an alternative method. Some recombinant prolamin allergens have already been produced and characterized.[20,21,27−31] Well-defined protein fragments can also be produced by heterologous expression, and such recombinant fragments have been valuable for studying the antigenicity of LMW glutenin subunits and their two domains[30] as well as fragments of high-molecular-weight (HMW) glutenins.[27]

The present work aims to further characterize the structural features linked to allergenicity of proteins belonging to the prolamin superfamily by focusing on two members: α- and γ-gliadins. Different forms (natural/recombinant; native/reduced-alkylated) and fragments (Nt- and Ct-) of α- and γ-gliadins were produced to study the contribution of their structural domains and disulfide bonds to IgE binding. Their secondary structure was determined using synchrotron radiation circular dichroism (SRCD); sera from patients with food allergies to wheat (FAW) or baker's asthma (BA) were used to analyze IgE binding.

MATERIALS AND METHODS

PCR Cloning of Whole Gliadins cDNA and of Their Moieties. rAG has been previously cloned.[29] The same procedure was used for cloning recombinant γ-gliadin. Briefly, total RNA was extracted from immature (14 days before anthesis) wheat grains (Neepawa cultivar). α-Gliadin (rAG) and γ-gliadin (rGG) cDNA were cloned by RT-PCR. First-strand cDNA was synthesized with using Superscript II system (Invitrogen, Carlsbad, CA, USA) containing an oligo-dT and adapter primers. PCR was done using primers described in ref 29 chosen according to dETS sequences found in databases or already cloned γ-gliadin (ID Q94G94) cDNA.[32] Their Nt- and Ct-moieties (rNt-AG, rAG-Ct, rNt-GG, and rGG-Ct) were cloned by PCR using full-length α- and γ-gliadin cDNA clones as templates. The DNA polymerase used was AccuPrime Pfx (Life Technologies). Fragments were cloned with restriction enzymes into a pET28b (Novagen) plasmid in frame with a sequence encoding the hexahistidine Tag (His-Tag). The sequences of the specific primers used in the cloning procedure are described in Supporting Information Table S.1 and in ref 29.

Production and Purification of Natural or Recombinant Gliadins and Fragments. Natural α- and γ-gliadins (nAG and nGG) were prepared from wheat flour (cultivar Recital) by several steps of chromatography.[15] The γ-gliadin corresponded to the γ46 fraction described in ref 33. When indicated, natural proteins were used after reduction/alkylation. After solubilization in 0.5 M Tris-HCl, pH 8.5, containing 8 M urea and 0.01 M EDTA, they were reduced with 0.1 M dithiothreitol (DTT) for 2 h at 37 °C and then alkylated with iodoacetamide in 0.5 M NaOH for 30 min in the dark. *Escherichia coli* strain Rosetta (Novagen) was used for recombinant protein production. Bacteria were transformed with a plasmid encoding rAG or rGG or their domains. Protein production and purification were performed as described in ref 30 with standard culture and IPTG induction, lysis with lysozyme in the presence of RNase A and DNase I (Qiagen), and precipitation steps. Proteins were reduced with DTT and alkylated with iodoacetamide during purification to increase extraction yields except when native full-length proteins were produced.

The purity of materials was checked by SDS-PAGE on a 15% w/w gel; 5 μg of proteins was loaded on the gel, and the proteins were stained with Coomassie Brilliant Blue R250. The presence of Nt-histidines in recombinant proteins was confirmed by Western blot using an anti-HisTag antibody.

Natural α-Gliadin Identification by Mass Spectrometry. nAG (3 mg/mL) was hydrolyzed at 37 °C for 6 h using porcine trypsin (Sigma T8253) and chymotrypsin (Sigma C 4879) in 25 mM NH_4HCO_3 at an enzyme/substrate ratio of 1:100 w/w. The resulting hydrolysate was reduced by mixing with 10 mM DTT and 10% acetonitrile at 45 °C for 1 h in the dark with gentle stirring. After the sample had cooled to room temperature, 0.5 M iodoacetamide was added, and the sample was incubated for 30 min in the dark. Following reduction/alkylation, the peptides were diluted 10 times in water; subsequently, 50 μL of the solution was eluted on C18 resin pipet tips (ZipTip, Millipore Corp, Billerica, MA, USA), cleaned, and finally extracted from the Zip Tip with 30 μL of 1:1 acetonitrile/water containing 0.05% formic acid. The acetonitrile was then evaporated on a Speed Vac, and the sample was stored for mass spectrometry analysis.

Nanoscale capillary liquid chromatography−tandem mass spectrometry (LC-MS/MS) analyses were performed using an Ultimate U3000 RSLC system (Dionex) coupled with an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fisher). Chromatographic separation and mass data acquisition were performed as described in ref 34. Protein identification was performed using the Mascot database search engine and the UniProt databank, which was restricted to *Triticum aestivum* sequences (release 2015_01). Mass tolerance for peptide precursors was set at 5 ppm, with the fragment mass tolerance set at 0.5 Da. Enzyme specificity was set to trypsin and chymotrypsin with a maximum of one missed cleavage. Proteins were identified with at least three peptides according to the above specifications.

Synchrotron Radiation Circular Dichroism (SRCD). Measurements were taken using the DISCO beamline at Soleil synchrotron (Gif-sur-Yvette, France). The samples were prepared in 50 mM sodium phosphate buffer, pH 8, containing 2% SDS and 10 mM sodium sulfate (typical range = 0.5−1.5 g/L). Data acquisition and processing were recorded as previously described.[35] Ratios of secondary structures were determined using the ContinLL program[36] in Dichroweb[37] and SP175 or Set5 as reference sets on the mean spectra from five measurements. Normalized root-mean-square deviation (NRMSD) indicated the most accurate fit for each spectrum; values <0.25 were considered significant.

Sera from Allergic Patients. Sera from 25 patients with FAW and from 5 patients with baker's asthma (BA) were selected on the basis of the presence of gliadin (nAG or nGG) specific IgE as determined by ELISA (Supporting Information Table S.2). All patients with BA were adults and presented rhinitis and asthma. Except for one adult, all patients with FAW were children. Their symptoms included atopic eczema dermatitis syndrome, anaphylaxis, asthma, urticaria, or gastrointestinal disorders. Sensitization to wheat proteins was assessed in patients using skin prick tests (SPT) to wheat flour (Moulins Soufflet), gluten (ALK-Abello), and natural gliadins and/or using specific IgE assays to wheat flour or gluten (Phadia ImmunoCAP). A wheat allergy was confirmed by a positive challenge or evident effect of wheat avoidance. The double-blind placebo-controlled food challenge and the inhalation challenge with wheat flour are described in refs38 and 4, respectively. Blood collection, SPT, and challenges were performed with the informed consent of the patients or their parents and after receiving approval from the biomedical research by the Ethics Committee of Ile de France III and AFSSAPS (Authorization 2008-A01565-50).

IgE Binding to Gliadins and Their Domains. Specific IgE concentrations toward the different forms of AG and GG (natural/recombinant; native/reduced-alkylated) and their Nt- or Ct-domains were determined by F-ELISA as described in ref 39 using alkaline phosphatase-conjugated goat anti-human IgE and 4-MUP as a substrate (Sigma A3525 and A3168, respectively, Saint-Quentin Fallavier, France). Proteins solubilized in PBS were coated at 5 mg/L in carbonate buffer on white 384-well plates (NUNC 460372, Fischer Scientific).

IgE Binding to Solid-Phase Synthetic Peptides (Pepscan). Sera containing high levels of specific IgE toward the gliadins and their domains ($n = 6$ for AG and $n = 5$ for GG) and three control sera from nonatopic subjects were used in the Pepscan analysis as described in ref 39. Decapeptides, overlapping on eight amino acid residues, spanned the full-length sequences of α- and γ-gliadins (UniProt ID P04725 and P08453, respectively), which were already used in previous Pescan analysis.[4,22]

Activation of RBL Cells by α-Gliadins and Their Domains. Rat basophil leukemia (RBL SX38) cell degranulation was expressed as the percentage of β-hexosaminidase release with a positive threshold set at

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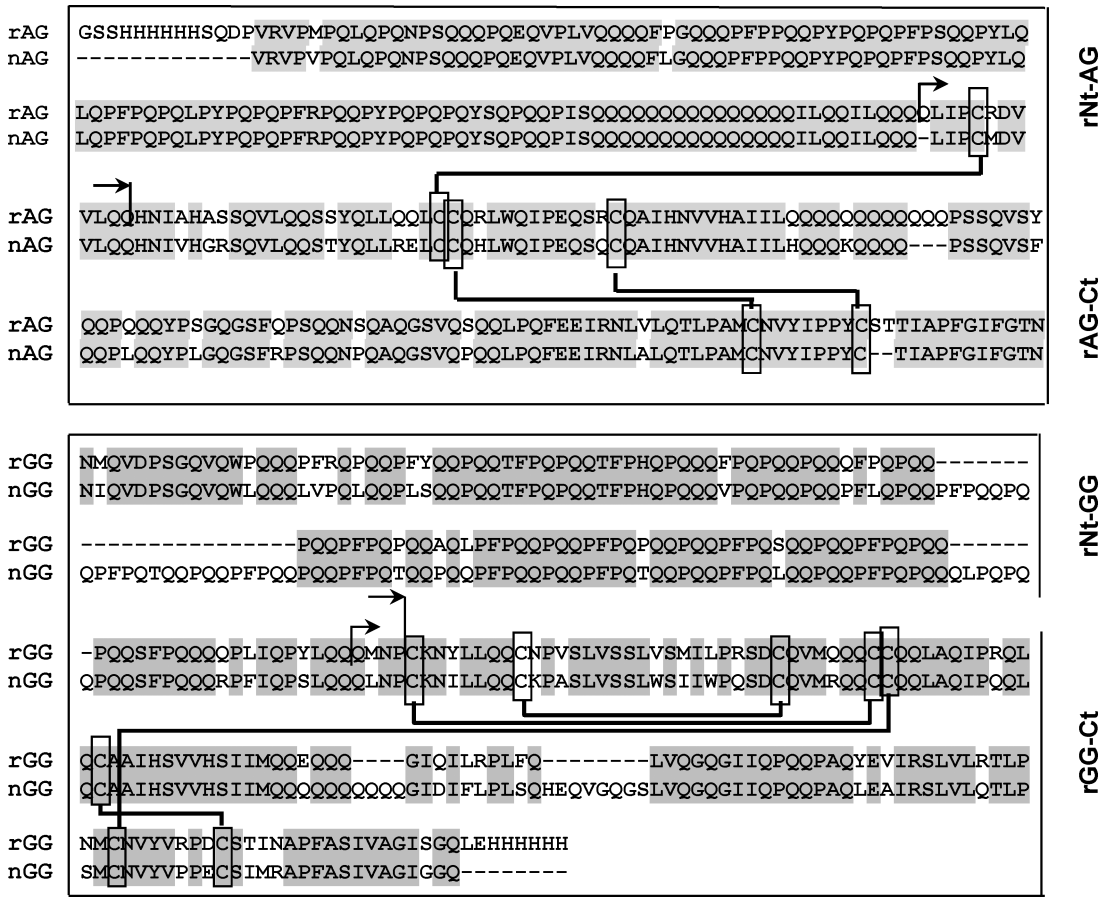

Figure 1. Comparison of the sequences of the recombinant proteins, α-gliadin (rAG) and γ-gliadin (rGG), and of the natural proteins, Uniprot ID Q3S4 V7 for α-gliadin and P08453 for γ-gliadin. The connections correspond to disulfide bonds between surrounded cysteine residues. The end of rNt-domains and the beginning of rCt-domains, (rNt-AG, rAG-Ct) and (rNt-GG, GG-Ct), are indicated by arrows. Identical amino acid residues are highlighted in gray.

11% as described in ref 39. Gliadin samples were tested at 2, 20, 200, and 2000 ng/mL with a pool of two sera (899 + 907) diluted 1:10.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA). For the ELISA results, paired tests were performed, and each pairing was confirmed to be significantly effective. Both specific IgE distributions and their paired differences failed the d'Agostino−Pearson omnibus normality test. Wilcoxon signed rank tests or Friedman tests with Dunn's multiple comparisons were then performed to compare the medians ; p values <0.05 were considered significant.

RESULTS

Characterization of Natural and Recombinant Gliadins and Their Domains. The full-length sequences of the recombinant and natural gliadins are shown in Figure 1. The recombinant α-gliadin (rAG) sequence (ID 1834759) shares 96% identity with the protein that was recorded under UniProt ID P02863.2. MS analysis of the natural α-gliadin (nAG) indicated that it contained in majority the α-gliadin ID Q3S4 V7, in addition to several minor isoforms of this protein. Sequence alignment revealed 95% similarity between rAG and nAG. In SDS-PAGE under reducing conditions, rAG migrated to approximately 45 kDa, whereas the nAG migrated to approximately 40 kDa (Figure 2). The sequence of recombinant protein rGG was deduced from its cloned cDNA sequence. It is identical to UniProt ID Q94G94.[32] The natural protein nGG corresponded to a γ46-gliadin type

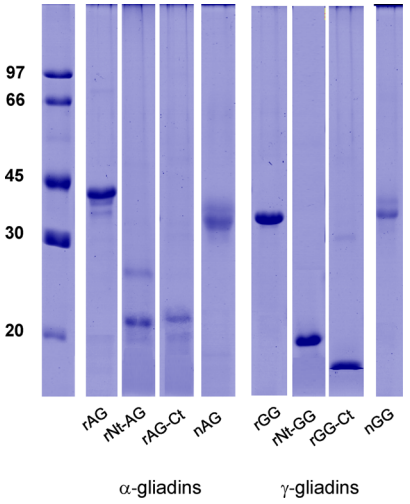

Figure 2. SDS-PAGE analysis of the proteins under reducing conditions colored with Coomassie Brilliant Blue R250. rAG and rGG, recombinant α-gliadin and γ-gliadin; rNt-AG, rNt-GG, rAG-Ct, and rGG-Ct, recombinant Nt- and Ct-domains of α-gliadin and γ-gliadin; nAG and nGG, natural α-gliadin and γ-gliadin.

according to its electrophoretic mobility in acid-PAGE and was previously described as P08453.[33] The rGG and nGG sequences displayed 80% similarity. Their mobility in SDS-PAGE was approximately 40 kDa. The Nt- and Ct-recombinant

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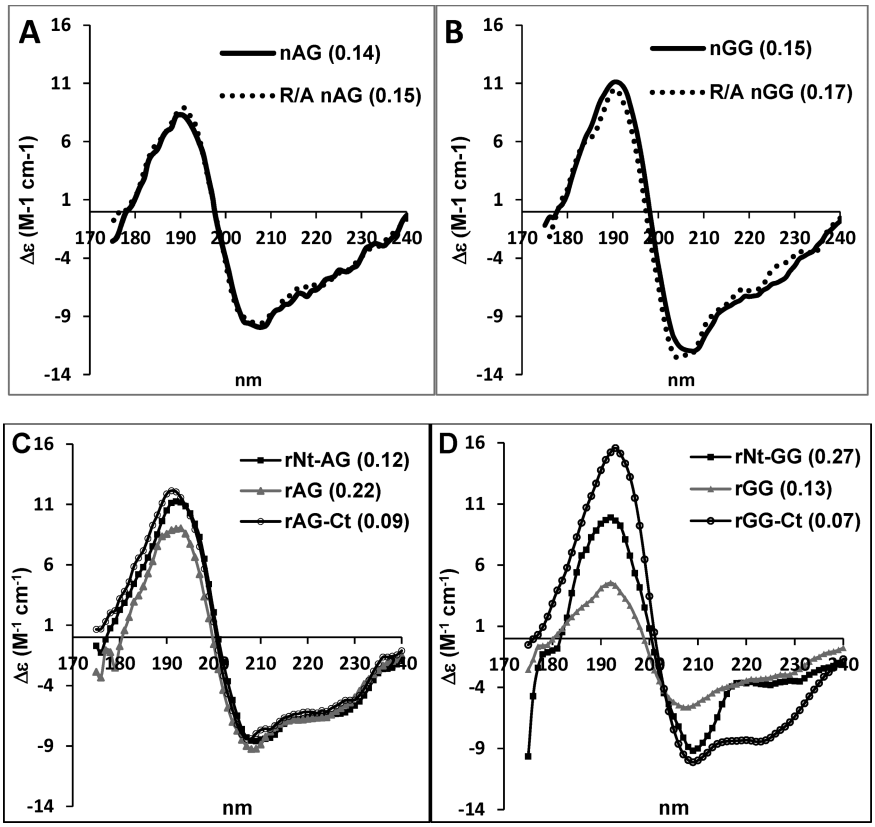

Figure 3. SRCD on natural and recombinant gliadins: spectra of native and reduced alkylated (R/A) natural α-gliadin (nAG) (A) and natural γ-gliadin (nGG) (B); spectra of recombinant full-length, N-terminal (Nt) and C-terminal (Ct) domains, (C) rAG and (D) rGG. NRMSDs are indicated in parentheses.

domains each constitute approximately half of a gliadin protein and migrated approximately 15−22 kDa. Despite their similar sizes, the mobility of the rNt-GG fragment (133 residues) differed that of the rGG-Ct fragment (136 residues). The molecular weights of the prolamins are often overestimated by SDS-PAGE, which has been attributed to the high content of proline in their Nt-repetitive domains.[10]

Secondary Structures of Natural and Recombinant Gliadins and Their Domains. The secondary structure contents of gliadins and of their domains were calculated after the deconvolution of the CD spectra. Low NRMSD values were obtained except for the rNt-GG domain (0.27).

The CD spectra of α-gliadins presented minimal values in the 206−208 and 220−224 nm regions, which reflects the α-helical structures. nAG contained approximately 35% α-helix, 18% turns, and a low level of β-strand. Reduction/alkylation of the protein did not affect the dichroic signal (Figure 3A). rAG and its domains displayed similar dichroic signals (Figure 3C) corresponding to a higher level of α-helix (52−58%) and a lower level of turn structures (11%) compared with nAG.

The secondary structure contents of nGG were approximately 25% α-helix and 16% β-strand and turns, and the reduction/alkylation of nGG did not change the CD spectrum (Figure 3B). The recombinant rGG contained slightly more α-helix (37%) and fewer strands (11%) than did nGG. Unlike rAG, full-length rGG and its Nt- and Ct-domains displayed different spectra (Figure 3D). The rGG-Ct domain is notably folded in α-helices (67%). The CD spectrum of the repetitive rNt-GG domain showed an unusual negative peak at 210 nm, which is characteristic of turn structures. This spectrum could be only poorly deconvoluted with the currently available sets (NRMSD >0.25); hence, its secondary structure was not evaluated.

IgE Binding to Natural and Recombinant Gliadins and the Involvement of Disulfide Bonds. Sera from 28 patients with FAW or BA were used to compare IgE reactivity toward different forms of α-gliadins in an ELISA. As shown in Figure 4 (top), compared with nAG, the IgE binding to rAG was significantly reduced. The mean of the specific IgE concentration was reduced by 60%, but 25 of the 28 sera retained binding to rAG. Reduction/alkylation highly decreased the IgE binding to nAG: the mean reduction was approximately 70%, and 67% of the sera exhibited a reduction of >70%. Reduction/alkylation had a lower but significant impact on IgE binding to rAG, with a 30% reduction.

Sera from 20 patients with FAW were used to compare IgE reactivity toward γ-gliadins. The sera from the five patients with BA had no or very weak IgE reactivity toward nGG. As shown in Figure 4 (bottom), compared with nGG, IgE binding to rGG was reduced. The reduction in the mean specific IgE concentration was approximately 30%, and 18 of the 20 sera retained IgE binding after nGG had been replaced with rGG. The decrease in the mean specific IgE concentration induced by the reduction/alkylation of nGG was approximately 60%, and 37% of the sera exhibited a reduction >70%. In the case of rGG, a significant decrease of approximately 60% was also observed.

Involvement of Nt- and Ct-Domains in IgE Binding to Gliadins. The involvement of the two structural domains of gliadins in IgE binding was analyzed with recombinant

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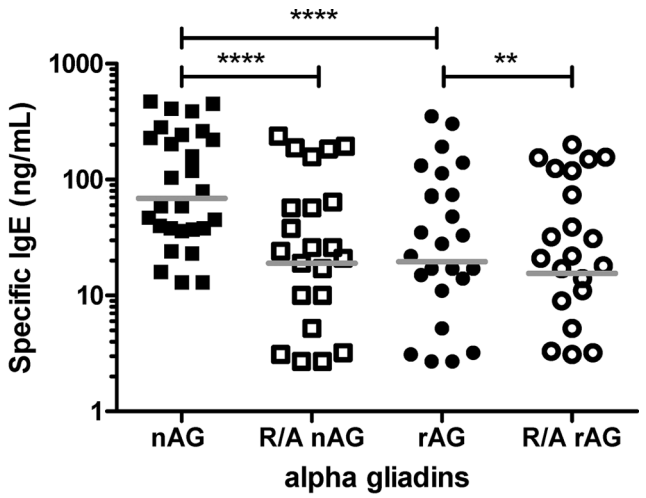
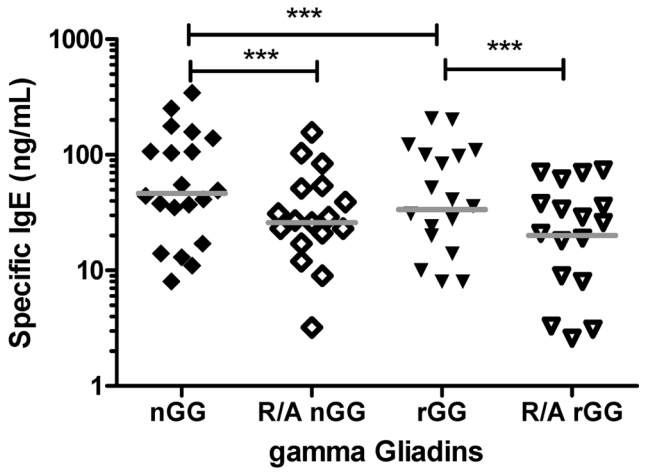


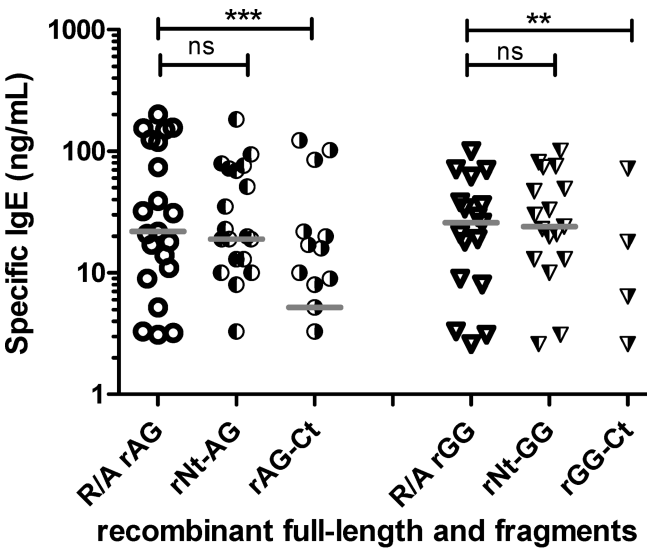
Mean	147	50	62	43
Median	69	19	20	16



Mean	88	37	60	26
Median	47	26	34	20

Figure 4. Effect of heterologous expression and reduction/alkylation of natural and recombinant α-gliadins (top) and γ-gliadins (bottom) on IgE binding measured by ELISA. Means and medians per sample are indicated under the graph. Medians, plotted as gray bars, are significantly different (**, $0.001 < p < 0.01$; ***, $0.0001 < p < 0.001$; ****, $p < 0.0001$; Wilcoxon signed rank test, $n = 28$ for AG and $n = 20$ for GG). Fifteen data points (5 for R/A nAG, 3 for rAG, 7 for R/A rAG) and seven data points (2 for R/A nGG, 2 for rGG, 3 for R/A rGG) were below the detection limit and not included on the graphs.

repetitive domains (rNt) and nonrepetitive domains (rCt). rCt domains were extracted after the reduction/alkylation of cysteines. Because it was shown above that reduction/alkylation affected IgE binding to gliadins, comparisons were then performed with reduced/alkylated full-length recombinant gliadins, and only sera with positive IgE binding to R/A recombinant gliadins were considered (Figure 5). The IgE binding data were analyzed by a Friedman test, which indicated significant difference among full-length gliadins and their fragments ($p < 0.0001$; $F = 19.4$, $n = 21$ for AG and $F = 26.8$, $n = 17$ for GG). For both gliadins, a subsequent Dunn's comparison test comparing the domains with the full-length protein revealed that the differences for the Ct-domain (difference in rank sum = 28.0 for AG, 20.0 for GG) were significant but that the differences were not significant ($p > 0.05$) for the Nt-domain.



Mean	57	39	20	32	36	6
Median	22	19	5	26	24	0

Figure 5. Comparison of IgE binding to the full-length gliadins and to their N-terminal (Nt) or C-terminal (Ct) domains measured by ELISA. Means and medians per sample are indicated under the graph. Statistical differences between medians of specified groups using Friedman and Dunn's multiple comparison post test are indicated. Medians, plotted as gray bars, are significantly different (**, $0.001 < p < 0.01$; ***, $p < 0.001$; $n = 21$ for AG and 17 for GG) or not (ns, $p > 0.05$). Twenty-four data points (2 for rNt-AG, 9 for rAG-Ct, 13 for rGG-Ct) were below the detection limit and not included in the graph.

For the α-gliadins, the mean specific IgE concentration was twice as high for the rNt-AG as it was for rAG-Ct (39 and 20 ng/mL, respectively); IgE from 43% of sera did not bind the Ct-domain, whereas most sera reacted the Nt-domain. The sum of the mean specific IgE concentrations for the two domains was equivalent to the mean concentration for the full-length recombinant gliadin after reduction/alkylation (57 ng/mL). For the γ-gliadins, the rGG-Ct domain was rarely bound by specific IgE (only 25% of the sera). Conversely, all sera displayed IgE binding to the rNt-GG. The mean concentrations of specific IgE for rNt-GG and R/A rGG binding were equivalent (36 and 32 ng/mL).

The involvement of the two structural gliadin domains in IgE binding was also analyzed by searching for continuous epitopes within these sequences. As shown in Supporting Information Table S3, IgE from all six tested sera bound sequences belonging to the Nt-domain of α-gliadin, whereas two sera (no. 451 and 590) did not bind any sequence located in the Ct-domain. Generally, more spots were detected in the Nt-domain than in the Ct-domain. Stretches of peptides were detected by several sera: the sequences ^{1}VRVPVPQLQP^{10}, ^{25}VQQQQFPG^{32}, ^{53}QPYLQLQPFPQQPFPPQLP^{72}, and ^{77}QSFPPQQPYPQQ^{88} in the Nt-domain and the sequence ^{219}QQQPSSQVSFQQ^{230} in the Ct-domain.

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The five sera tested on the γ-gliadin sequence displayed IgE binding to several peptides located in the Nt-domain, whereas only one serum (no. 899) displayed IgE binding to a sequence in the Ct-domain. The numerous epitopes in the Nt-domain corresponding to the consensus sequences PQQPFPQQPQ, LSQQPQQTFP, or QPQQ(S/T)FPQ were found with all sera, reflecting a higher homogeneity of repetition in γ-gliadin than in α-gliadin. There is no relationship between the number of peptides detected by Pepscan analysis and the IgE binding measured by ELISA toward the respective domains.

Triggering Potential of Natural, Recombinant, and Nt- and Ct-Fragments of α-Gliadin. The triggering potential of α-gliadin samples was assessed in an RBL assay using a pool of two sera that displayed high levels of specific IgE toward nAG (approximately 400 ng/mL), rAG (approximately 250 ng/mL), R/A nAG (approximately 200 ng/mL), R/A rAG (approximately 150 ng/mL), and the gliadin domains (70 ng/mL for rNt-AG and 100 ng/mL for rAG-Ct). Both nAG and rAG were able to induce cell degranulation in the presence of the pool of sera (Figure 6). However, maximum degranulation (approximately 35%) was obtained at a lower protein concentration for nAG (20 ng/mL) than for rAG (200 ng/mL). Reduced/alkylated nAG or rAG induced low degranulation at similar concentration (200 ng/mL), and none of the recombinant domains were able to induce degranulation above the positive threshold.

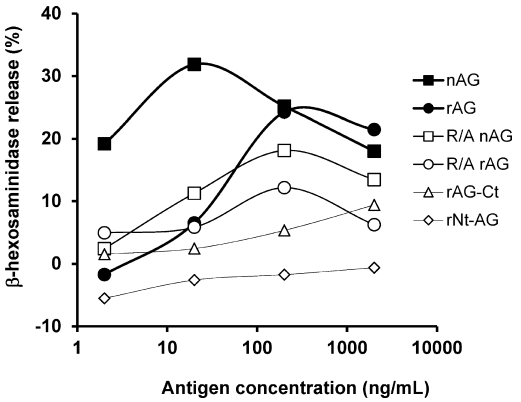

Figure 6. Triggering potential of natural and recombinant α-gliadins and domains tested in an RBL assay with a pool of two sera. Duplicate measurements were performed, and means are represented on the graph.

DISCUSSION

Wheat gliadins are frequently involved in FAW and occasionally involved in BA. This work aimed to elucidate the molecular structures responsible for IgE binding to α- and γ-gliadins by using a collection of sera from patients with FAW and BA and different preparations of gliadin proteins: natural and recombinant full-length proteins (native or reduced), recombinant fragments corresponding to the structural Nt- and Ct-domains, and synthetic peptides.

Like all wheat storage proteins, α- and γ-gliadins are polymorphic and have closely related sequences. The recombinant gliadins rAG and rGG displayed good sequence homology to the isoforms present in the natural AG and GG purified fractions. These recombinant proteins displayed higher contents in α-helical secondary structure (52% for rAG and 36% for rGG) compared with the natural fractions (36% for nAG and 25% for nGG). The results from SRCD are in accordance with the published data indicating that nAG contains primarily α-helical structure,[8,9,29] whereas nGG contains large amounts of α-helical and turn structures.[10,40]

The recombinant α-gliadin rAG showed reduced IgE binding compared with natural α-gliadin nAG, but 90% of the sera were still reactive. We also observed that rAG retained the capacity to induce basophil activation. A smaller reduction in IgE binding was observed for rGG compared to nGG. It is unlikely that these differences arise from the presence of post-translational modifications such as glycosylation, as no evidence of significant levels of glycosylation was found on the natural gliadins.[41] Different folding and a possibly incorrect formation of disulfide bonds by the heterologous expression in *E. coli* may be partly responsible for the different IgE reactivity. The small differences in sequences between the recombinant and natural isoforms (Figure 1) may also partly explain the difference in IgE reactivity. However, recombinant gliadins maintained the IgE reactivity for most sera, as recently reported for recombinant LMW glutenins.[28,30]

The disruption of disulfide bonds in AG and GG led to reduced IgE binding to these allergens for many sera. This disruption also reduced the degranulation potency of AG. Disulfide bonds are located in the Ct-domain of both proteins. Protein reduction likely disrupted some of the conformational epitopes. Only one sera (no. 901) bound to a cysteine-containing peptide of AG by Pepscan analysis. However, reduction may have an indirect but drastic effect on the global folding of the protein. When the proteins were reduced/alkylated, no significant effect was observed on the secondary structures of natural AG and GG. Reduction/alkylation likely modified the tertiary structures of these proteins but, unfortunately, all attempts to crystallize them failed. These results indicate that gliadins with their disulfide bonds are more IgE-reactive for many sera than are gliadins without disulfide bonds. The importance of disulfide bonds for IgE binding has already been shown for other allergens of the prolamin superfamily. For example, wheat LTP and peanut allergen Ara h 6 were poorly recognized by patient IgE after reduction of their disulfide bonds.[4,5] However, in the case of LTP and Ara h 6, secondary structures were also greatly affected by disulfide bond disruption, whereas in the present study, the CD spectra of α and γ-gliadins were not affected by this modification.

IgE binding to peptides and recombinant domains of AG indicated the presence of epitopic regions in both domains of this protein. Nevertheless, stronger binding to the Nt-domain compared with the Ct-domain was observed both by the Pepscan technique and using the recombinant domains. The secondary structure contents of the Nt- and Ct-domains were equivalent, with the high level of α-helix most likely due to the clusters of 13−15 glutamines present in both domains. Recombinant rNt-AG polypeptide and synthetic peptides may represent, to a certain extent, the natural Nt-domain folding, allowing IgE to cross-react with these different entities. Conversely, IgE cross-reactions between the natural Ct-domain and the corresponding (reduced) recombinant domain and synthetic peptides should be less likely. In the case of GG, this phenomenon was more pronounced because very low binding to the Ct-domain was observed by both Pepscan and ELISA using the recombinant domain, whereas numerous continuous epitopes were detected in the Nt-domain. The CD spectra clearly indicated that the two domains have different secondary

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structures: the rNt-GG primarily contains turn structures, whereas the rGG-Ct contains a very high content of α-helix.

Despite sequence homologies and similarities in their organization, differences were observed in patients' reactivity toward α- and γ-gliadins: allergenicity of GG was less affected by the reduction of disulfide bridges and more pronounced toward its Nt-domain than in the case of AG. This could be linked to differences in the number and homogeneity of repeated motifs between gliadins.

The continuous epitopes identified in this study by Pepscan in AG confirmed some of the epitopes identified in previous works: PLVVQQQQFPGQQQQ, YLQLQPFPQP, and PAMCNVYIPP.[4,22] The present work supports the hypothesis that AG appears to contain both types of epitopes, with continuous epitopes primarily located in the repetitive sequence domain and discontinuous epitopes most likely in the Ct-domain.[4] Nt-repetitive domains were previously shown to be highly resistant to pepsin hydrolysis;[42] they could more efficiently stimulate the production of specific IgEs compared with the nonrepetitive domains. However, both domains and correctly formed disulfide bonds in the Ct-domain appeared to be required for AG to induce basophil degranulation. This result differs from the reported presence of continuous repetitive IgE epitopes in deamidated ω2-gliadins or fragments from the repetitive domain of HMW glutenin that could induce basophil degranulation.[27,43]

Using recombinant domains of gliadins is a complementary approach to synthetic peptides and expands the possibilities of epitope research by accounting, to a certain extent, for protein folding. The proteins in their native form should be preferentially used for IgE reactivity detection. As for other allergens of the prolamin superfamily, disulfide bond formation appears to be of importance for IgE binding to α- and γ-gliadins. Disulfide bonds in the Ct-domain most likely play a role in generating conformational epitopes within this domain or formed by a combination of residues from both domains. Indeed, studies with Nt- and Ct-fragments of LMW glutenin subunits demonstrated interactions between both parts and their significance in IgE binding.[30]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables S1−S3. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01922.

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Author Contributions

S.D.-P. conceived and coordinated the study; S.D.-P., H.M., J.-C.G., and J.S. participated in the design of the study; E.P., E.B., D.-A.M.-V., and M.D. recruited patients and performed clinical tests; H.M. carried out the experiments; V.S. and C.L. participated in the production/characterization of natural proteins; R.L. performed RBL studies; Y.G. and F.W. supervised CD studies; C.B. analyzed Pepscan results and performed the statistical analysis; S.D.-P., H.M., and C.B. wrote the manuscript. All authors helped to revise the manuscript and concur with the submission.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AG, α-gliadin; GG, γ-gliadin; nAG, natural α-gliadin; rAG, recombinant α-gliadin; nGG, natural γ-gliadin; rGG, recombinant γ-gliadin; Nt, N-terminal; Ct, C-terminal; rNt-AG, N-terminal domain of α-gliadin; rNt-GG, N-terminal domain of γ-gliadin; rAG-Ct, C-terminal domain of α-gliadin; rGG-Ct, C-terminal domain of γ-gliadin; ATI, α-amylase/trypsin inhibitors; LTP, nonspecific lipid transfer proteins; SRCD, circular dichroism synchrotron radiation; FAW, food allergy to wheat; BA, baker's asthma; RBL cells, rat basophil leukemia cells; LMW or HMW, low or high molecular weight; SPT, skin prick test; R/A, reduced and alkylated

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