Molecular Interactions between a Recombinant IgE Antibody and the β-Lactoglobulin Allergen

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SUMMARY

Allergies are caused by the immune reaction to commonly harmless proteins, allergens. This reaction is typified by immunoglobulin E (IgE) antibodies. We report the crystal structure of an IgE Fab fragment in complex with β-lactoglobulin (BLG), one of the major allergens of bovine milk. The solved structure shows how two IgE/Fab molecules bind the dimeric BLG. The epitope of BLG consists of six different short fragments of the polypeptide chain, which are located especially in the β strands, covering a flat area on the allergen surface. All six CDR (complementary-determining region) loops of the IgE Fab participate in the binding of BLG. The light chain CDR loops are responsible for the binding of the flat β sheet region of BLG. The IgE epitope is different from common IgG epitopes that are normally located in the exposed loop regions of antigens and observed also in the two recently determined allergen-IgG complexes.

INTRODUCTION

More than 25% of the human population in industrialized countries suffers from type I hypersensitive reactions like allergic asthma, rhinitis, food and skin allergy, and even anaphylaxis. This type I allergy is based on the formation of immunoglobulin E (IgE) antibodies against, in principle, harmless proteins, allergens. The symptoms of type I allergy are mediated when an allergen molecule binds to two IgE antibodies bound to the high-affinity FcεRI receptors on a mast cell or basophil surface and induces cross-linking of the IgE-FcεRI complexes. This triggers the degranulation of biological mediators like histamine and lipid mediators that cause inflammatory reactions (Larché et al., 2006). IgE is a large molecule that consists of two identical light and heavy chains. The heavy chains of IgE contain four constant domains (C1–C4) and one variable domain, whereas the light chain contains one constant domain and one variable domain (Figure 1A). Recently solved crystal structures have revealed the structures of heavy-chain domains C1–C4 and the binding mechanism for the FcεRI receptor. The binding of IgE requires substantial conformational changes in C1 that lead to the very stable IgE-FcεRI complex (KD = 10−9 M), which means that IgE can be fixed to FcεRI even in the absence of a bound allergen (Garman et al., 2000; Wan et al., 2002).

In the last few years, the three-dimensional structures for a large number of different allergens have been determined. Structurally, these allergens vary considerably, and no common structural motif that could explain the capability of these allergens to cause synthesis of IgE antibodies has been identified (Aalberse, 2000). However, by comparing plant food and pollen allergies, it has been found that allergenicity is restricted to only a few protein families, thus raising evidence that structural features of proteins could also have a role in allergenicity (Jenkins et al., 2005; Radauer and Breiteneder, 2006). Some recent experimental studies have also raised evidence that there would be differences between IgG and IgE antibodies in the binding of allergens. For instance, it has been found that polyclonal IgE antibodies against Amb a 1 and Der p 1 bind especially strongly to allergens (KD = 10−8–10−11 M) and are directed toward only a few epitopes (Pierson-Mullany et al., 2000). Whilst studying binding strength against pollen allergens Phi p 5a and Bet v 1a, it was found that IgE antibodies bound allergens tighter (KD = 10−10–10−11 M) than IgG antibodies (KD = 10−7–10−8 M) (Hantusch et al., 2005).

The essential question when studying allergenicity involves the so-called B cell epitope, the IgE antibody-binding site of an allergen that unfortunately cannot be deduced directly from the three-dimensional structure. To date, B cell epitopes have been sought by analyzing fragments or peptides of an allergen that react with polyclonal IgE serum pools from allergic patients. In addition, site-directed mutagenesis of allergens, use of epitope mimics (mimotopes), and bioinformatics modeling studies have been used (Bannon and Ogawa, 2006; Riemer et al., 2004). However, the only rational method by which to determine the complete epitope of any allergen involves measuring the crystal structure of an allergen in complex with an IgE antibody (Laver et al., 1990). So far, no structural data exist describing the structures of IgE antibodies complexed with allergens, due to the fact that the
production of monoclonal IgE antibodies has not been successful through hybridoma technology.

At present, there are over 80 crystal structures of antigens complexed with immunoglobulins in the Protein Data Bank. The majority represent Fab-fragment complexes. These structures include only one structure of an IgE antibody, SPE7 (Fv fragment) raised against a 2,4-dinitrophenyl hapten in mouse. SPE7 was found to exist in different conformations and is able to bind different ligands, which one of them was found to be the thioredoxin of E. coli, which is not an allergen (James et al., 2003). There are also two published crystal structures of allergens complexed with mouse IgG1 Fab fragments, the allergens in these immunocomplexes are birch pollen allergen Bet v 1 and bee venom Api m 2 (hyaluronidase) (Mirza et al., 2000; Padavattan et al., 2007). In this study, we report the first, to our knowledge, structure of an allergen complexed with a Fab fragment of a recombinant IgE (D1/Fab) antibody at 2.8 Å diffraction resolution. The allergen is β-lactoglobulin (BLG, Bos d 5), which is a well-known bovine milk allergen, and its immunogenicity has been studied extensively (Wal, 1998). To our knowledge, the solved structure gives first insight into the molecular interactions between an IgE antibody and allergen, revealing the structure of an IgE epitope of BLG.

RESULTS

D1/Fab Isolation, Production, and Purification

The D1 IgE antibody was isolated from a combinatorial scFv phage display library that contains the IgE VH gene pool combined with either the κ or λ VL pools. The IgE-specific heavy chain and the light chains were amplified from the total RNA isolated from lymphocytes of peripheral blood of a milk-allergic, adult patient. The IgE-specific cDNA was amplified by using primers specific to the ε CH2 region and to all Ig human V regions. The IgE specificity of the amplified cDNA pool was verified by sequencing ten individual cloned heavy-chain cDNA fragments. The scFv libraries were constructed by combining the amplified VH-encoding regions of the IgE-specific cDNA with the VL-encoding regions of the κ and λ light chains. The D1 IgE scFv fragment was selected from the library by using biotinylated BLG immobilized on streptavidin wells. The isolated D1 IgE scFv was converted to a Fab fragment by cloning the human Fd chain (Brownlow et al., 1997). An elongated electron density was found in both lipid-binding cavities of BLG monomers; this density was interpreted to be that of n-dodecyl-β-D-maltoside, which is used as a detergent in crystallization. The maltoside part of the detergent is outside the cavity, but far away from the Fab fragment; therefore, the binding of ligands would probably not affect antibody binding. The overall structures of D1/Fab fragments are clearly bent. The calculated elbow angles were 141° and 150° for Fab fragments H/L and I/M, respectively. These values are typical of Fab fragments in which the L chain is of type κ. In the antibodies that have type λ L chains, elbow angles are usually larger (>195°) (Stanfield et al., 2006).

IgE Epitope of BLG

The IgE-binding site of BLG is explicitly located in one monomer and does not extend to the second monomer or to the dimer interface (Figure 2A). In total, 27 residues of BLG (17% in total) are at least partially buried in the complex structure; the buried area in BLG is 890 Å².

Overall Structure of the Immunocomplex

Crystals were obtained at pH 5.5, in which the BLG protein exists predominantly in the dimeric form (Sakurai and Goto, 2002); consequently, the solved structure shows two D1/Fab molecules (chains L, H and chains M, I) bound to the dimeric BLG (chains A, B) (Figure 1B). The structure shows a 2-fold symmetry axis between two BLG monomers. The location of the axis is similar to that of the native dimeric BLG, and ~6% (530 Å²) of the monomer surface is buried on the dimer interface. The conformation of BLG in the immunocomplex is also similar to the structure of native BLG, except for a slight difference in the position of the C terminus (Brownlow et al., 1997). An elongated electron density was found in both lipid-binding cavities of BLG monomers; this density is interpreted to be that of n-dodecyl-β-D-maltoside, which is used as a detergent in crystallization. The maltoside part of the detergent is outside the cavity, but far away from the Fab fragment; therefore, the binding of ligands would probably not affect antibody binding. The overall structures of D1/Fab fragments are clearly bent. The calculated elbow angles were 141° and 150° for Fab fragments H/L and I/M, respectively. These values are typical of Fab fragments in which the L chain is of type κ. In the antibodies that have type λ L chains, elbow angles are usually larger (>195°) (Stanfield et al., 2006).
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Figure 2. The D1/Fab IgE Epitope of BLG
(A) Location of two monovalent epitopes in the BLG dimer.
(B) The IgE-binding epitope of BLG. The residues making contacts are shown in red, and the residues that are buried upon binding of the IgE are shown in orange. The different segments of the epitope are numbered 1–6.

CDR Loops of D1/Fab

The overall structure of the two variable domains of D1/Fab is similar to that of the known IgG structures. All three CDR loops from the light chain and CDR loops H1 and H2 from the heavy chain belong to the common canonical classes of immunoglobulins (Al-Lazikani et al., 1997). However, CDR-H3 is an unusually long loop. It has 17 residues and a bulged conformation in the torso region (Morea et al., 1998). The head region of CDR-H3 packs against all other CDR loops, extensively filling the groove between the two variable domains (Figure 4). This groove is usually the central part of the antigen-binding site in IgG antibodies. As it is common in immunocomplexes, the buried area is higher in the H chain (550 Å²) than in the L chain (385 Å²). In total, 14 residues of D1/Fab make contact with BLG, and only 2 of them (Y49 and Y91 from the light chain) are aromatic, which is unusual (Davies and Cohen, 1996). Three light-chain loops contain 7 residues that are all in contact with the β sheet of BLG (segments 2, 3, and 4). CDR-L1 and CDR-L2 are both in contact with 3 residues of BLG: S30, S31, and R32 and Y49, A50, and S52, respectively. CDR-L3 makes only one hydrogen bond with BLG through the phenolic hydroxyl group of Y91. Three heavy-chain CDRs also make seven contacts with the BLG. H31 of CDR-H1 binds to the loop on the edge of the epitope (segment 5), and CDR-H2 binds to the C-terminal helix (segment 6) via residues S52 and S54. In the middle of the D1/Fab-binding area of BLG, there is a small depression between the C-terminal α helix and β strands A and B. CDR-H3 utilizes this cavity by placing Arg101 between Y20 from strand A and E40 from strand B (segments 1, 2, and 5). V102 of the CDR-H3 loop is also important for binding to Y20 from epitope segment 1 and to P126 from epitope segment 5 (Figure 4).

The comparison of 52 germline VH sequences with the VH region of D1/Fab revealed that the VH3-23 is highly identical (83%) to D1/Fab. This germline sequence has two identical interacting residues with the D1 sequence in the CDR-H2 loop (S52 and S54). H31 from the CDR-H1 loop of D1 was replaced by a smaller side chain, serine in VH3-23. This suggests that S31H mutation could represent a somatic mutation that improves the binding affinity and selectivity of D1/Fab.

DISCUSSION

D1/Fab and Homology in the IgE Light Chains

D1/Fab is a recombinant human antibody with the VH region from the IgG Fd-specific cDNA combined in vitro to the Vκ region, and, thus, it is not necessarily a pairing that occurred naturally in the patient in response to the BLG allergen. However, we found that the CDR loops of the D1 light chain are highly homologous with CDR loops of a few available IgE antibodies raised against different allergens, which indicates that the selected VH/VL combination might present an authentic pairing of an IgE antibody. Very few amino acid sequences of IgE antibodies are available today. Comparison of the sequences of the heavy chains is typical for antibodies, and, as one might expect, the largest differences can be found in the CDR-H3 regions. However, some sequences of the IgE light chains showed very high identity (85%–90%) with the D1 light chain. In particular, CDR-L1 and CDR-L2 loops were quite conserved. The light chain of D1 shares high homology with light chains of IgEs, which bind allergens Phl p 1, Phl p 2, Phl p 5, and Hev b 6, which all belong to structurally different protein families (Flicker et al., 2002, 2006; Laukkonen et al., 2003; Steinberger et al., 1996). This suggests that a flat surface of a β sheet or a similar flat patch could be characteristic for other IgE epitope structures as well. However, in the absence of structural data for these allergen-IgE immunocomplexes, it is difficult to estimate the significance of the high L chain identity. However, at least in the case of the D1 antibody, CDR-L loops are essential for the binding of the β sheet surface of BLG allergen (Figure 4).
Epitope Mapping

The epitopes of BLG have been characterized in many studies during the last two decades. In a recent study, Järvinen et al. (2001) used 77 overlapping decapeptides to define 6 IgG and 7 IgE epitopes of BLG against polyclonal human sera. The identified IgE epitope peptides cover 62% of the polypeptide chain of BLG. The two strongest epitopes were located in a loop (K75-D85) and in the α-helix (E127-P144), which are not found in the epitope structure of BLG-D1/Fab. Three weaker epitope peptides (L31-P48, K47-K60, and L57-I78) extend over segments 2, 3, and 4. The epitope L31-P48 was the only peptide that was not recognized by IgG antibodies (Järvinen et al., 2001). In another study, two methods were used: a PEPSCAN assay with 151 overlapping dodecapeptides, which was used to investigate the binding of rabbit polyclonal antisera to BLG; and the mimotypes identified by peptide phage display, which were used to study the binding to anti-BLG IgG. By these methods, four continuous epitopes, which are all located in loops and are not found in the epitope of the BLG-D1/Fab-immunocomplex structure, were identified (Williams et al., 1998). There is limited correlation between the BLG-D1/Fab epitope obtained from the immunocomplex structure of BLG-D1/Fab and the results from the peptide scanning. The peptide scanning is based on the use of polyclonal IgE antibodies to recognize linear peptides that do not necessarily exist in the same conformation as in the native allergen.

Clement et al. (2002) have characterized the IgG epitope map of BLG by using 52 monoclonal antibodies in a competitive immunoassay. The 12 identified antigenic regions were further characterized by site-directed mutagenesis. According to their study, the most antigenic regions were located in the α-helix and in the external loops. The highest decrease in affinity of antibodies toward mutated BLG (over 1000-fold) was found for the mutants E127A, E127Q, D130N, and E134Q; E127 is located in segment 5 on the outer edge of the D1 epitope. However, according to the BLG-D1/Fab structure, the effect of the substitutions E127A and E127Q on the binding would only be small. On the other hand, a minor antigenic area was found to be located in segment 2 in the β-strand (mutants E44A, E44G-E45G) (Clement et al., 2002).
Thus, the epitope of the BLG-D1/Fab is not located in the most antigenic area accessible for IgG antibodies, but it may have overlapping areas with minor antigenic sites.

There are several computer programs for the prediction of B cell epitopes. Using a number of amino acid propensity scales, most of them are based on protein sequences and the use of hydrophilic properties, flexibility, β turns, and surface accessibility. In the case of the BLG-D1/Fab structure, these methods fail to predict the correct epitope because the most important epitope region is located in the β sheet structure. Recently, a new program, DiscoTope, that uses the data from experimentally determined antigen-antibody structures to predict discontinuous B cell epitopes, has been developed (Andersen et al., 2006). This program was used in the context of the three-dimensional structure of BLG. The analysis resulted in 13 putative B cell epitope residues that were located in the loops and in the two C-terminal α helices. Only one of them was the same as in the BLG-D1/Fab structure, namely, T154 in the C-terminal α helix. Because DiscoTope suggests epitopes located in the protruding areas, it does not identify the epitope located in the β sheet.

**Crossreactivity**

BLG belongs to the lipocalin protein family, which contains several known animal allergens from cow, horse, dog, rat, mouse, and cockroach. The overall three-dimensional structures within the family are very similar (Rouvinen et al., 1999). However, a sequence comparison reveals that the interacting residues are not conserved, suggesting that these allergens are not crossreactive with D1/Fab (Figure 3). This is in accordance with the low sequence identity (8%–25%) between BLG and the above-mentioned lipocalin allergens. However, β-lactoglobulin from reindeer milk has recently been reported to be partially crossreactive with BLG-specific IgE sera, and the observed crossreactivity is in accordance with the structure of BLG-IgE/Fab. There are only 9 amino acid residues that are different between these two proteins (94% identity) (Oksanen et al., 2006). Structure comparison showed that E65 of BLG, which is located just before epitope segment 4, is replaced by lysine in the reindeer β-lactoglobulin. This substitution would bring the side chain of K65 very close to His92 from the CDR-L3 loop in the complex, which might decrease the binding to D1. The last two residues, H161 and I162, are also different in the reindeer β-lactoglobulin, causing changes in the position of the small C-terminal α helix and, thus, in segment 6.

**The Dimeric Nature of an Allergen**

The dissociation constant for the BLG dimer formation is low (Kd = 5.0 × 10⁻⁶ M); however, BLG exists predominantly as a dimer at neutral pH (Sakurai and Goto, 2002). From the crystal structure of BLG-D1/Fab (Figure 1B), we can see that Fab fragments are located in positions such that the dimeric BLG is able to crosslink two IgE-FcRI receptor complexes.

Allergens require at least two IgE epitopes for crosslinking. This presumes the development of at least two different IgE antibodies for monomeric allergens. In addition, the crosslinking requires that two IgEs are able to bind simultaneously to the epitopes of the antigen. If the allergen is able to form dimers, oligomers, or even aggregates, the development of only one IgE may be sufficient for crosslinking. Therefore, the capability of an allergen to form dimers, such as in the case of BLG, could increase the potency of proteins to operate as allergens. BLG is a member of the lipocalin protein family, which contains many animal allergens. Many allergens of the lipocalin protein family, such as Aca s 13, Can f 1, Can f 2, Can f 3, Cav p 2, Equ c 1, Mus m 1 and Rat n 1, are able to form dimers (Eriksson et al., 1999; Kamata et al., 2007; Fahibusch et al., 2003; Lascombe et al., 2000; Böckeki et al., 1992). In fact, dimeric or tetrameric allergens can also be found in many other allergen classes. So far, the significance of allergen dimerization for crosslinking has been studied very little. Schöll et al. (2005) have recently shown that the dimeric,
not the monomeric, form of the major birch pollen allergen Bet v 1 was able to crosslink IgE antibodies on mast cells.

Comparison of Immunocomplexes

When comparing the BLG-D1/Fab structure to those of other immunocomplexes, it must be kept in mind that the analysis is based on only one example of an allergen-IgE complex. The most striking feature of the structure of the BLG-D1/Fab immunocomplex is the light-chain interaction with the flat surface of the BLG formed mainly by a β sheet. This kind of epitope structure is very unusual among the known crystal structures of immunocomplexes. We found 82 different protein-antibody immunocomplexes in the Protein Data Bank that almost exclusively represent antigen-IgG complexes. We have looked at the structural elements in epitopes (α helices, β strands, and loops) and the shapes of epitopes (convex, planar, and concave) (Figure 5). Half of the epitopes were formed by loops alone, and the other half contained both loops and secondary structure elements. The majority of the epitopes (68%) were located in the convex or protruding areas of antigens.

The published immunocomplex structures include only two IgG1 Fab fragments complexed with allergens, birch pollen allergen (Bet v 1; 1FSK), and bee venom hyaluronidase (Api m 2; 2J88) (Mirza et al., 2000; Padavattan et al., 2007). Both of these IgG antibodies competed for allergen binding with IgE sera, suggesting that the IgG and IgE epitopes at least partially overlap. In both cases, antibody is bound to the protruding region of the allergen. In Bet v 1, the epitope is formed mainly by the β hairpin, and, in Api m 2, the epitope is formed by the helix-turn-helix motif. Thus, the epitopes are very different than in the BLG-D1/Fab complex (Figures 5A, 5C, and 5D). On the other hand, the protruding shapes of these two IgG1 epitopes are similar to those commonly observed in the antigen-IgG immunocomplexes that have been predicted to be the most antigenic (Thornton et al., 1986). Lysozyme, complexed with the HYHEL-10 IgG antibody (3HFM), partially shows binding to a smaller flat face, but the overall shape of the epitope is convex (Figure 5F) (Padijan et al., 1989).

Concave epitopes represent the second largest group of immunocomplexes (23%). Typical examples are neutralizing antibodies of HIV or single-domain antibodies of dromedaries, lamas, or sharks. In these antibodies, the exposed CDR-H3 loops bind larger clefts or cavities on the antigen surfaces (Desmyter et al., 1996; Saphire et al., 2007).
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2001; Stanfield et al., 2004; De Genst et al., 2006). A similar type of binding can be seen only in one previously determined IgE immunocomplex, in which the CDR-H3 loop binds to the concave area of thioredoxin formed by three loops (Figure 5B). The crystal structure of a monomeric, soluble form of CD28 in complex with the Fab fragment of a mitogenic antibody (1YJD), which shows the only example in which the epitope is partly formed by a flat face of a β sheet, also has a concave epitope shape. (Figure 5E) (Evans et al., 2005).

A smaller group of immunocomplexes (9%) represent the antibody binding to the flat or to the quite flat area. These seven structures contain four structures in which the epitope is predominantly formed by loops (1G9M, 1DVF, 1OSP, 1YNT) and three structures in which the epitope is formed by helices and loops (1W72, 1AR1, 1EZV). Among the known immunocomplex structures, there is no example of a flat epitope formed by β structures, which is seen in the BLG-D1/Fab complex.

Nature of the IgE Epitope
Some experimental evidence exists that suggests that allergen-specific IgE may be directed to epitopes different from those recognized by IgG. In addition, it has been demonstrated that allergenicity is limited to a small number of protein families, which indicates that structural features could contribute to the allergenicity. However, structurally, allergens present different folds, which make direct comparison between allergen structure and putative IgE epitopes difficult. Another point of view comes from the findings that some allergens are weak immunogens for the development of IgG antibodies (Larche et al., 2002; Saarelainen et al., 2002), and that IgG antibodies have a smaller affinity with allergens. This suggests that allergens have less preferable binding sites for IgG antibodies. It is known that IgE is made in response to parasitic worms (helminths) and arthropods (Yazdanbakhsh et al., 2002). Thus, it is possible that an IgE antibody response is developed to detect unusual structural features of molecules from these parasites that are less attainable for IgG antibodies.

To our knowledge, the BLG-D1/Fab structure is the first determined allergen-IgE structure. It reveals the binding of the IgE antibody to the epitope, which is planar and consists mainly of a β sheet structure. It is therefore different from typical epitopes for IgG antibodies, which are located in loops that protrude from the surface of the antigen. However, it is clear that we need more allergen-IgE complex structures to prove that there is a difference in the epitope preference between IgE and IgG.

EXPERIMENTAL PROCEDURES
Crystallization and Data Collection
Microcrystals (about 70 × 50 × 50 μm) of BLG-D1/Fab were obtained with a vapor-diffusion method by mixing 2 μl D1/Fab solution (concentration 1.4 mg/ml in 20 mM phosphate buffer [pH 7.0]), 1 μl BLG solution (2 mg/ml in pure water), 0.5 μl n-dodecyl-D-maltoside solution, and 2.5 μl reservoir solution (14% [w/v] polyethylene glycol 3350, 0.1 M BTP [1,3-bis(tris(hydroxymethyl)methylamino)propane]-hydrochloric acid] buffer [pH 5.5]). The diffraction data set was collected from a single crystal at beamline ID29 in ESRF (wavelength 1.000 Å) at 100 K. The crystal belonged to the space group P212121 with unit cell dimensions of a = 67.0, b = 100.6, c = 168.1 Å. The data set was collected at 2.8 Å resolution (Table 1). A more detailed description of crystalization will be published elsewhere (M.N. et al., unpublished data).

Structure Determination
The structure was solved with the molecular replacement method by using the Molrep program implemented in the CCP4 program package (CCP4, 1994). A BLG monomer (PDB code: 1B8E) and Fab fragment of the IgG antibody against GP41 of HIV virus (1DFB) (identity 92% for light chain, and 79% for heavy chain) were used as search models. Because of a low number of unique reflections, restraints were used for atomic coordinates and individual B factors to keep both D1/Fab fragments and BLG monomers similar during the whole refinement. BLG exists in two isoforms, and the electron density suggested that we have glycine at position 64 and alanine at position 118. No water molecules were added, but the long electron density in the lipid-binding pocket of BLG was modeled as an n-dodecyl-D-maltoside. The final structure has an R value of 24.5% and an Rfree value of 29.9%. In the Ramachandran plot, 82.5% of the residues were in the most favored regions, and 0.6% of the residues were in the most favored regions.

Table 1. Data Collection and Refinement Statistics

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*a The highest-resolution shell is shown in parentheses.
residues were in the disallowed regions (Table 1). Buried surface area calculations were conducted with the Areaimol program (CCP4, 1994). All figures were generated with PyMol (DeLano, 2002).

Supplemental Data

Supplemental Data include one figure showing the representative electron density map available at http://www.structure.org/cgi/content/full/15/11/1413/DC1/.

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Accession Numbers

The structural coordinates for the BLG-D1/Fab immunocomplex have been deposited in the Protein Data Bank with accession code 2R56.