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Identification of conformational core epitope Lys[68] in C5a based on the 3-D modeling complex C5a and its functional antibody F20

Huawei Wei[a,1], Zhou Lin[a,1], Jiannan Feng[a,1], Hui Peng[a], Renfeng Guo[b], Gencheng Han[a], Shusheng Geng[c], Xiaoling Lang[c], Yingxun Sun[a,c], Beifen Shen[a], Yan Li[a,*]

[a] Department of Immunology, Institute of Basic Medical Sciences, Beijing, China
[b] Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA
[c] Beijing Mabworks Biotech Co., Ltd, Beijing, China

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ABSTRACT

Inhibition of C5a by antibodies has been demonstrated to dramatically improve survival in various sepsis models in mice and rats. The structural basis of C5a mediated bioactivity and C5a antibody mediated neutralization are of interesting to be investigated. In the previous study, we obtained a novel functional mouse antibody named as F20. With computer-guided modeling method, the 3-D theoretical structure of F20 Fv fragment was constructed. Using the crystal structure of C5a, the 3-D complex structure of C5a and F20 Fv fragment was modeled with molecular docking method. Based on distance geometry method and intermolecular interaction theory, the key residue Lys[68] in C5a identified by F20 was predicted. The mutant experimental results showed that the residue Lys[68] was the critical residue of C5a for it's bioactivity and F20 binding activity. The present study shed new light on the structural basis of C5a mediated bioactivity. The identification of the critical residue will provide useful information for human complement C5a targeted therapeutic intervention.

1. Introduction

Among the complement activation products, C5a is one of the most potent inflammatory peptides with a broad spectrum of functions. C5a cannot only be generated systemically through complement activation pathways but it may also be produced locally through a phagocytic cell-related serine protease with C5 convertase activity. C5a causes an oxidative burst in neutrophils and enhances phagocytosis and release of granule enzymes (Goldstein and Weissmann, 1974; Sacks et al., 1978; Mollnes et al., 2002). Widespread up-regulation of C5aR expression occurs during onset of sepsis, and blockade of C5a/C5aR interaction by anti-C5a, or anti-C5aR antibodies, or C5aR antagonists renders highly protective effects in rodent models of sepsis (Czermak et al., 1999; Huber-Lang et al., 2001; Riedemann et al., 2002). However, previously generated anti-C5a antibodies exhibited only moderate blocking activities on biological effects induced by C5a. Identifying the structural basis of C5a antibody mediated neutralization will provide useful information for increasing the antibody affinity or designing novel antagonist against C5a.

The physical interactions between antigen and antibody in 3-D antigen–antibody complex structure provided crucial insights into antigen or antibody function. It is precisely the 3-D antigen–antibody complex structures that enable researchers to study interactions in residue's details, and find out, for example, how a few residue modifications in parent antibody might to a more effective and affinity-higher antibody.

In the past several experimental techniques have been developed for mapping antibody interacting residues in antigen that includes identification of interacting residues from 3-D antibody–antigen complex structure (Van, 1989). One of the popular approaches is overlapping peptide synthesis covering the entire antigen sequence, which identifies mainly sequential epitopes (Frank, 2002). However, mapping of antibody interacting residues was hampered by the costly and time taking process of 3D structure determination. With development of bioinformatics, many methods, which based on features like flexibility, solvent accessibility (Novotny et al., 1986; Kulkarni-Kale et al., 2005) and amino acid propensity scales (Haste et al., 2006), are available for antibody interacting residues identification if the 3-D structure of antigen or its homolog is known.

In the previous work, we obtained a mouse anti-C5a antibody F20 using hybridoma technique (data not shown in the study). According to the crystal structure of C5a and modeling structure of F20 Fv fragment, the interaction mode between C5a and F20 was

* Corresponding author. Tel.: +86 10 66931325.
E-mail address: liyan62033@yahoo.com.cn (Y. Li).
[1] These authors contributed equally to this work.

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studied theoretically. The key residue in C5a identified by F20 was predicted and tested by mutant experiment.

2. Methods and materials

2.1. Antibody Fv fragment structure modeling

The amino-acid residue sequences of the light and heavy chains variable domains of anti-C5a antibody, named as F20 (a novel functional anti-C5a antibody, obtained in our lab using hybridoma technique, data not shown) (V_L and V_H) were compared with the primary sequences of all immunoglobulins deposited in the Protein Data Bank (Berman et al., 2000) using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/, Altschul et al., 1997). The CDR (Complementary Determinant Region) definition of anti-C5a antibody F20 variable domains was studied using Kabat method (http://immuno.bme.nwu.edu/, Kabat et al., 1991). The best match for the V_H of anti-C5a antibody F20 was the V_H of mouse monoclonal antibody 10E5 Fab fragment (PDB code: 1ty3, Xiao et al., 2004), where there was 82% identity of residue sequences. The most homologous V_L of anti-C5a antibody F20 was the V_L of Fab1583 fragment neutralizing a broad range of HIV-1 (PDB code: 1nld, Davies et al., 1997). To obtain the F20 V_H and V_L complex structure, the crystal structure of anti-prion protein scFv fragment (PDB code: 2hh0, Luginbuhl et al., 2006) was selected as template. Consequently, the framework of 1NLD V_L and 1TY3 V_H was superimposed on the corresponding 2HH0 V_L and V_H framework, using a rigid-body superimposition program (Zuker and Somorjai, 1989). A composite Fv domain was thereby created with V_L of 1NLD and V_H of 1TY3 in which most of the 'key' residues and their interactions in the V_L:V_H interface were conserved.

Using Homology and Docking methods (Molecular Simulations, San Diego, CA), the complex structure of F20 V_H and V_L was modeling. In order to minimize steric clashes and ensure correct bond lengths and angles after modeling, the model structure of CDRs were subjected to 10,000 steps of energy minimization using Discover program, while the alpha carbons of FRs were held fixed in position.

2.2. Computer-guided molecular docking method

A model structure of C5a was generated from its crystal structure (PDB code: 1kjs, Zhang et al., 1997). Using Docking method, the original C5a–F20 complex structure was constructed.

There was an obvious similarity in shape of the inter-domain groove of the variable region of antibodies. Inspection of the molecular surface of the binding sites F20 indicated that a pocket in the bottom of center could provide an anchor point for the epitopes of the C5a. In order to minimize steric clashes and ensure correct interaction non-bond distances, angles and hydrogen bonds after molecular docking, the interaction domains between C5a and F20 were subjected to 5000 steps of energy minimization, while the remainder was held fixed in position.

To avoid the conformation of the interaction domains trapped in a local potential energy minimum, residues at the base of the interaction domains were held fixed while the remainder of the interaction domains was subjected to simulated heating and molecular dynamics at elevated temperatures followed by slow cooling to a low energy conformation. The interaction domain residues were initially assigned a temperature of 300 K and slowly heated to 500 K in increments of 25 K, with 50 dynamics steps at each temperature using a timestep of 1 fs. The structure was similarly heated to 1000, 2000, 3000 and 4000 K. At each temperature the interaction domains were subjected to a 100 ps dynamics run followed by slowing to 300 K, and two series of minimizations, first for 500 steps, then 3000 steps.

Based on the 3-D optimized complex structure of C5a–F20, the binding sites between C5a and F20 were predicted and a series of C5a mutants were designed.

2.3. Recombinant C5a and C5a mutant preparation

DNA sequences encoding human C5a was obtained by reverse transcriptase polymerse chain reaction (RT-PCR) using RNA isolated from peripheral blood leukocytes. C5a mutants (i.e., C5aM1 (Asn^{64} was replaced by alanine), C5aM2 (Ile^{65} was replaced by alanine), C5aM3 (Ser^{66} was replaced by alanine), C5aM4 (Lys^{68} was replaced by alanine), C5aM5 (Asn^{64} was replaced by alanine, and $Ser^{66}$was replaced by alanine), C5aM6 (Asn^{64} was replaced by alanine, Lys^{68} was replaced by alanine), C5aM7 (Ser^{66} was replaced by alanine, Lys^{68} was replaced by alanine), C5aM8 (Asn^{64} was replaced by alanine, $Ser^{66}$was replaced by alanine, and Lys^{68} was replaced by alanine) were generated using overlapped PCR methods by introducing the GCT (coding alanine) into the mutation site. All the plasmids constructed above were confirmed by DNA sequencing. The C5a or mutants were expressed in BL21 and purified with nickel-chelated affinity column by standard methods from the supernatant of cell lysate. Then the purified proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Western-Blotting of F20 binding activity of human C5a or C5a mutant

RhC5a protein and rhC5a mutants (2 μg) were subjected to 15% SDS-PAGE followed by transfer to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences) and probing with the anti-C5a antibody F20 (2 μg/mL). The goat anti-mouse antibody labeled with horseradish peroxidase (HRP) was used as the secondary antibody. The binding activity of F20 with human rhC5a or rhC5a mutant was detected by the enhanced chemiluminescence (ECL) Western blotting detection system.

2.5. ELISA analysis of F20 binding activity to human C5a or C5a mutant

ELISA was performed using plates coated with 100 nM of Trx, rhC5a, rhC5a mutants and human C5a (sigma), respectively. Anti-C5a antibody F20 (0.08, 0.4, and 2 μg/mL) prepared with dilution buffer was then added to each well. The goat anti-mouse antibody labeled with horseradish peroxidase (HRP) was used as the secondary antibody. Binding signals were visualized using OPD substrate and analysed at 492 nm. The OD value for recombinant C5a was set as 100% binding activity. The binding capability of C5a mutant is calculated by $OD_{C5a\ mutant}/OD_{rhC5a}$.

2.6. Binding kinetics assays

Furthermore, we measured the binding kinetics of the anti-C5a antibody F20 to C5a and its mutants, using Bio-Layer Inter-Ferometry on Octet RED (ForteBio, USA). All interaction analyses were conducted at 30 °C in PBS buffer unless stated otherwise. Sensor tips were prewet for 5 min in buffer immediately prior to use, and the microplates used in the Octet were filled with 200 μL of sample or buffer per well and agitated at 1000 rpm. Anti-C5a antibody F20 (25 μg/mL) was loaded to saturation onto anti-mouse IgG capture biosensors, then we washed the loaded biosensors in buffer for 120 s and transferred to wells containing C5a and its mutant proteins at concentrations of 300 nM, 100 nM, 30 nM in