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Identification of the Key Functional Domain of BAFF for Binding TACI by Computer-Guided Molecular Modeling Method

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Abstract

As an important member of the Tumor Necrosis Factor (TNF) super family, B cell activating factor (BAFF: also known as BLyS, TALL-1, THANK and zTNF4 or TNFSF13b) induces B cell proliferation and differentiation both *in vivo* and *in vitro*. A series of experimental results showed that the soluble form of a BAFF receptor, trans-membrane activator and CAML-interactor (TACI), could alleviate the autoimmune symptoms of NZBWF1 and MRL-lpr/lpr mice. Furthermore, the fusion protein TACI-Fc, as a novel BAFF antagonist, was used to the treatment of relapsing multiple sclerosis. However, it is not clear how BAFF interact with its receptor TACI. In this study, using the crystal structures of BAFF and its receptor TACI, the binding mode and the key domains of BAFF interaction with TACI were analyzed based on the computer-guided molecular modeling method. According to the theoretical predictions, a series of mutants of BAFF, including M1 (from Ile^{158} to Phe^{165}), M2 (from Asp^{203} to Leu^{211}), M3 (from Ser^{225} to Arg^{231}) and M4 (from Ile^{233} to Glu^{238}), were designed and evaluated with biological experiments. The results showed that the domains M2 and M4 of BAFF were the key domains interacting with TACI, which was in accord with our theoretical results. The results will highlight the clues for further development of novel BAFF inhibitors.

Keywords: BAFF; TACI; Domain; Molecular modeling

Introduction

B cell Activating Factor (BAFF: also known as BLyS, TALL-1, THANK and zTNF4 or TNFSF13b), identified in 1999, is an important member of the TNF family of cytokines and plays a major role in B cell survival, proliferation and differentiation [1-4]. BAFF is a type II transmembrane protein, and its extracellular domain can be cleaved at the cell membrane to yield biologically active soluble homotrimeric BAFF. BAFF is mainly produced by innate immune cells such as macrophages/monocytes, neutrophils, Dendritic cells (DCs), activated T cells and malignant B cells, and by non-lymphoid cells like epithelial cells as well [5,6]. Its biological role is mediated by three distinct receptors: B-cell Maturation Antigen (BCMA), Transmembrane Activator, calcium modulator and Cyclophilin Ligand Interactor (TACI) and BAFF receptor (BAFF-R, also known as BR3) [7,8].

A series of research works showed that, BAFF plays an important role not only in B-cell survival and tolerance, but also in B-cell maturation and diversification, including IgG and IgA class switching [9]. Besides, it is now clear that BAFF can also modulate T cell function *in vitro* and *in vivo* [10]. Thus, BAFF plays an important role in humoral immunity. Most studies demonstrate that overproduction of BAFF is associated with human autoimmune diseases and B cell malignancies [11].

Several lines of evidence indicate that TACI may be the most versatile receptor responsible for BAFF-mediated function [12-15]. TACI is emerging as an unusual TNF receptor-like molecule with a sophisticated mode of action [16,17]. Recently, the soluble protein TACI was shown to alleviate the autoimmune phenotype of NZBWF1 and MRL-lpr/lpr mice [18] and its fusion protein (TACI-Fc) was used as a novel BAFF antagonist to treat the relapsing multiple sclerosis induced by BAFF and APRIL.

As well known, protein-protein recognition plays an essential role in structure and function [19]. Specific non-covalent interactions stabilize the structure of macromolecular assemblies and sequential signaling pathways essential for cell function [20]. The number of protein-protein interactions far exceeds the number of proteins themselves and their experimental characterization, while improving, remains relatively slow [21,22]. With the development of computational biology, it becomes possible to understand the physical basis of protein-protein interaction and constrain the molecular basis of their specificity based on the computational prediction.

Till now, the binding mode and domains between BAFF and its receptor TACI were completely understood. In the present study, the key domains of BAFF identified by TACI were determined.

Methods and Materials

Computer-guided molecular modeling and docking

Based on the crystal structure of BAFF (PDB code: 1kd7) and its receptor TACI (PDB code: 1xu1), the hydrogen atoms were added and optimized using molecular mechanism method under AMBER forcefield. Based on the computer-guided molecular docking method, the 3-D complex structure of BAFF and TACI was constructed and optimized using molecular dynamics method [23,24]. Using computer graphics technology, distance geometry, intermolecular hydrogen

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bond and the difference of the solvent accessible areas between complex and monomer methods, the identified domain of BAFF by TACI was predicted.

Recombinant BAFF and BAFF mutant preparation

The cDNA encoding extracellular domain of human BAFF was obtained by reverse transcriptase polymerse chain reaction (RT-PCR) using RNA isolated from peripheral blood leukocytes, inserted into pET-32a to construct a recombinant plasmid pET-32aBAFF.

According to the predicted key domains of BAFF identified by TACI, the residues were substituted with alanine by over-lapped PCR to construct recombinant plasmids of BAFF mutants. Four domain mutants of BAFF (*i.e.* BAFFM1 (from Ile^{158} to Phe^{165} were replaced by alanines), BAFFM2 (from Asp^{203} to Leu^{211} were replaced by alanines), BAFFM3 (from Ser^{225} to Arg^{231} were replaced by alanines), BAFFM4 (from Ile^{233} to Glu^{238} were replaced by alanines) were generated using overlapped PCR methods by introducing the GCT (coding alanine) into the mutation sites. All the plasmids constructed above were confirmed by DNA sequencing.

Protein purification

The recombinant plasmids mentioned above were transformed into *E.coli* BL21. The bacteria carrying the recombinant plasmids were induced with IPTG (0.1 mM) at 20°C overnight. After centrifuged at 5000 rpm for 5 mins, the pellets were resuspended in 20 mmol/L sodium phosphate buffer (PB) plus 500 mmol/L sodium chloride (NaCl, pH 8.0). The bacteria were broken by sonication and centrifuged at 9000 rpm for 15 mins to remove cell debris. The supernatant of cell lysate was purified with a nickel-chelated affinity column by standard methods. Then the purified proteins were analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA analysis

ELISA was performed using plates coated with 200 nM of Thioredoxin (Trx), rhBAFF, rhBAFF mutants and standard human BAFF (Peprotech) respectively. BAFF receptor TACI-Ig (0.01, 0.1, 1 and 10 μg/mL) prepared with dilution buffer was then added to each well as the first antibody. The peroxidase-conjugated affinipure goat anti-human IgG was used as the secondary antibody. Binding signals were visualized using TMB substrate and analyzed at 450 nm. The OD value for recombinant BAFF was set as 100% binding activity. The binding capability of BAFF mutant was calculated by $OD_{BAFF\ mutant}/OD_{rhBAFF}$.

Binding kinetics assays

The binding kinetics of the TACI-Ig to BAFF and its mutants were measured 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 pre-wet for 5 mins 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. TACI-Ig (30 μg/mL) was loaded to saturation onto anti-human IgG capture biosensors, and then we washed the loaded biosensors in buffer for 120 seconds and transferred to wells containing BAFF and its mutant proteins at concentrations of 900 μg/mL, 600 μg/mL, 300 μg/mL in buffer. We measured BAFF and its mutants association and dissociation for 15 mins, respectively. Kinetic parameters (k_{on} and k_{off}) and affinities (K_D) were calculated from a non-linear global fit of the data between antigen and antibody using the Octet software. Multiple independent measurements were performed.

Mouse splenocyte survival assay for BAFF bioactivity

B cells were isolated from the spleens of C57BL/6 mice (4-6weeks). Mice were killed by cervical dislocation and spleens were collected under sterile conditions. Splenocytes of mouse spleen were isolated by density gradient centrifugation over Ficoll-Paque™ PLUS (TBD Sciences). Cells were washed twice with medium (RPMI 1640, HyClone) and then B cells were isolated by a mouse B Cell Isolation Kit (Miltenyi Biotech, Germany) according to the Mini-Macs protocol (Miltenyi). At the end of the purification procedure, mouse B cells were re-suspended at 1×10^6 cells/ml in RPMI1640 plus 10% FBS, dispensed into 96-well plates at 0.1 ml/well and incubated at 37°C in a 5% CO_2 humidify incubator. After 2 hours, several concentrations (i.e. 10 μg/ml, 1 μg/ml and 0.1 μg/ml) of BAFF and mutants were added and the cell suspensions were incubated for 72 hrs at 37°C in a 5% CO_2 humidify incubator. The same concentrations of thioredoxin were used as the negative controls. 3 days later, 10 μl MTS (Promega Co, US) was added to all wells and incubation continued for a further 4hrs. The MTS absorbance values were tested at 492 nm. These plots were representative of at least five experiments.

Results

The predicted key domains of BAFF identified by TACI

The optimized 3-D complex structure of BAFF and TACI was obtained and shown in (Figure 1). According to the theoretical 3-D complex structure of BAFF and TACI, based on the difference of solvent accessible area of the monomer and complex, considering the intermolecular hydrogen bond forming, the interaction mode between BAFF and TACI was analyzed. The potential key domains of BAFF identified by TACI were predicted.

Comparing the monomer BAFF and complex of BAFF-TACI, the accessible solvent areas of the domain from Asp^{203} to Leu^{211} of BAFF decreased 26.03 $Å^2$ when the monomer BAFF interacted with TACI, from Ile^{233} to Glu^{238} of BAFF decreased 42.41 $Å^2$ when the monomer BAFF interacted with TACI. In addition, the accessible solvent areas

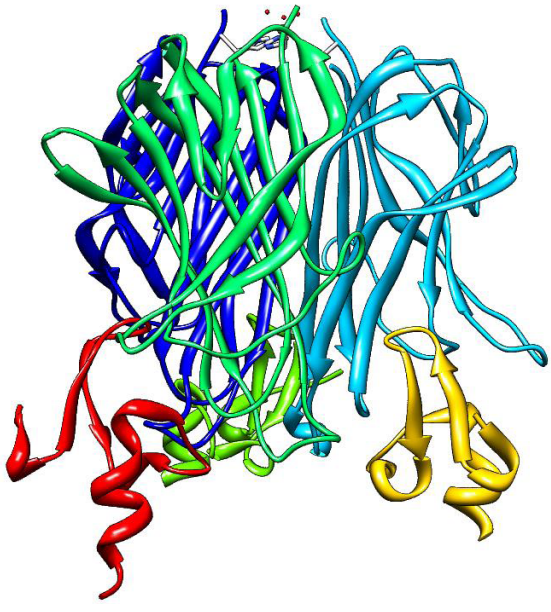

Figure 1: The 3-D optimized complex structure of BAFF and TACI based on computer-guided molecular docking method.

of the domain from Ile^{158} to Phe^{165} of BAFF decreased 4.26 Å^2 when the monomer BAFF interacted with TACI, from Ser^{225} to Arg^{231} of BAFF increased 5.29 Å^2 when the monomer BAFF interacted with TACI. The results showed that the domains from Asp^{203} to Leu^{211} and from Ile^{233} to Glu^{238} of BAFF were the major domains interacting with TACI, while the domains from Ile^{158} to Phe^{165} and from Ser^{225} to Arg^{231} were not the binding sites of BAFF for TACI.

Characterization of the key domains in BAFF identified by TACI

With the theoretical analysis of the interaction between BAFF and its receptor TACI, five domain mutants of BAFF (i.e. BAFFM1, BAFFM2, BAFFM3, and BAFFM4, as mentioned above) were designed. The residues of the mutant domains were replaced by alanines. BAFF and mutants were purified and analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The binding activities between TACI and BAFF, BAFF mutants were measured by ELISA as shown in (Figure 2). The results showed that the M2 and M4 mutants lost all binding ability to TACI.

To evaluate the binding activity of the receptor (i.e.TACI) and the ligand (i.e. BAFF and its mutants) further, we detected the interacting kinetics of TACI and BAFF or BAFF mutants. As shown in (Figure 3) and Table 1, the BAFF mutants, include (M2, M4) lost the binding ability with TACI almost.

Biological effect of the key domains in BAFF

The biological effect of the BAFF mutants (mentioned above) was assessed by BAFF-induced mouse splenocyte survival assay as shown in (Figure 4). It was shown that the mutants, which included M2 and M4, lost the biological effect of BAFF mostly. The results showed that the key domains in BAFF identified by TACI were the functional domains and acted very important roles in BAFF functions.

Discussion

Many proteins have evolved to form specific molecular complexes and the specificity of this interaction is essential for their function [25]. Protein-protein interactions are facilitated by myriad of residue-residue contacts on the interacting proteins. Identifying the sites of interaction in the proteins is a key for deciphering its functional mechanisms, and is crucial for drug development.

Serum levels of BAFF have been found to be elevated in patients with autoimmune diseases, such as Systemic Lupus Erythematosus (SLE) [26], rheumatoid arthritis [27], and sjogren's syndrome [28]. A

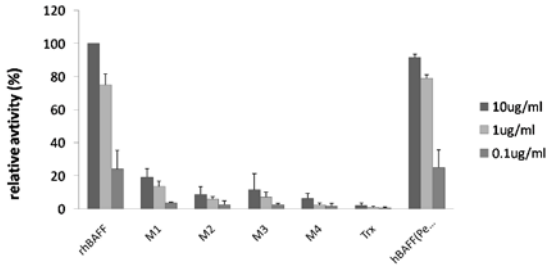

Figure 2: The binding activity of TACI to BAFF and its domain mutants were tested with ELISA method. ELISA was performed using plates coated with 100nM of Trx 、 rhBAFF 、 rhBAFF mutants and human BAFF (PEPROTECH) respectively. TACI-Ig prepared with dilution buffer was then added to each well. The HRP-GAH was used as the secondary antibody. Binding signals were visualized using TMB substrate and analyzed at 450 nm. The OD value for recombinant BAFF was set as 100% binding activity. The binding capability of BAFF mutant was calculated by $OD_{BAFF\ mutant}/OD_{rhBAFF}$.

rhBAFF: recombinant human BAFF; Trx (Thioredoxin) was a BAFF-unrelated protein and used as the negative control. BAFF mutants (i.e. BAFFM1 (from Ile^{158} to Phe^{165} were replaced by alanines), BAFFM2 (from Asp^{203} to Leu^{211} were replaced by alanines), BAFFM3 (from Ser^{225} to Arg^{231} were replaced by alanines), BAFFM4 (from Ile^{233} to Glu^{238} were replaced by alanines)); human BAFF(peprotech): positive control.

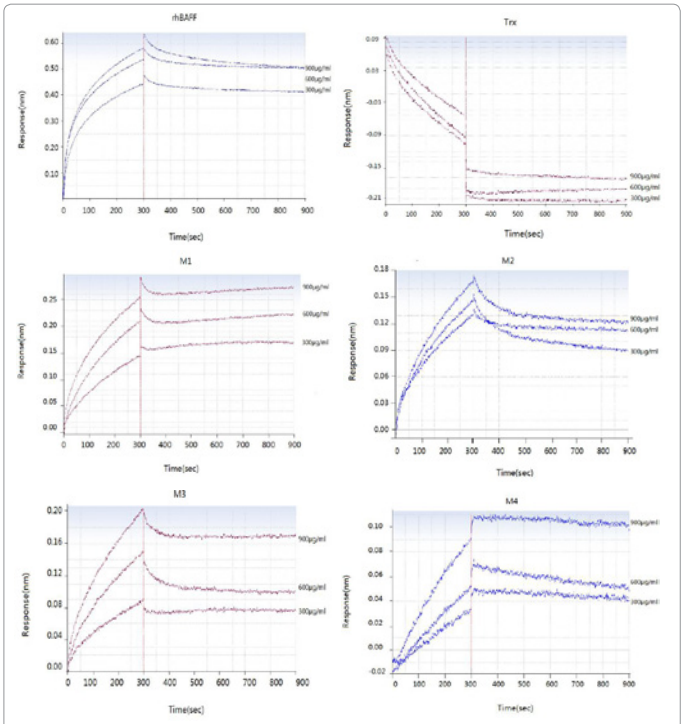

Figure 3: The kinetic activity measurement of TACI and BAFF or its doman mutant. Affinity measurement on the sensor surface using ForteBio: cruve of the experimental data corresponding to phases of association and dissociation of proteins (Trx, rhBAFF, rhCBAFF mutants) at various concentrations on the TACI-Ig anchored to the sensor chip. Starting from the bottom the protein concentrations are: 900, 600, 300 μg/mL. These curves were used to determine K_D, K_{on} and K_{off}.

Trx 、 rhBAFF 、 M1 、 M2 、 M3 and M4 (Figure 2).

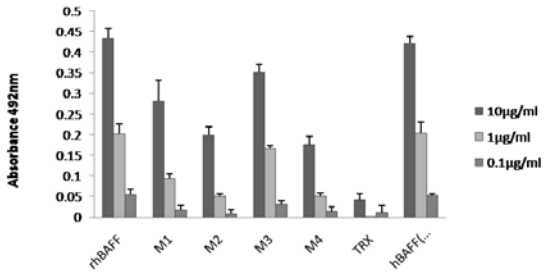

Figure 4: Evaluation of proliferative activity of BAFF in mouse splenocytes.

Induction of mouse splenocyte surivival is an important biological feature of BAFF.

Recombinant hBAFF and hBAFF mutants stimulated the proliferation of mouse splenic B cells in a dose-dependent manner. The results indicated that rhBAFF and rhBAFF mutants could promote the survival of mouse splenic B cells *in vitro*. The control protein trx did not have any survival effect. Among these BAFF mutants, the proliferative activity of M2 and M4 had obviously decrease, which confirmed that the mutated residues in M2 and M4 domains were significant in BAFF function. Trx, rhBAFF , M1, M2, M3 and M4: see Figure 2.

human BAFF(peprotech): positive control.

Variant	K_D (M)	K_{on} (1/Ms)	K_{off} (1/s)	R^2
rhBAFF	3.63E-08	9.64E+02	3.50E-05	0.872721
Trx	---	---	---	0*
M1	3.18E-07	2.93E+02	9.34E-05	0.951368
M2	1.17E-06	4.18E+02	4.88E-04	0.914619
M3	5.33E-07	4.60E+02	2.45E-04	0.959939
M4	---	---	---	0*

Table 1: The binding kinetics of the BAFF receptor TACI to BAFF and BAFF mutant using the ForteBio system Off-rate kinetics (koff) were measured by saturating the chip with TACI and then monitoring dissociation after switching to buffer. On-rate kinetics (kon) was measured using 3 kinds of dilutions of BAFF or BAFF mutant. K_D, the equilibrium dissociation constant, was calculated as K_{off}/K_{on}. Trx、rhBAFF 、M1、M2、M3 and M4: see Figure 2.

The bar (---) means the data is too limited to be analyzed.

*Curve equations could not be established to fit with the experiment data and fitting accuracy (R^2) showed only zero.

series research works showed that the preferred antagonist to BAFF was its soluble receptor TACI [18].

Recently, the 3-D crystal structures of BAFF and TACI were obtained. In this study, using molecular docking method and molecular dynamics stimulation, the 3-D complex structure of BAFF and TACI was constructed and the interaction domains were determined. The key domains in BAFF identified by TACI were the domains from Ile^{233} to Glu^{238} and from Asp^{203} to Leu^{211}, and the biological experiment results confirmed that the domain was the important site. In this research work, we had brought together several experimentally determined protein-protein interaction data and assessed the prediction accuracy of protein function based on them.

The results highlight the novel antagonists design and virtual screening based on the computer-guided molecular design method.

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