

Two Observed Regions in B Lymphocyte Stimulator Important for Its Biological Activity

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Abstract B lymphocyte stimulator (BLyS), a member of the tumor necrosis factor superfamily of ligands, is a crucial survival factor for B cells. We successfully constructed seven mutants of the functional soluble fragment of human BLyS (named cBLyS, amino acid 134–285), including three deletion mutants and four site-directed mutants. All the mutant proteins were expressed in *Escherichia coli* and purified by Ni-NTA affinity chromatography. The biological activities of these mutants were assessed by the ligand-receptor binding assay, B cell proliferation assay and immune effect response *in vivo*. Our results indicated that four residues, H²¹⁸, F²²⁰, T²²⁸ and L²²⁹, are indispensable for the biological activity of cBLyS, whereas two regions, amino acid 134–148 and amino acid 271–285, are related to the biological activity of BLyS. The protein of deletion of amino acid 134–148 leads to a complete defection in raising the antigen-specific IgM titer. The deletion of amino acid 271–285 reduces the effectiveness compared with the native cBLyS. This indicates that the region of amino acid 134–148 is indispensable for cBLyS to function normally.

Key words B lymphocyte stimulator (BLyS); deletion mutant; biological activity

B lymphocyte stimulator (BLyS), known as BAFF, THANK, TALL- α and zTNF4 [1–4], is a member of the tumor necrosis factor (TNF) superfamily of cytokines, which play an important role in B cell immunity. Produced predominantly by myeloid cells, BLyS is one of the B cell survival factors and a co-stimulator of B cell proliferation and antibody secretion. BLyS, a type II membrane protein, is produced first as a 285-amino acid membrane bound precursor, and then cleaved by a furin-like protease to produce a 152-amino acid soluble protein (cBLyS) [1,2,5]. BLyS is a trimeric molecule, including a globular extracellular TNF-homologous domain, an extracellular stalk, a short transmembrane segment and a small cytoplasmic domain [6].

The BLyS receptor system is rather complex because two of the receptors, transmembrane activator and CAML interactor (TACI) [7] and B cell maturation antigen

(BCMA) [8], interact with a sTNF family ligand known as APRIL [9–11]. The third receptor, termed BAFF-R, does not interact with APRIL or any other TNF family member other than BLyS [12,13].

The human BLyS homotrimer crystal structure, typical of the TNF family, has been determined previously [6,14,15]. The BLyS monomer is a jellyroll β -sandwich and oligomerizes to form a homotrimer. The receptor-binding region in BLyS is a deeper, more pronounced groove than that in other cytokines. The conserved elements on the “floor” of this groove allow cytokine recognition of several structurally related receptors, whereas variations on the walls and outer rims of the groove confer receptor specificity.

In this paper, partly based on the results of Oren *et al.* [6], four amino acids, H²¹⁸, F²²⁰, T²²⁸ and L²²⁹, which were residues to surface accessible on the “floor”, were chosen as mutation sites to explore their importance in the cBLyS-BCMA interactions. We also obtained three deletion mutants of cBLyS. Our results indicate that these deletions might be important in the biological activity of cBLyS,

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whereas H²¹⁸, F²²⁰, T²²⁸ and L²²⁹ are not critical residues to cBLyS.

Materials and Methods

Materials

BALB/c mice were obtained from Shanghai Experiment Animal Center, the Chinese Academy of Science (Shanghai, China). pT7450-C-BLyS was constructed, as described previously [16]. All primers were synthesized by SBS Genetech (Beijing, China). T4 DNA ligase, restriction enzymes and Pyrobest DNA polymerase were purchased from TaKaRa (Dalian, China). The paired, purified and HRP-conjugated anti-mouse IgG, IgM and anti-human IgG were purchased from Dingguo (Beijing, China). Recombinant His-cBLyS protein, BCMA-Fc protein, other plasmids and strains were stored in our laboratory.

DNA construction

Using pT7450-C-BLyS as a template, DNA of three deletions (Δ N15, Δ C15 and Δ NC) and four mutants (T1, T2, T3 and T4) of human cBLyS were constructed by polymerase chain reaction (PCR) or overlap-extension PCR. The PCR products were purified, digested with *Nde*I and *Xho*I, and ligated to the expression vector pT7-450, which was digested with the same enzymes. The ligation mixture was transformed into *Escherichia coli* BL21(DE3) and positive clones were screened by double enzyme digestion and DNA sequencing. Primers used for deletion or site-directed mutagenesis and the mutated or deleted amino acids are listed in **Table 1**.

Expression and purification of the mutants

The transformants were grown at 37 °C in 500 ml LB medium containing 100 µg/ml ampicillin. IPTG was added to the final concentration of 0.5 mM when the culture turbidity at 600 nm reacted 0.6. After 8 h of incubation at 20 °C, cells were harvested by centrifugation at 6000 *g* at 4 °C for 10 min and the pellet was resuspended in 50 ml buffer A (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl). The cells were disrupted by sonication. After centrifugation (15,000 *g*) at 4 °C for 20 min, the supernatants containing soluble cytoplasmic proteins were loaded onto a 5-ml Ni-NTA affinity column. Unbound proteins were removed by buffer A containing 80 mM imidazole. Bound proteins were eluted with buffer A containing 200 mM imidazole. The eluted fraction was pooled and dialyzed against phosphate-buffered saline (PBS), then stored at -80 °C until use.

Receptor binding assayed by enzyme-linked immunosorbent assay (ELISA)

The 96-well plates were coated with 5 µg/ml different mutants in PBS overnight at 4 °C and blocked using 4% skimmed milk (300 µl). After washing with PBS, BCMA-Fc was added and incubated for 1 h at 37 °C. The ligand-receptor interactions were detected with horseradish peroxidase (HRP)-conjugated mouse-anti-human antibody (1:1000). Tetramethylbenzidine (TMB) substrate was used for detection and the reaction was stopped with 2 M H₂SO₄. The absorbance at 450 nm (*A*₄₅₀) was determined using a microplate reader.

Murine B cell proliferation

Spleen cells were isolated from 6- to 8-week old female

Table 1 Mutants of cBLyS

Name	Position	Mutant type	Primer sequence (5' → 3')
Δ N15	134–148	Deletion	CAAGAATTCCATATGCTGATTGCAGAC CAAGCTTCTCGAGCAGCAGTTTCAATG
Δ C15	271–285	Deletion	ATGGGAATTCCATATGGCCGTTTCAGGGTCCAG CTCCATCCTCGAGTATTTGTGCATTT
Δ NC	134–148, 271–285	Deletion	CAAGAATTCCATATGCTGATTGCAGAC CTCCATCCTCGAGTATTTGTGCATTT
T1	220/228/229	Mutation	ATGGGAATTCCATATGGCCGTTTCAGGGTCCAG
T2	220/228/229	Mutation	CACCAGACTCAATTCATCCCAN(ACGT)CTCN(ACGT)GACCTTC
T3	218/220/228/229	Mutation	TGGGGATGAATTGAGTCTGGTGN(ACGT)CTCN(ACGT)GTTTCGATGT
T4	218/228/229	Mutation	CAAGCTTCTCGAGCAGCAGTTTCAATG

BALB/c mice by Ficoll centrifugation. B cells (purified by polyamide fiber [17], 1×10^5 cells/well) were stimulated with recombinant protein in the presence of 20 $\mu\text{g/ml}$ anti-IgM. Proliferation was measured by incubating the cells with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) for the final 5 h of the 72 h culture. Then, the cells were incubated overnight at 37 °C with 15% sodium dodecyl sulfate (SDS), 15 mM HCl. The absorbance was measured at 450 nm.

Immunization

Female BALB/c mice aged from 8 to 10 weeks (18–21 g) received intraperitoneal immunization with 100 μg lysozyme in incomplete Freund's adjuvant solution on day 0. Then mice were randomized into several groups of five mice each. The mutated proteins (ΔN15 , ΔC15 and ΔNC), cBLyS or PBS were administered intravenously once per day within the following 7 d. Lysozyme-specific IgM and IgG titers were determined on sera collected on day 8 by ELISA. The 96-well plates were coated with 5 $\mu\text{g/ml}$ lysozyme in PBS overnight at 4 °C and blocked with 4% skimmed milk (300 μl). After washing with PBS, serial dilutions of different mice sera were added and incubated for 1 h at 37 °C. The lysozyme-specific IgM and IgG were detected with HRP-conjugated anti-mouse IgG, anti-mouse IgM antibody (1:1000).

Results and Discussion

Production and affinity purification of the mutant BLyS

The mutants were constructed by PCR (Fig. 1) and

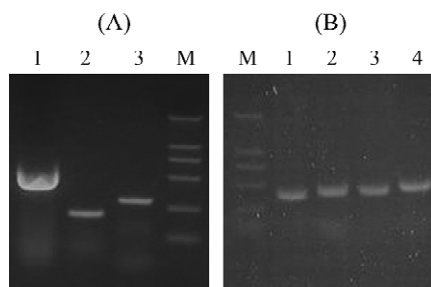


Fig. 1 Polymerase chain reaction (PCR) products of the mutants on 1% agarose gel

(A) Overlap PCR product bands of the site-directed mutants. 1, mutant cBLyS; 2, left fragment of cBLyS; 3, right fragment of cBLyS; M, DL2000 DNA marker. (B) PCR product band of the deletion mutants. M, DL2000 DNA marker; 1, ΔNC fragment; 2, ΔN15 fragment; 3, ΔC15 fragment; 4, cBLyS fragment.

expressed in host BL21(DE3) cells. Using the low culture temperature and inducer concentration, 40% of the mutant proteins could be obtained in the supernatants and 60% in intracellular precipitate. Only the proteins in the supernatants were purified.

Expression vector pT7-450 was used because the target protein was expressed as a fusion protein with a His₆-tag to facilitate subsequent purification. So, the mutant proteins were purified in a one-step procedure by Ni-NTA affinity sepharose column. Different concentrations of the imidazole were tested in equilibrium buffer. The most nonspecific bounded proteins could be eluted by 80 mM imidazole. Mutants were eluted with buffer A containing 200 mM imidazole. The result of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the purity was no less than 85% (Fig. 2). No attempts were made to remove the impurities by further purification steps. The molecular weight of ΔC15 , ΔN15 and ΔNC was determined by MALDI-TOF-MS (ΔN15 , 16636.8 Da; ΔC15 , 16521.8 Da; ΔNC , 15058.8 Da), which complied with our expectation (ΔN , 16,635.9 Da; ΔC , 16520.7 Da; ΔNC , 15058.1 Da). But the results of both normal SDS-PAGE and SDS-PAGE with 8 M urea were abnormal, in which ΔN15 showed an apparently lower molecular weight than anticipated. The reason for this remains unknown.

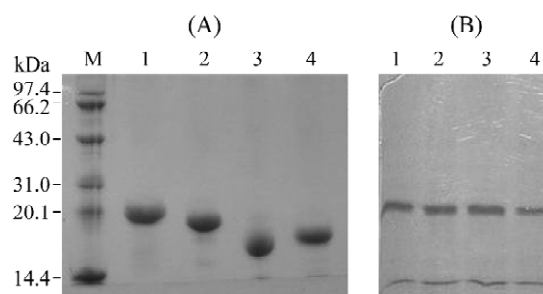


Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified mutant proteins

(A) SDS-PAGE analysis of deletion mutants with 12% polyacrylamide under denatured conditions. M, marker; 1, recombinant cBLyS; 2, ΔC15 mutant; 3, ΔN15 mutant; 4, ΔNC mutant. (B) SDS-PAGE analysis of site-directed mutants. 1, purified T1; 2, purified T2; 3, purified T3; 4, purified T4.

Receptor binding studies

The biological activity of mutant cBLyS was evaluated by testing its binding ability to the receptor (BCMA). BSA and another member of the same TNF superfamily were

used as controls. ELISA was performed by coating with different ligands, and the results showed that all of the mutants cBLyS bound to the receptor (BCMA-Fc) with higher affinity. However, the controls were negative (Fig. 3). Therefore, all mutants were biologically active. Thus the 45-bp deletion on amino acid 134–148 or/and amino acid 271–285 of cBLyS does not affect its binding ability to BCMA. There were no remarkable differences between the four site-directed mutants and cBLyS.

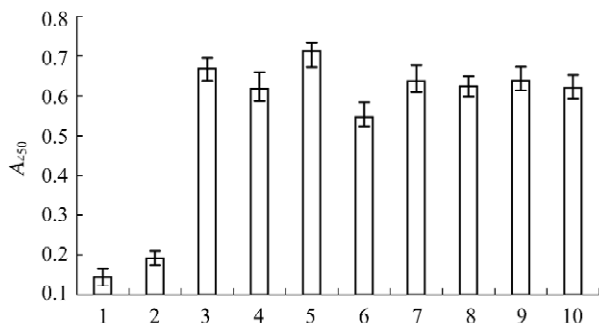


Fig. 3 Specific interaction of mutant proteins and BCMA-Fc

The interaction of mutant proteins and BCMA-Fc. 1, BSA+BCMA-Fc; 2, TNF+BCMA-Fc; 3, cBLyS+BCMA-Fc; 4, ΔC15+BCMA-Fc; 5, ΔN15+BCMA-Fc; 6, ΔNC+BCMA-Fc; 7, T1+BCMA-Fc; 8, T2+BCMA-Fc; 9, T3+BCMA-Fc; 10, T4+BCMA-Fc.

In vitro murine B cell proliferation

Although the four site-directed mutants have the similar binding ability to the BCMA with recombinant cBLyS, we investigated their ability to induce B cell proliferation. When they were used in a co-stimulation assay with anti-IgM, all of the mutants and cBLyS were able to stimulate the proliferation of B cells. T1, T2, T3 and T4 induced murine B cell proliferation at concentrations similar to those observed with recombinant cBLyS (Fig. 4). The results showed that site-directed mutation on H²¹⁸, F²²⁰, T²²⁸ and L²²⁹ of cBLyS could change neither the binding affinity to the BCMA nor the ability of induction of proliferation on murine B cells.

In vivo humoral immune response

BLyS, a growth factor that promotes B cell proliferation and differentiation, is being developed to increase the production of endogenous immunoglobulins in patients with certain hypogammaglobulinemias. A potential consequence of increased B cell representation *in vivo* is a relative increase in serum Ig titers [18]. Accordingly, serum IgM and IgG concentrations were compared between

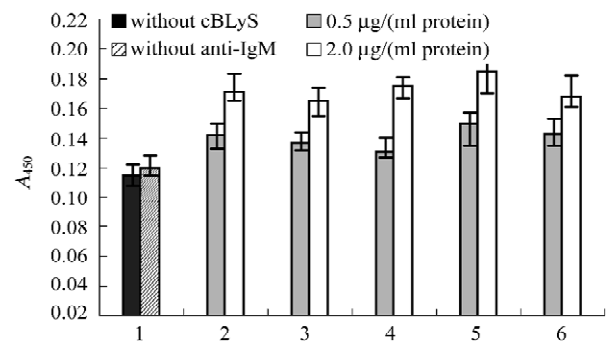


Fig. 4 Induction of B cell proliferation by the mutant proteins

Murine splenic B cell proliferation assay in the presence of 20 μg/ml anti-IgM. 1, control; 2, recombinant cBLyS; 3, mutant T1; 4, mutant T2; 5, mutant T3; 6, mutant T4.

control and mutant cBLyS-treated mice (Fig. 5). Three mutants (ΔN15, ΔC15 and ΔNC) and recombinant cBLyS administration resulted in a high increase in serum IgG. Statistical analysis indicated that, compared with cBLyS, ΔC15 showed lower inducing ability on serum IgM.

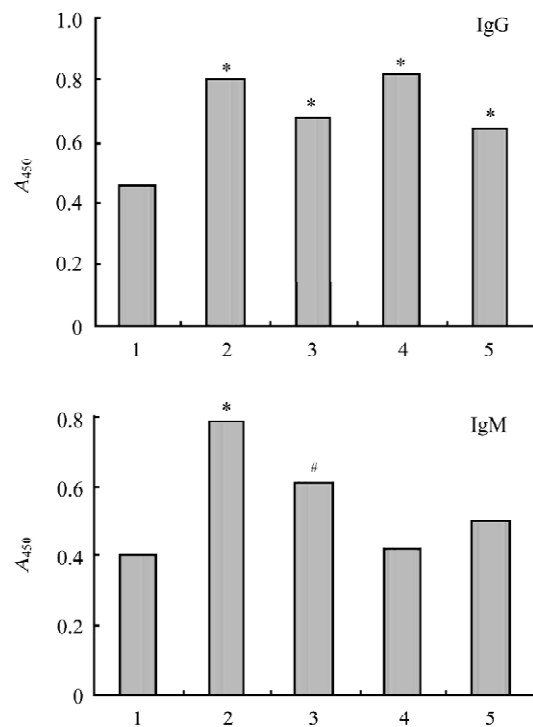


Fig. 5 Effects of i.v. administration of cBLyS and its three deletion mutants on serum immunoglobulins in BALB/c mice

At day 8 after immunization, sera were assayed for lysozyme-specific IgG and lysozyme-specific IgM. 1, PBS; 2, 0.3 mg/kg cBLyS; 3, 0.3 mg/kg ΔC15; 4, 0.3 mg/kg ΔN15; 5, 0.3 mg/kg ΔNC. Statistically significant differences (* P <0.05) between target proteins and control. Statistically significant differences (# P <0.05) between ΔC15 and cBLyS or control.

Circulating IgM concentrations did not increase after 7 d treatment with ΔN15 and ΔNC proteins.

The structure of BLYS revealed a distinct binding groove formed by adjacent monomers within the trimer that permitted the cytokine to discriminate among closely related receptors. One ‘wall’ of the groove contains loop DE with some residues of loops aa’ and GH, and the other wall contains loops EF, Aa and a’A’’ (Fig. 6). Modeling potential interactions of BLYS with TNF-R suggested that the outer rim of the groove (loops DE and the β-hairpin of loop AA’’) would lead to steric conflict [6]. We mutated H²¹⁸, F²²⁰ from strand D and conservative T²²⁸, L²²⁹ from strand E. The results showed that these site-directed mutations of cBLYS could change neither the binding affinity to the BCMA nor the ability to induce murine B cells proliferation. These four sites may not be critical for cBLYS biological

activity. Mutant ΔC15 slightly reduced the biological activity of cBLYS with deletion on strand H, but mutant ΔN15 could not increase the antigen-specific IgM titer which destroys the loop AA’’. Therefore, both regions, amino acid 134–148 and amino acid 271–285, could influence the biological activity of BLYS, of which the former would more important for BLYS activity. Interestingly, ΔBAFF, which lacks 57 nt encoding the loop Aa and is co-expressed with BLYS in many mouse and human myeloid cells, can diminish BLYS bioactivity and release [19]. This suggests that loop Aa probably plays an important role in BLYS activity.

Conclusively, we have successfully constructed three cBLYS mutants with fragment deletion of amino acid 134–148, amino acid 271–285 and co-deletion of these two regions. Four residues (H²¹⁸, F²²⁰, T²²⁸ and L²²⁹) intended

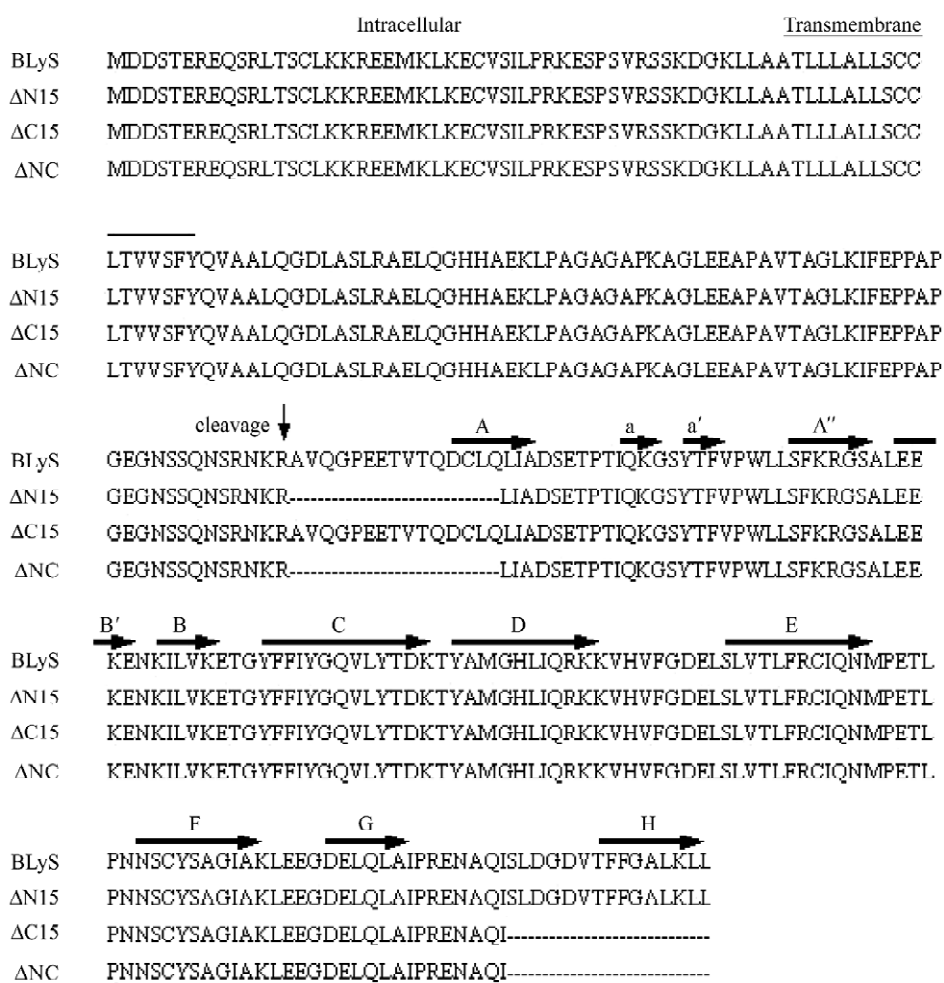


Fig. 6 Amino acid sequence of BLYS and the deletion mutants

The strand and loop designations given to the extracellular domains are according to the crystal structure of human BLYS [6]. Residue deletions on the mutants are shown as dashed lines.

to be surface accessible on the “floor”, have been mutated. The assessment of B cell proliferation *in vitro* suggests that different mutants of H²¹⁸, F²²⁰, T²²⁸ and L²²⁹ had no effect compared with native cBLyS. Statistical analysis of immune response *in vivo* indicated that deletion of amino acid 271–285 might be harmful to the biological activity of cBLyS, whereas deletion of amino acid 134–148 leads to a complete defection of cBLyS in raising the antigen-specific IgM titer.

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