Lamprey buccal gland secretory protein-2 (BGSP-2) inhibits human T lymphocyte proliferation

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Abstract Lamprey is a representative of the agnathans, the most ancient class of vertebrates. Parasitic lampreys secrete anticoagulant from their buccal glands and prevent blood coagulation of host fishes. We identified a buccal gland secretory protein-2 (BGSP-2) from a buccal gland cDNA library of Lampetra japonica. The full-length BGSP-2 gene was cloned and the recombinant BGSP-2 protein was generated. The role of BGSP-2 on lymphocyte proliferation was studied by examining its effects on human T lymphocytes. We found that lamprey BGSP-2 was able to effectively block the proliferation of T cells in vitro by inducing G1/S cell cycle arrest. Furthermore, it inhibited the proliferation of human T lymphocytes stimulated by phytohemagglutinin (PHA) at a minimum concentration of 0.1 μg/ml. Our data suggest that lamprey BGSP-2 is able to block the mitosis of human T lymphocytes at the G1/S point, and has the potential of anti-proliferative effect on PHA-activated T lymphocytes [Current Zoology 56 (2): 252–258, 2010].

Key words Lampetra japonica, BGSP-2, Cell cycle, T lymphocyte, Proliferation

The cysteine-rich secretory protein (CRISP) superfamily comprises a large number of single-chain secretory proteins with different evolutionary origins and functions. CRISPs contain 16 highly conserved cysteine residues and 10 of them locate at the C-terminal region (Eberspaecher et al., 1995). Up to now, most identified CRISPs of non-mammal vertebrates are from the reptile venom. The reptile venom CRISPs have been shown to block multiple ion channels and the hyperpolarization-activated cyclic nucleotide-gated channel (Morrissette et al., 1995). In addition, it has been found that several members of CRISP superfamily, including Shk, Margatoxin, and Correolide, inhibit T lymphocyte mitosis, making them novel immunosuppressant that block the Kv1.3 potassium channel on T lymphocyte (Koo et al., 1999; Bao et al., 2005). T lymphocyte is a key molecule in human immunity and its death is crucial to lymphocyte homeostasis. The proliferation of T lymphocyte, which is induced by the antigen receptor stimulation (Beeton et al., 2006; Kappos et al., 2000; Naik et al., 2004; Ott et al., 2005), is involved in many pathophysiological processes including transplantation, asthma, and many other immune diseases (Song et al., 2004). The voltage-gated potassium channel, Kv1.3, is specifically expressed in human lymphocytes. It controls membrane potential and calcium influx (Wulff et al., 2003; Viglietta et al., 2002; Rus et al., 2005; Ellis and Krueger, 2001; Rinaldi et al., 2006). The blocking of Kv1.3 channels has been found to prevent T cell activation and attenuate immune responses in vivo (Koo et al., 1999; Damjanovich et al., 2004). Thus, the CRISPs, which act as immunosuppressants, could be applied to the immune therapy for autoimmune disease.

Lampreys are the most primitive vertebrates. They represent the only extant members of the oldest class Agnatha dating back over 530 million years (Sower et al., 2006). Lampreys attach to the host fishes by using their sucker-like mouth and feed on the blood. Lampreys secrete anticoagulant called lamphredin from their paired buccal glands preventing the host’s blood from clotting (Gage and Gage-Day, 1927). Two major protein components, buccal gland secretory protein-1 (BGSP-1) and buccal gland secretory protein-2 (BGSP-2), had been identified in secretion of Lampetra japonica buccal gland (Xiao et al., 2007). BGSP-1 possesses fibrinogenolytic activity (Xiao et al., 2007), whereas BGSP-2 is a member of CRISP superfamily. BGSP-2 can block the depolarization-induced contraction of rat-tail arterial smooth muscle, and inhibit high K⁺-induced contraction of rat-tail arterial smooth mus-
cle without toxicity (Ito et al., 2007), which are in accordance with snake venom CRISP proteins (Yamazaki and Morita, 2004). Hence, it can be concluded that BGSP-2 plays an important part in lamprey parasitic feeding due to its vasodilator effect on the blood vessels of hosts.

So far, many researches focus on the function of reptilian venom CRISPs. For lampreys, however, little work has been done on the relationship between BGSP-2 and T lymphocyte proliferation. In the present study, we purified the recombinant *L. japonica* BGSP-2 (rLj-BGSP-2), and examined its effects on the activation and proliferation of human T lymphocyte.

1 Materials and Methods

1.1 Animals
Lampreys *L. japonica* were caught in Tong River, a branch of Songhua River in Heilongjiang Province of China in December.

1.2 EST analysis
The cDNA library of *L. japonica* buccal gland was constructed and more than 1100 expressed sequence tags (ESTs) were sequenced (Gao et al., 2005). The ESTs were analyzed with BLAST searching at NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the putative BGSP-2 was identified. The BGSP-2 sequence is the same as that in Swiss-Prot/TrEMBL database (http://www.ebi.ac.uk/TrEMBL/).

1.3 Sequence alignments
Sequence alignment of the CRISP cysteine-rich domain (CRD) from horse (*Q8HXA1*), human (*P16562*), bovine (*Q3ZCL0*), Erabu sea snake (*Q8JI38*), Inland Taipan (*Q3SB06*), Chinese cobra (*Q7T1K6*), Green snake (*Q09G39*), and lamprey (*A4PIZ5*) was performed with ClustalX software (Jeanmougin et al., 1998).

1.4 Expression vector construction
Total RNA of buccal gland was extracted by using Trizol protocol (GIBCO BRL). RT-PCR was carried out with RT-PCR kit (TaKaRa) to obtain the full-length open reading frame of *BGSP-2* gene. The forward primer: 5’`XXatatgagaggctgtaggagccaa3`; the reverse primer: 5’`XXaagcttagcattggtaggacct3`’. The PCR product, which contained a *Nde* I restriction site and a *Hind* III restriction site flanking the *BGSP-2* cDNA sequence, was cut with *Nde* I and *Hind* III and cloned into pET23b expression vector. The nucleotide sequence of *BGSP-2* gene was sequenced (TaKaRa).

1.5 Expression and purification of the recombinant protein
The recombinant plasmids were transformed into *Escherichia coli* Rosetta and the bacteria was cultivated overnight in LB medium. When OD₆₀₀ of the medium reached to 0.6, 1 mM isopropyl-1-thio-β-D- galactopyranoside (IPTG) was added to the cultures to induce the expression of the fusion protein for 5 h at 37°C. Subsequently, cells were centrifuged at 7000 rpm for 15 min at 4°C, and the pellet was re-suspended in 20 mM Tris-HCl buffer containing 5 mM imidazole and 500 mM NaCl (pH7.9). The cell suspension was sonicated for 3 min interruptedly on ice, centrifuged again at 5000 rpm for 15 min at 4°C. The pellet was re-suspended in 1× Binding buffer (20 mM sodium phosphate, 500 mM NaCl, 45 mM imidazole, pH 7.4) with 8 M Urea and 90 mM DL-Dithiothreitol (DTT), and then incubated at room temperature for 1 h, and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was collected and the histidine tagged fusion protein was purified by His-Bind affinity chromatography (Novagen, USA). The recombinant protein was confirmed in 10% SDS-PAGE. The concentration of the recombinant protein was measured using a Bicinchoninic Acid (BCA) Protein Assay kit (BEYOTIME).

1.6 Human T cell preparation
Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of healthy volunteers by Lymphocyte Separation Medium-H (Sigma, USA). PBMCs were then washed for three times in PBS buffer (without Ca²⁺ and Mg²⁺) containing 0.1% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.4. T lymphocytes were isolated from PBMCs by Dynal T Cell Negative Isolation Kit Version II (Invitrogen Dynal California) (Mandrekar et al., 2004; Harriague and Bismuth, 2002). T lymphocytes were cultured at a concentration of 1×10⁶/mL in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Sigma, USA), 4 mM glutamine, 1 mM HEPES, and penicillin (200 IU/mL), streptomycin (50 mg/mL; Sigma, USA) at 37°C in 95% humidity and 5% CO₂.

1.7 T cell proliferation assay with MTT method
After incubation for 2 h, 100 μL aliquots of cell suspension were added to wells of a 96-well culture plate. After being cultured for 24 h, the rLj-BGSP-2 at a concentration of 0.075, 0.225, 0.375, 0.53 and 0.75 μg/mL with 5 μg/mL PHA were added to each well. The well with T cell suspension only was used as a negative control, and the well with T cell suspension and PHA was used as a positive control. After incubation for 24 h, 100 μL of 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) was
added to each well, incubated in the dark at 37°C for 4 h. The MTT solution was drawn out from the plate and 200 μL DMSO (Amresco, USA) was added. The plate was read with a Multiskan MK3 Microplate Reader (Thermo, China) at a wavelength of 570/620 nm (Lim et al., 2006). The data represented three independent experiments. Proliferation index (PI) and inhibition ratio of proliferation (IRP) were calculated with the following equations: \( PI = \frac{OD_{\text{experimental groups or positive group}} - OD_{\text{negative group}}}{OD_{\text{negative group}}} \times 100\% \) and \( IRP = \left(1 - \frac{PI_{\text{experimental groups}}}{PI_{\text{positive group}}} \right) \times 100\% \).

1.8 Cell cycle assays of T cell proliferation treated with rLj-BGSP-2

The purified T cells were incubated for 2 h, and the rLj-BGSP-2 and PHA were added to each well as described above. After incubation for 24 h, the cells were collected and washed twice with PBS. Cells were then fixed in 70% cold ethanol and stored at 4°C over night. Cells were stained by 0.5-1 mL PI working solution (50 μg/mL PI, 0.1% Triton X-100, 0.1 mmol/L EDTA and 50 μg/mL RNAse in PBS) (Sigma, USA) for 30 min. The 488 nm line of an argon laser provided excitation of PI. The red fluorescence of PI was analyzed on a fluorescence activated cell sorter (FACS) by using the CellOuest software (Falk et al., 2004). The experiment was repeated for three times, and the percentage of each cell cycle phase was calculated with the mean value of three independent experiments.

1.9 Statistical analysis

The data were also analyzed by using One-Way ANOVA with SPSS 13.0 for windows (SPSS Inc., USA). P values < 0.05 were considered to be statistically significant.

2 Results

2.1 Sequence analysis

The cysteine-rich domain (CRD) is a common motif of the CRISP family. The alignment of BGSP-2 CRD domain to multiple species demonstrated that there are six conserved cysteines and a C-terminal long loop (loop I) (Fig. 1). Three disulfuric bonds are established between Cys1 and Cys6, Cys2 and Cys4, and Cys3 and Cys5.

2.2 Expression of recombinant BGSP-2

The full-length open reading frame of L. japonica BGSP-2 was cloned into pET23b expression vector, and the recombinant protein was generated by His-Bind affinity chromatography. The recombinant BGSP-2 was confirmed in 10% SDS-PAGE. The rLj-BGSP-2 had approximately 30 kDa molecular mass (lane 2, Fig. 2). The concentration of the recombinant protein was 0.193 mg/mL by BCA Protein Assay.

2.3 The inhibitory effect of BGSP-2 on PHA-induced T lymphocyte proliferation

Human T lymphocytes were isolated and cultured. The rLj-BGSP-2 at a concentration of 0.075, 0.225, 0.375, 0.53 and 0.75 μg/mL plus PHA (5 μg/mL) were added to the proliferative T cells. After 24 h, 100 μL of 5 mg/mL MTT was added to each well, and incubated in the dark at 37°C for 4 h. The plate was read 570/620 nm, and the cell Proliferation index (PI) and inhibition ratio of proliferation (IRP) were calculated. As expected, T lymphocytes were stimulated with PHA in vitro, and the proliferation increased after 24 h. The PI and IRP decreased as the concentration of BGSP-2 increased (Table 1). But rLj-BGSP-2 with doses greater than 1.0 μg/mL had no effect on suppressing the proliferation.

![Fig. 1 Sequence analysis of the CRD of CRISP family](image)

Invariant residues are marked with asterisks. Six conserved cysteines are covered with vertical bars. Disulfide bridges in the CRD are indicated by lines. Loop I is indicated by a bar filled with biases.
of T lymphocyte (data not shown). Within the concentration range from 0.225 to 0.75 μg/mL, the rLj-BGSP-2 inhibited the proliferation of T lymphocyte significantly (One-way ANOVA, $F_{3,8} = 235.347, P < 0.0001$) in a dose-dependent manner.

### 2.4 The recombinant BGSP-2 affects Cell cycle of PHA-induced T lymphocytes

T cells were incubated with various concentrations of rLj-BGSP-2 plus PHA for 24 h, and the cells were fixed and stained by 0.5-1 mL PI working solution. The 488 nm line of an argon laser provided excitation of PI. The red fluorescence of PI was analyzed on a fluorescence activated cell sorter (FACS) using the CellOuest software (Fig. 3). The number of T cells in S phase decreased with increasing concentrations of rLj-BGSP-2 (Fig. 3, C-F). The negative control group had nearly no S phase T cells, while the positive control showed the highest number of S phase T cells (Fig. 3, A and B). Our result further indicated that the rate of cell debris increased as the concentration of rLj-BGSP-2 increased. In addition, we found that the rLj-BGSP-2 within the concentration range from 0.3 to 0.7 μg/mL significantly inhibited the proliferation of T lymphocyte (One-Way ANOVA, $F_{3,8} = 170.872, P < 0.0001$) and blocked the cell cycle at the G1/S point (Fig. 4).

### 3 Discussion

We cloned and expressed a full-length BGSP-2 gene from a buccal gland cDNA library of *L. japonica*. Lj-BGSP-2 contained 16 conserved cysteine residues, suggesting that it belongs to the CRISP superfamily. In addition, we found that the recombinant BGSP-2 can inhibit T lymphocyte proliferation in vitro, and block the cell cycle at the G1/S point. The effective concentration of rLj-BGSP-2 for inhibition of T lymphocyte proliferation was at the microgramme level. CRISPs contain 16 highly conserved cysteine residues, with the N-terminal pathogenesis related protein-like domain, and the C-terminal cysteine-rich domain. The two domains are separated by a short peptide segment (Roberts, et al., 2006). Ito et al. (2007) reported that the lamprey CRISP had several 2-5 amino acids insertions when aligned with reptilian venom CRISPs, possibly due to the distant evolutionary relatedness between them. CRISPs such as Shk, Margatoxin, and Correolide have been shown to selectively block Kv1.3 channel to inhibit T lymphocyte proliferation (Koo et al., 1999; Bao et al., 2005). Earlier functional assays also suggested that CRISPs from animal venom can also block Ca$^{2+}$, K$^+$ and cyclic nucleotide-gated ion channels. The potassium channel, as one of the basic membrane structures in the organisms, has high selective affinity to K$^+$. This channel is a key molecule in physiological

### Table 1 The inhibitory function of rLj-BGSP-2 on T cell proliferation (means ± SD, n = 3)

| Group             | rLj-BGSP-2 (μg/mL) | PHA (μg/mL) | OD     | PI     | IRP (%) |
|-------------------|--------------------|-------------|--------|--------|---------|
| Negative control  | 0                  | 0           | 0.101±0.001 | 0   | 100     |
| Positive control  | 0                  | 5           | 0.144±0.002 | 0.43±0.009 | 0      |
|                   | 0.075              | 5           | 0.137±0.005 | 0.36±0.026 | 16.34±6.04 ** # |
|                   | 0.225              | 5           | 0.129±0.003 | 0.28±0.008 | 34.90±1.66 ** # |
| rLj-BGSP-2        | 0.375              | 5           | 0.123±0.003 | 0.22±0.009 | 48.86±1.98 ** # |
|                   | 0.53               | 5           | 0.115±0.005 | 0.14±0.002 | 67.50±5.96 ** # |
|                   | 0.75               | 5           | 0.108±0.001 | 0.07±0.005 | 83.71±1.29 ** # |

** $P<0.001$, (the concentration of rLj-BGSP-2 range from 0.075 to 0.75 μg/mL: $F_{1,4} = 383.969, F_{1,4} = 3070.273, F_{1,4} = 1334.321, F_{1,4} = 59.553, F_{1,4} = 316.878$, respectively, vs negative control group), # $P<0.05$ (the concentration of rLj-BGSP-2: 0.75 μg/mL: $F_{1,4} = 14.640$. vs positive control group), ** $P<0.01$ (the concentration of rLj-BGSP-2 range from 0.225 to 0.75 μg/mL: $F_{1,4} = 882.142, F_{1,4} = 1217.671, F_{1,4} = 256.812, F_{1,4} = 8367.668$. respectively, vs positive control group), One-Way ANOVA.
Fig. 3  Cell cycle of T lymphocyte treated with rLj-BGSP-2 Detected by FACS via PI staining
Cells were cultured with (panel B) or without (panel A) PHA. Cells were cultured with PHA in the presence of BGSP-2 0.1 (panel C), 0.3 (panel D), 0.5 (panel E) and 0.7 μg/ml (panel F) respectively.

processes, e.g., the action of lymphocyte (Harriague and Bismuth, 2002). Consequently, the blocking effect of CRISP on potassium channel plays a critical role in the inhibition of T lymphocyte proliferation.

The lamprey CRISP shows the ion channel-blocking activity too. Previously, Ito et al. (2007) found that the lamprey CRISP could suppress frog muscle contraction. They inferred that the suppression of muscle contraction was probably through blockage of Ca$^{2+}$ channel, but did not give directly evidence. Chi et al. (2009) demonstrated that the lamprey CRISP probably reduced the neuronal excitability through the inhibition of the voltage-dependent Na$^+$ channels of neurons. The lamprey CRISP possesses a conserved CRD domain which is essential for K$^+$ channel inhibitor-like fold in reptile CRISP (Guo et al., 2005). Given that the reptile CRISPs can inhibit T lymphocyte proliferation by blocking the Kv1.3 potassium channel, we could not exclude the possibility that the lamprey BGSP-2 inhibitory effect on T cells may also due to the blockage of Kv1.3 potassium
channel. Of course, further studies are needed to clarify this question. Furthermore, the Kv1.3 potassium channel plays an essential role in effector memory T cells and has been implicated in several important autoimmune diseases including rheumatoid arthritis, psoriasis and type-1 diabetes mellitus (Beeton et al., 2006). The high selectivity of Kv1.3 blockers provides a new therapeutical approach for autoimmune disease (Damjanovich et al., 2004). Hence, we expect that rLj-BGSP-2 will be a candidate for autoimmune disease if BGSP-2 can be proven to be a Kv1.3 channel blocker in the future.

In summary, the rLj-BGSP-2 was shown to inhibit PHA-activated T lymphocyte proliferation at a low concentration range, and block the cell cycle at the G1/S point. In addition, our results also indicate that the rLj-BGSP-2, as a candidate immune inhibitor, may be a more attractive therapeutic target than those classical immune inhibitors, due to its higher selectivity and less harmful effect.

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