Site-directed removal of N-glycosylation sites in BST-1/CD157: effects on molecular and functional heterogeneity

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Cyclic ADP ribose (cADPR) is a novel second messenger that releases calcium from intracellular calcium stores, but works independently of inositol 1,4,5-trisphosphate. In mammals ADP-ribosyl cyclase function is found in two membrane proteins, CD38 and bone marrow stromal cell antigen 1 (BST-1)/CD157. These enzymes are exposed extracellularly and also possess cADPR hydrolase activity, but an intracellular soluble ADP-ribosyl cyclase has been reported in human T-cells. Previously, a soluble form of BST-1/CD157 (sBST-1), which lacked the glycosylphosphatidylinositol-anchored portion, was expressed by a baculovirus–insect-cell system. In this study, we have purified the sBST-1, and it migrated as two major bands by SDS/PAGE, suggesting that it is post-translationally modified. BST-1 contains four putative N-glycosylation sites. Tunicamycin treatment reduced sBST-1 expression in the culture medium, indicating that N-glycosylation is essential for secretion. Site-directed mutagenesis was performed to generate sBST-1 mutants (N1–N4), each preserving a single N-glycosylation site. N1, N3 and N4 were well secreted into the medium, and were each detected as a single band. Although N3 and N4 retained the ADP-ribosyl cyclase activity, the cADPR-hydrolase activity was retained only in N4. We conclude that N-glycosylation of sBST-1 facilitates the folding of the nascent polypeptide chain into a conformation that is conductive for intracellular transport and enzymic activity. Furthermore a crystal has been obtained using the N4 mutant, but not the wild-type sBST-1. Thus the artificial engineering of N-glycosylation sites could be an effective method to generate homogeneous material for structural studies.

Key words: ADP-ribosyl cyclase, crystallization, cyclic ADP ribose, glycosylphosphatidylinositol-anchored protein, post-translational modification.

INTRODUCTION

Cyclic ADP ribose (cADPR) is considered to be a new second messenger that elicits calcium release from intracellular calcium stores [1]. Evidence suggests that this calcium mobilization system exists from protozoans and plants to humans [2–4]. Soluble ADP-ribosyl cyclase, an enzyme which synthesizes cADPR from NAD^+ by a cyclization reaction, was discovered in an extract of sea urchin eggs and was purified from *Aplysia californica* ovotestis [5,6]. The mammalian ADP-ribosyl cyclases include CD38 [7], a type-II membrane protein on human lymphocytes, and bone marrow stromal cell antigen 1 (BST-1/CD157) [8], a glycosyl-phosphatidylinositol (GPI)-anchored protein that was identified from cell lines derived from the bone marrow stromal cells of rheumatoid patients. These two mammalian ADP-ribosyl cyclases catalyse the cyclization of NAD^+ to cADPR and also catalyse the hydrolysis reactions of cADPR and NAD^+. Thus these enzyme reactions are catalytically novel. The oxo-carbenium-ion intermediate has been considered to be a common intermediate for the dual reaction pathways [9,10]. On the other hand, covalent binding of the substrate to the enzymes has been proposed recently [11]. However, the precise reaction mechanism remains to be elucidated. Furthermore it also remains to be answered how the extracellularly produced cADPR functions intracellularly. Two mechanisms are postulated; the membrane ectoenzymes may be internalized [12] or they may possess channel or transporter properties [13]. Notably, a soluble ADP-ribosyl cyclase has been recently reported in Jurkat human T-cells, and its role for the regulation of calcium signalling is clearly demonstrated [14].

Previously, we expressed a soluble form of BST-1 (sBST-1) without the GPI-anchored portion in a baculovirus–insect-cell system, and isolated the peptide inhibitor of BST-1 cyclase by a phage display method [15]. The binding stoichiometry of the peptide to sBST-1 was also determined [16]. In this study, we have purified sBST-1. The purified sBST-1 was detected as two major bands by SDS/PAGE, suggesting a post-translational modification. BST-1 contains four potential N-linked glycosylation sites in the extracellular region. To understand the relationship of the glycosylation and the molecular heterogeneity, we have prepared sBST-1 mutants lacking selected N-linked glycosylation sites. The resulting mutants were expressed in

Abbreviations used: ADPR, ADP ribose; BST-1, bone marrow stromal cell antigen 1; cADPR, cyclic ADP ribose; cGDPR, cyclic GDP ribose; CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; MES, 4-morpholine-ethanesulphonic acid; NGD^+, nicotinamide guanine dinucleotide; pfu, plaque-forming unit; PNGase F, peptide N-glycosidase F; sBST-1, soluble BST-1.

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insect cells, and their enzyme activities were analysed. Our results indicated that the oligosaccharides attached to the distinct sites on BST-1 have different roles. Furthermore, crystal formation experiments using the mutant proteins are presented here.

EXPERIMENTAL

Construction of the transfer vectors for the baculoviruses

The *Eco*RV–*Xba*I fragment (nt residues 24–1018 of the human BST-1 cDNA) of pΔ63-BOS [8] was ligated into the *Sma*I–*Xba*I sites of the baculovirus transfer vector, pVL1393 (Pharmingen, San Diego, CA, U.S.A.). sBST-1 mutants containing amino acid substitutions for the asparagine residue in the recognition signal for N-linked glycosylation Asn-X-Ser/Thr [17,18] were generated by oligonucleotide-directed mutagenesis as described by Kunkel et al. [19]. The replacement of asparagine with a different amino acid depended on the amino acid sequences of the BST-1 homologues. The mutagenic oligonucleotides used were as follows (codons are underlined): 5′-GCGGAACAAGGACTGCACAGCC-3′ converts Asn^{66} into Asp; 5′-CTTTTTATTACCTTGTCCAGGC-3′ converts Asn^{95} into Thr; 5′-GTCGACAGAAAGCTGACTCTGG-3′ converts Asn^{148} into Ala; 5′-TCATGCTGGATGGTTCAGAGCC-3′ converts Asn^{192} into Asp. Construction of the mutants was done using the Mutan®-Super Express Km kit (Takara, Osaka, Japan). The amino acid mutations were confirmed by DNA sequencing. The resulting constructs contained three amino acid substitutions: mutant N1 contains Thr^{95}, Ala^{148} and Asp^{192}; mutant N2 contains Asp^{66}, Ala^{148} and Asp^{192}; mutant N3 contains Asp^{66}, Thr^{95} and Asp^{192}; mutant N4 contains Asp^{66}, Thr^{95} and Ala^{148}. In mutant N0, all four N-glycosylation sites were removed by substitution of the asparagine residues at amino acid positions 66, 95, 148 and 192 by Asp, Thr, Ala and Asp, respectively.

Cell culture and isolation of recombinant baculoviruses

Spodoptera frugiperda (Sf9) cells were propagated either as suspension or monolayer cultures in 1 × Grace's insect medium (Pharmingen), 0.4 % (v/v) yeastolate, 0.4 % (v/v) lactalbumin hydrolysate and 0.1 % (v/v) pluronic F-68 (Life Technologies, Rockville, MD, U.S.A.), supplemented with 10 % (v/v) heat-inactivated fetal calf serum at 27 °C. Recombinant baculoviruses were generated by co-transfecting Sf9 insect cells with 1 μg of plasmid DNA and 0.1 μg of linearized AcNPV baculovirus DNA (Pharmingen) with the Lipofectin reagent (Life Technologies). After 6 days of incubation, the virus in the supernatant was titrated and individual plaques were isolated by plaque purification. High-titre virus stocks were prepared in suspension cultures of Sf9 cells infected at a multiplicity of 0.1 plaque-forming unit (pfu)/cell.

Expression of sBST-1 by recombinant baculovirus-infected High Five cells

The purified recombinant baculoviruses were used to infect confluent monolayers of High Five cells at a multiplicity of 1.0 pfu/cell. The baculoviruses, suspended in Express Five serum-free medium (Life Technologies) supplemented with 18 mM L-glutamine, were added at a concentration of 1×10^6, and the cultures were then incubated for 6 days.

Western blotting

The blot was probed with a rabbit anti-(BST-1) antibody [20], and then the bound antibody was detected with alkaline-phosphatase-conjugated anti-(rabbit-IgG) as the secondary antibody. The production of sBST-1 from Chinese hamster ovary (CHO) cells has been described previously [21].

Purification of sBST-1 and sBST-1 mutants

The culture medium of the recombinant baculovirus-infected High Five cells was collected by centrifugation, and was diluted 5-fold with 20 mM acetate, pH 5.0. The sample was applied to an ion-exchange chromatography column (POROS HS gel matrix; PerSeptive Biosystems, Cambridge, MA, U.S.A.). After washing with 10-column volumes of 20 mM acetate, pH 5.0, the bound material was eluted with 20 mM acetate, pH 5.0, containing 2 M NaCl. The fractions that stained positive by immunoblotting were collected and applied to a dye ligand chromatography column (HiTrap Blue; Amersham Pharmacia Biotech). After the column was washed with 20 mM acetate, pH 5.0, containing 200 mM NaCl, the bound sample was eluted with 20 mM acetate, pH 5.0, containing 500 mM NaCl. The positive fractions were collected and dialysed against 10 mM 4-morpholine-ethanesulphonic acid (MES), pH 6.0.

For the purification of the sBST-1 mutants, the samples eluted from POROS HS were dialysed against 20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl. The monoclonal antibody (mAb) BEC7 [22] (10 mg) was coupled to 1 ml of HiTrap NHS-activated column resin (Amersham Pharmacia Biotech) in buffer L [200 mM $NaHCO_3$ (pH 8.3)/500 mM NaCl] at 4 °C overnight. The column was washed several times alternately with buffer W1 [0.5 M ethanolamine (pH 8.3)/500 mM NaCl] and buffer W2 [100 mM acetate (pH 4.0)/500 mM NaCl]. After neutralization with 20 mM Tris/HCl, pH 7.5, the samples were dialysed and subjected to immunoaffinity column chromatography. After the column was washed with 20 mM HCl, pH 7.5, containing 0.5 M NaCl, the sample was eluted with 0.1 M glycine, pH 3.0. The eluted material was neutralized with 100 mM Tris/HCl, pH 7.5, and was dialysed with 10 mM MES, pH 6.0.

Tunicamycin treatment

High Five cells (3×10^6 cells) were infected with the recombinant virus for sBST-1, and were cultured for four days in a 50 ml flask with a culture surface area of 25 cm^2 with Express Five medium containing 18 mM L-glutamine. Tunicamycin (0.1, 1 and 10 mg/ml) was added post-infection. After an incubation at 27 °C for 4 days, the medium was concentrated 5-fold by Centricon 10 ultrafiltration (Millipore), and the cell lysates were subjected to Western blotting. The culture medium of the recombinant baculovirus-infected High Five cells was collected by centrifugation. The cells were solubilized by the addition of ice-cold lysis buffer [10 mM Hepes (pH 7.5)/200 mM NaCl/1 % (v/v) Triton X-100], and the lysates were incubated on ice for 30 min. The cell extracts were centrifuged at 17000 ***g*** for 10 min and the supernatant was used as the cell lysate.

N-glycosidase treatment

Purified sBST-1 and sBST-1 mutants were denatured by boiling with 0.1 % (w/v) SDS for 2 min. The reaction buffer was then adjusted to 20 mM sodium phosphate, pH 7.0, containing 50 mM EDTA, 10 mM NaN_3 and 0.5 % (v/v) Nonidet P40, and the solution was boiled for an additional 2 min. Half of the reaction mixture was incubated for 24 h with 0.2 unit of peptide N-glycosidase F (PNGase F; Boehringer Mannheim, Mannheim, Germany). One unit is the enzyme activity that hydrolyses 1 nmol of dansyl-fetuin glycopeptide within 1 min at 37 °C. The remaining half was not treated with the enzyme and was used as

a control. Both mixtures were subjected in parallel to SDS/PAGE.

Assay for ADP-ribosyl cyclase and cADPR hydrolase

The ADP-ribosyl cyclase activity was determined by the conversion of an NAD^+ analogue, nicotinamide guanine dinucleotide (NGD^+; Sigma, St. Louis, MO, U.S.A.) to the fluorescent product cyclic GDP ribose (cGDPR) [15,23]. The cADPR hydrolase activity was assayed by HPLC, as described previously [15,21].

Measurement of the apparent K_m values of sBST-1

For the measurement of the apparent K_m value for NGD^+, concentrations of 200, 280, 360, 440, 640 and 800 μM NGD^+ were used. The concentration of sBST-1 in the reaction mixtures was 1.5 μg/ml. Triplicate experiments were performed. The v (nmol/min) values at each substrate concentration were averaged, and the K_m values were estimated from Lineweaver–Burk plots. Each value is an average $\pm$ S.D. of three independent determinations.

sBST-1 crystallization

For the crystallization trial, sBST-1 (mutant N4) in 10 mM MES buffer, pH 6.0, was concentrated to 10 mg/ml using a Centriprep unit (Millipore). Crystallization of purified sBST-1 (mutant N4) was performed using the vapour-diffusion hanging-drop method at 293 K. Optimum conditions were screened with precipitating agents (polyethylene glycol 4000, ammonium sulphate and 2-methyl-2,4-pentanediol), the buffers at various pH values and additives such as metal ions. For cryoprotection during data collection, the crystals were briefly soaked in a harvest buffer containing 20% (v/v) glycerol as a cryoprotectant, and were flash-frozen to 100 K in a nitrogen stream using a Cryostream Cooler (Oxford Cryosystems, Oxford, U.K.).

sBST-1 homology model

The model of the sBST-1 structure was built by using SWISS-MODEL [24], an automated protein-modelling server at Glaxo-SmithKline Experimental Research (Geneva) (http://www.expasy.ch/swissmod/SWISS-MODEL.html). The first approach mode was used for the modelling with the amino acid sequence of the extracellular domain of human BST-1 as the query. The X-ray structure of *Aplysia* ADP-ribosyl cyclase (Protein Database accession number 1LBE, [25]) was used as the template for the model.

RESULTS

Expression and purification of sBST-1 in insect cells

We expressed sBST-1 in baculovirus-infected insect cells as a soluble and secretory protein. The High Five cells were infected with the recombinant baculovirus containing the cDNA corresponding to the extracellular region of BST-1 under the control of the polyhedrin promoter (Figure 1A). After an incubation for 6 days, the culture medium was collected and diluted. The sample was applied to a cation exchange column and then to a Blue Sepharose column (Table 1), as described in the Experimental section. From 500 ml of culture medium, we obtained 6 mg of purified sBST-1. Two major species, 34 and 32 kDa bands, were detected by SDS/PAGE (Figure 1B). The results of over-loaded gels showed that the purity of the sample was over 99.9%. The molecular mass by MS (results not shown) was not consistent with the predicted molecular mass of 30.2 kDa as deduced from the primary amino acid sequence, suggesting the presence of post-translational modifications which constituted approx. 12% of the total mass of the protein. This was confirmed by N-glycosidase treatment of sBST-1, which produced a band of

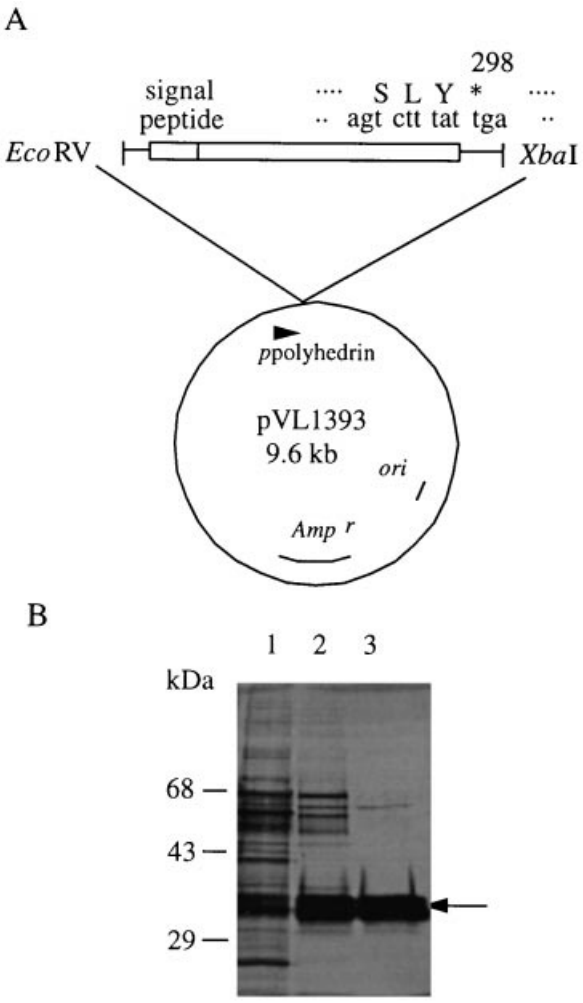

Figure 1 Expression of sBST-1 in insect cells

(**A**) Representation of the transfer vector for insect cells. sBST-1 was prepared by introducing a stop codon at Thr^{298}. The resulting virus was expressed under the control of the polyhedrin promoter (*p*olyhedrin) in insect cells. (**B**) Silver-stained PAGE of sBST-1 expressed in insect cells. An aliquot of culture medium from insect cells infected with the recombinant virus for sBST-1 (lane 1), an aliquot of an ion-exchange column fraction (lane 2), and an aliquot of a Blue-Sepharose column fraction (lane 3) were subjected to SDS/PAGE [10% (v/v) gel]. The arrowhead indicates sBST-1.

Table 1 Purification of sBST-1 from insect cell culture medium

Protein concentrations were determined using the Bradford method [31]. The yield was calculated starting from the culture medium purification step.

Step	Volume (ml)	Total protein (mg)	Yield (%)
1. Culture medium	500	55.0	100
2. Cation exchange (POROS HS)	75	7.8	14
3. Blue Sepharose (HiTrap Blue)	50	6.0	11

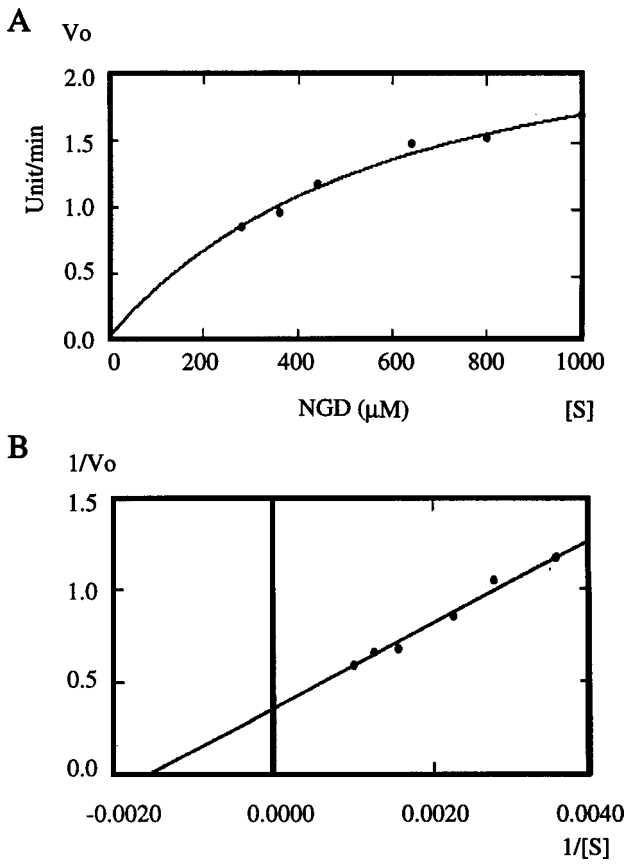

Figure 2 Kinetic analysis of sBST-1

(**A**) The effect of substrate concentration (200–800 μM NGD^+) on the initial velocity of the ADP ribosyl cyclase activity of sBST-1 (1.5 μg/ml) is shown. All cyclase reactions were performed for 5 min at 25 °C in the presence of NGD^+. The results shown represent the mean values obtained from three separate determinations. (**B**) A Lineweaver–Burk plot of 1/[S] versus $1/V_0$ for the product, cGDPR.

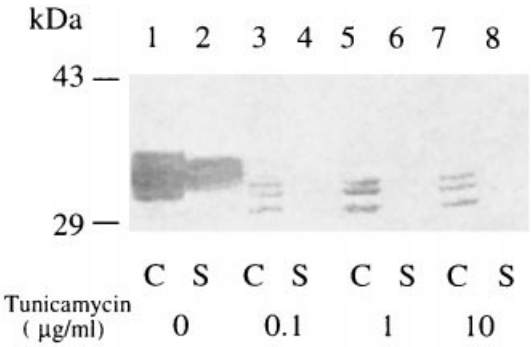

Figure 3 Effects of tunicamycin on the secretion of sBST-1

Western blot analysis of sBST-1 expression with tunicamycin. High Five cells (3×10^6 cells) were cultured in Express Five medium containing 18 mM L-glutamine. Tunicamycin (0.1, 1 and 10 mg/ml) was added post-infection. After incubation at 27 °C for 4 days, the cell lysate and the cell culture supernatant were prepared as described in the Experimental section, and were subjected to Western blotting. The cell lysates (C) (lanes 1, 3, 5 and 7) and the cell culture supernatants (S) (lanes 2, 4, 6 and 8) were subjected to SDS/PAGE [10% (v/v) gel], and the blot was probed with a rabbit anti-(BST-1) antibody.

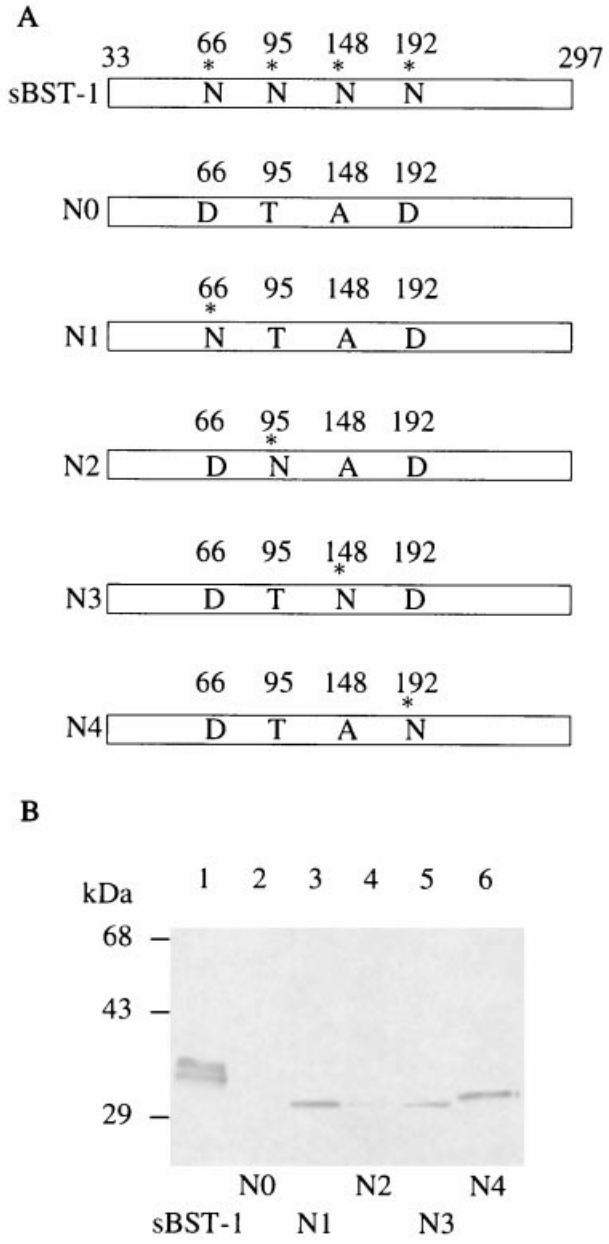

Figure 4 Expression of mutant forms of sBST-1 in insect cells

(**A**) Representation of mutant forms of sBST-1. sBST-1 mutants lacking potential N-glycosylation sites were generated by replacing the asparagine residues with the corresponding residue of the BST-1 homologues, CD38 and *Aplysia* ADP-ribosyl cyclase, in the consensus sequence Asn-X-Ser/Thr. The amino acid substitutions are defined using the following nomenclature: N0 is a mutant in which all four N-glycosylation sites have been removed by replacing Asn^{66}, Asn^{95}, Asn^{148} and Asn^{192} with Asp (D), Thr (T), Ala (A) and Asp (D), respectively. N1 is a mutant that preserves the asparagine (N) at position 66, while Asn^{95}, Asn^{148} and Asn^{192} were converted into Thr^{95} (T), Ala^{148} (A) and Asp^{192} (D), respectively. For details of the generation of these constructs, see the Experimental section. (**B**) Expression of sBST-1 mutants lacking potential N-glycosylation sites. Western-blot analysis of sBST-1 and sBST-1 mutants was performed. High Five cells were infected at 1.0×10^6 cells/ml with a multiplicity of 1.0 pfu/cell for each virus. Cells were harvested at 6 days post-infection, and then aliquots of the culture media of insect cells infected with the recombinant virus for sBST-1, N0, N1, N2, N3 and N4 (lanes 1–6) were subjected to SDS/PAGE [10% (v/v) gel] and were analysed with a rabbit anti-(BST-1) antibody. Media samples represented 4.5×10^4 cells.

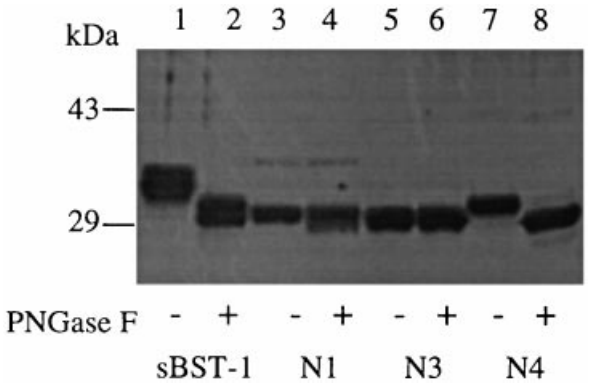

Figure 5 Enzymic deglycosylation of sBST-1 mutants

Each (30 mg) of the immunoaffinity-column-purified sBST-1 (lane 1), N1 (lane 3), N3 (lane 5) and N4 (lane 7) were denatured with 0.1% (w/v) SDS and were incubated with 0.2 unit of PNGase F in a total volume of 100 ml of the reaction buffer described in the Experimental section. The digestion was performed at 37 °C for 24 h. 20 ml of digested samples (+) (lanes 2, 4, 6 and 8) and control samples (−) (lanes 1, 3, 5 and 7) were subjected to SDS/PAGE [10% (v/v) gel]. The gel was silver-stained.

approximately 31 kDa. We also identified the first ten amino acids of purified sBST-1 as Arg-Trp-Arg-Ala-Glu-Gly-Thr-Ser-Ala-His. This N-terminal sequence is the same as that of sBST-1 expressed in CHO cells [21].

Kinetic studies of BST-1 were performed using the sBST-1 (Figure 2). The apparent K_m value of sBST-1 for NGD^+ is $610 \pm 6.0\ \mu M$. This value is consistent with that obtained with the sBST-1 expressed in CHO [21]. A V_{max} value of 560 ± 56.0 nmol/min per mg of protein was obtained.

Effect of tunicamycin on sBST-1 expression

To test the possibility that the heterogeneity of the purified sBST-1 by SDS/PAGE is caused by differential glycosylation, High Five cells were infected with recombinant virus in the absence or presence of different concentrations of tunicamycin, an inhibitor of N-glycosylation (Figure 3). The major bands (Figure 3, lane 2) observed in the supernatants in the absence of tunicamycin, were not present in the supernatants of cells incubated with tunicamycin (Figure 3; lanes 4, 6 and 8). Although immunoreactive bands were detected in the cell lysates, regardless of the presence of tunicamycin (Figure 3; lanes 3, 5 and 7), the slowest migrating band was smaller than the bands detected in the supernatants in the absence of tunicamycin. Thus, we concluded that N-glycosylation is necessary for the secretion of sBST-1 into the culture medium.

Expression of sBST-1 mutants in insect cells

Next, to examine the functional importance of the individual glycosylation sites of sBST-1, five N-glycosylation-site mutants were generated (Figure 4A) and were expressed in High Five cells. When all four potential N-glycosylation sites were removed, the sBST-1 (N0) was not detected in the medium, but remained intracellularly trapped in a membrane fraction (results not shown). A single N-glycosylation site is preserved in each of the sBST-1 mutants (N1–N4). N1, N3 and N4 were secreted efficiently, but N2 was poorly secreted into the culture medium (Figure 4B). N1 and N3 migrated equally, whereas N4 migrated slower than N1 and N3.

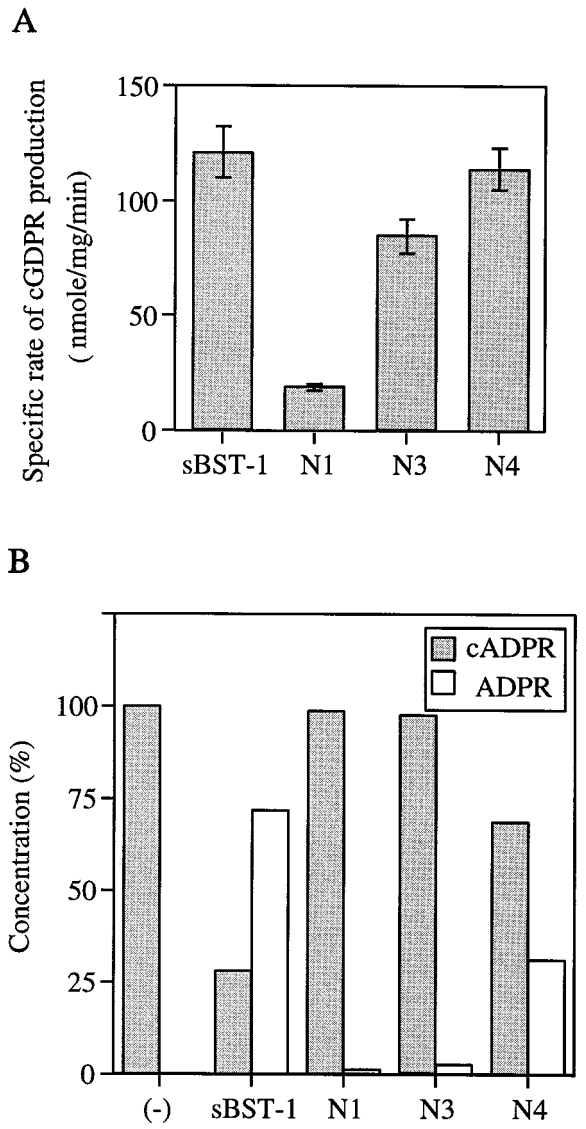

Figure 6 ADP-ribosyl cyclase (A) and cADPR hydrolase (B) activities of sBST-1 mutants

(**A**) sBST-1 mutants (10 μg/ml) were incubated at 25 °C with NGD^+ (200 μM) in a buffer containing 50 mM MES, pH 6.0, and 1 mM $ZnCl_2$, and the fluorescence at 410 nm (excited at 300 nm) was monitored. (**B**) sBST-1 (50 μg/ml) was incubated at 37 °C for 4 h with 20 μM cADPR. cADPR and ADP-ribose (ADPR) were separated on an anion-exchange column. Concentrations of cADPR and ADPR are represented as the percentage of their peak area on HPLC (100% represents the total area of the two peaks of cADPR and ADPR). Hydrolysed products during the incubation of cADPR alone are expressed as the background yield. The results shown are representative of three independent experiments.

Glycosylation analysis of purified sBST-1 mutants

To purify N1, N3 and N4, the culture media from the cells infected with the respective recombinant baculovirus were diluted and applied to cation exchange and immunoaffinity columns, as described in the Experimental section. Major bands for N1, N3 and N4 were detected at approx. 31–34 kDa, which were over 99% pure (results not shown). The purified sBST-1 and sBST-1 mutants were subjected to N-glycosidase treatment using PNGase F and were analysed by SDS/PAGE (Figure 5). The original

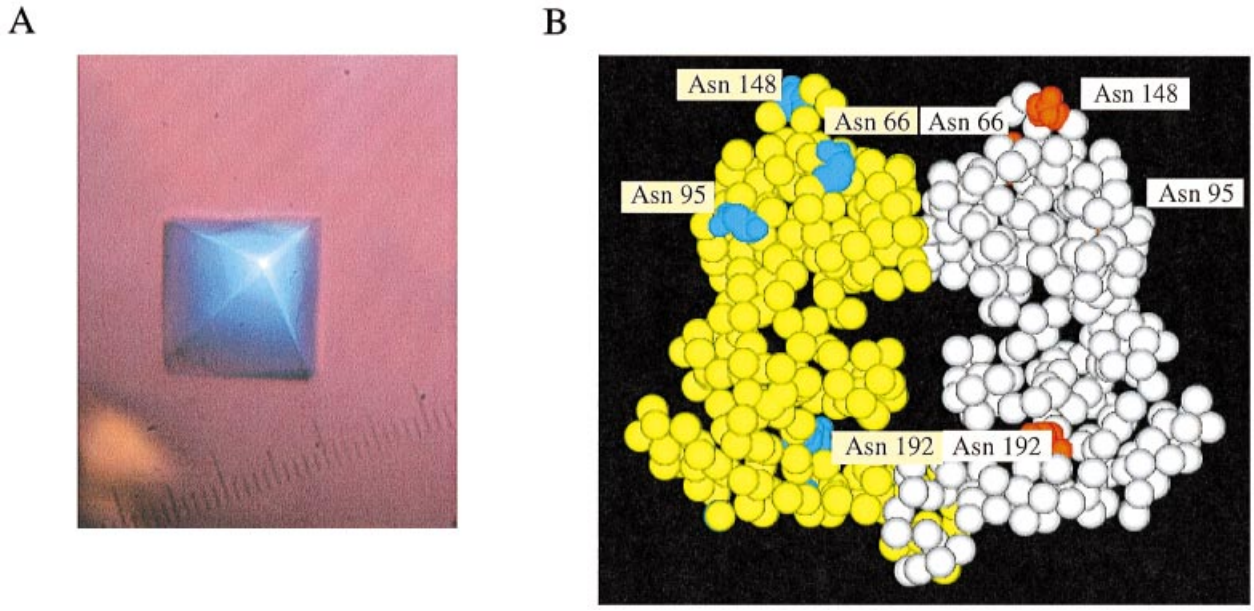

Figure 7 Crystal of N4, a mutant of sBST-1, and the model structure of BST-1

(**A**) Crystal of sBST-1 (mutant N4). Approximate dimensions are 0.30 mm × 0.40 mm × 0.30 mm. (**B**) The model of a dimer of sBST-1 based on the homology with *Aplysia* ADP-ribosyl cyclase. The four asparagine residues (Asn^{66}, Asn^{95}, Asn^{148} and Asn^{192}), which are possible N-glycosylation sites, are shown with their side chains in a space-filling (Corey–Pauling–Koltun) model (red and blue). For other residues, only the α-carbon backbones are shown (white and yellow).

sBST-1 showed two major bands, even after N-glycosidase treatment (Figure 5, lanes 1 and 2). The mobilities of N1 and N3 shifted slightly to lower molecular and broader mass positions after the 24 h incubation, whereas the mobility of N4 significantly shifted to a lower molecular mass position. At the end of the incubation, the sBST-1 and sBST-1 mutants exhibited similar electrophoretic mobilities, migrating as 30 kDa proteins. The carbohydrate moieties attached to N1 and N3 seemed to be incomplete and resistant to N-glycosidase treatment. N4 preserved the N-glycosylation moiety that was sensitive to the N-glycosidase.

Effect of N-linked carbohydrate moieties on the enzyme activity of sBST-1

The ADP-ribosyl cyclase activities of the purified N1, N3 and N4 were measured using NGD^+ as the substrate (Figure 6A). In comparison with the original sBST-1, the cyclase activity of N1 was about 15%. The cyclase activity of N3 was about 80% of that of sBST-1. N4 retained cyclase activity comparable with that of sBST-1. The cADPR hydrolase activity was also measured (Figure 6B). The activity of N4 was about 50% of that of sBST-1. The hydrolase activities of N1 and N3 were severely decreased.

Crystallization

The crystal of sBST-1 (mutant N4) was grown using 37.5 mM citrate, pH 5.0, 1.2 M ammonium sulphate, 50 mM $ZnSO_4$ and 5 mg/ml of protein. The crystal belongs to the orthorhombic space group $P2_12_12_1$ with cell dimensions a = 60.63 Å, b = 113.30 Å and c = 132.29 Å. The crystal diffracted beyond 3.5 Å resolution at room temperature. However, X-ray exposure caused a rapid decay in the diffraction quality. To overcome this radiation damage, we applied a cryogenic technique where the crystals were flash-cooled at 100 K. The cryoprotection improved the resolution up to 2.5 Å, and allowed us to collect complete data sets. The diffraction pattern, measured under the cryo-conditions, showed the unit cell dimensions of a = 58.72 Å, b = 112.61 Å and c = 130.09 Å.

DISCUSSION

We have expressed a soluble form of BST-1/CD157, which preserves the ADP-ribosyl cyclase activity, in insect cells using a recombinant baculovirus, and have purified it from the culture medium. Its property was characterized by applying a technique in which NGD^+ was used as a substrate for the synthesis of fluorescent cGDPR. This assay has been considered to mimic the physiologically relevant reaction in which the native substrate, NAD^+, is converted into non-fluorescent cADPR. Kinetic analyses of the purified cyclase showed a K_m value of 610 ± 6.0 μM for NGD^+. This relatively high value raises a question of how the high concentration of substrate is provided. A high local concentration of substrate might be supplied from lytic or apoptotic cells in some environments. Another proposed function of BST-1 is as a receptor molecule. Cross-linking of BST-1 with polyclonal anti-(BST-1) antibodies induced tyrosine phosphorylation of a 130 kDa protein (p130) in the human myeloid cell lines, U937 and THP-1 [22,26,27]. In fact, in the case of CD38, another mammalian homologue of *Aplysia* cyclase, CD31 has been identified as a putative ligand for CD38 [28]. However, a similar molecule that binds to BST-1 has not been reported yet. SNP-1, found as a peptide inhibitor for BST-1 by phage display, had no amino acid sequence homology to the known sequences [15].

The purified cyclase appeared to be heterogeneous by SDS/PAGE analysis. A similar finding was observed with sBST-1 expressed in yeast (results not shown). The sBST-1 expressed in *Escherichia coli* was inactive (results not shown). As we thought

that post-translational modifications may give rise to the heterogeneity, we produced N-glycosylation site mutants. Both tunicamycin treatment of insect cells infected with the virus encoding the wild type of sBST-1 and complete removal of the N-glycosylation sites by protein engineering, reduced the secretion of sBST-1. Three glycosylation mutants, each with a single intact N-glycosylation site, were secreted into the culture medium. The mutants, N1, N3 and N4, each migrated as a single band by SDS/PAGE, whereas N2 was poorly secreted into the medium. The results suggest that the carbohydrates attached to Asn^{66}, Asn^{148} and Asn^{192} are necessary for secretion, and that each N-glycosylation site attaches a carbohydrate of a different size. The original sBST-1 showed heterogeneity even after glycosidase treatment. This resistance to the glycosidase might be due to incomplete denaturation prior to incubation with the glycosidase or due to special types of glycosylation induced by an insect cell expression system. On the other hand, the mobilities of N1 and N3 hardly shifted indicating that the oligosaccharide moieties of N1 and N3 may be very small by nature. The purified mutants also showed ADP-ribosyl cyclase activity, although the cyclase activity of N1 was low. The cADPR hydrolase activities of N1 and N3 were scarcely retained. The results suggest that the carbohydrates attached to Asn^{148} and Asn^{192} are needed for the cyclase activity, and the carbohydrate attached to Asn^{192} may be important for the hydrolase activity. Interestingly, the glycosylation site of Asn^{192} is conserved between BST-1 and CD38, indicating its importance for biological function. Thus we speculate that each glycosylation site possibly has distinctive roles, including intracellular protein targeting to the surface, secretion at the plasma membrane, or protein folding etc. Our results are consistent with the report that the glycosylation of CD38 plays an important role to maintain the enzyme activity and substrate affinity [29].

Next we examined the crystal-forming capacities of the purified sBST-1s, including the series of mutant sBST-1s. Crystals were obtained with one of the glycosylation mutants, N4 (Figure 7A), but not with the wild-type sBST-1. The diffraction pattern showed Bragg reflections up to 2.5 Å resolution. One obstacle to the formation of glycoprotein crystals is thought to be due to interference by the surface carbohydrate moiety and the heterogeneity of the attached carbohydrate. We made a homology model of sBST-1, based on the published coordinates of *Aplysia* cyclase, as shown in Figure 7(B). In the model, the N-glycosylation sites of N1, N2 and N3 are exposed on the surface of the molecule, whereas that of N4 mutant faces toward the interior of the molecule. The oligosaccharide linked to N4 might not interfere with the crystal packing. In sBST-1, the results of a series of lectin columns (results not shown) are consistent with the notion that the glycosylation of proteins expressed in insect cells is incomplete, as compared with that in mammalian cells. There is insufficient information available to determine whether the N-glycosidase used in our experiment is relevant to the proteins expressed in insect cells. An actual atomic resolution analysis of sBST-1 is needed to probe the applicability of our methodology. A similar approach has been utilized in the structural analysis of the mannose-6-phosphate receptor [30]. Thus, we speculate that an artificial modification of the carbohydrate-moiety-attaching sites would be a reasonable procedure for making glycoprotein crystals.

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