Novel peptide inhibitor of ecto-ADP-ribosyl cyclase of bone marrow stromal cell antigen-1 (BST-1/CD157)

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cADP-ribose, which is produced by ecto-ADP-ribosyl cyclase of CD38 or bone marrow stromal cell antigen-1 (BST-1), is a key regulator of Ca^{2+} -induced calcium release in an $\text{Ins}(1,4,5)\text{P}_3$ -independent manner. In the present study, we have identified a specific peptide inhibitor for ADP-ribosyl cyclase of BST-1. A 15-mer random peptide phage library was screened with a soluble form of BST-1 expressed in baculovirus-infected insect cells. After biopanning, two potent sequences reactive towards BST-1 were isolated. The two synthetic peptides corresponding to the identified sequences were shown to antagonize each other's ability to inhibit the binding of the two isolated phage to BST-1, suggesting that they bind at a common binding site on BST-1.

One of the peptides (SNP-1) was shown to inhibit ADP-ribosyl cyclase activity of BST-1 in an uncompetitive manner with a K_i value of 180 ± 40 nM ($n = 3$). SNP-1 also inhibited cyclic ADP-ribose hydrolase activity of BST-1 dose-dependently. Selected phage did not cross-react with a soluble form of CD38. SNP-1 did not show any inhibitory effect on ADP-ribosyl cyclase activity of CD38, and therefore this peptide inhibitor will be useful to serve as a starting tool for understanding the roles of these intriguing ecto-enzymes. This is the first report of a specific ADP-ribosyl cyclase inhibitor.

Key words: cADP-ribose, ecto-enzyme, phage display.

INTRODUCTION

cADP-ribose acts as an agent potent in mobilizing Ca^{2+} from ryanodine-sensitive, $\text{Ins}(1,4,5)\text{P}_3$ -independent intracellular stores [1]. cADP-ribose is thought to be an endogenous modulator of ryanodine receptors [2]. Biological functions of cADP-ribose have been studied in sea-urchin eggs, as well as in a variety of mammalian cell systems. Convincing evidence is accumulating that cADP-ribose is involved in cardiac muscle contraction [3], glucose-induced release of insulin in endocrine pancreas [4] and contraction of intestinal longitudinal muscle [5]. Recently, in plants, cADP-ribose has been reported to be involved in a signalling pathway of stress response [6]. It is also suggested that cADP-ribose may mediate the effects of NO on calcium [7].

cADP-ribose-synthesizing enzyme was originally identified in the cytoplasmic extract of the ova-testis of the *Aplysia californica*, and the *A. californica* cyclase has been shown to be a soluble protein [8]. In mammalian cells, cADP-ribose is synthesized by the membrane-bound enzymes CD38 [9] and BST-1 [10]/BP-3 [11], which is now renamed CD157 [12]. Human BST-1/CD157, which was identified in a bone marrow stromal cell line, consists of 290 amino acids. The amino acid sequence corresponding to BST-1 cDNA contains an N-terminal signal sequence and a short C-terminal hydrophobic region, suggesting that BST-1 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein [10,12]. CD38 [9] is a type II membrane protein and is known to be an immunoregulatory molecule found on lymphocytes [13,14]. The amino acid sequence of BST-1 shows 29% homology with that of *A. californica* ADP ribosyl cyclase [15] and 33% homology with CD38. The genomic structures of the three proteins have

revealed that BST-1 [16] and CD38 [17,18] evolved from a common ancestor by gene duplication. The positions of ten cysteine residues of BST-1 are completely conserved between CD38 and the *A. californica* cyclase, suggesting that their tertiary structures are similar.

In contrast with the soluble *A. californica* ADP-ribosyl cyclase, both of these membrane proteins (BST-1 and CD38) have their catalytic regions on the extracellular side of the membrane. The extracellular portions of BST-1 [19] and CD38 [20–22] were isolated and shown to have both cADP-ribose synthesizing and hydrolytic activities. Thus BST-1 and CD38 are bifunctional ecto-enzymes, having both ADP-ribosyl cyclase and cADP-ribose hydrolase activities. However, both products, cADP-ribose and ADP-ribose, do not appear to cross the plasma membrane themselves. How cADP-ribose, which is presumably produced by extracellularly facing ADP-ribosyl cyclase, enters into the cell interior is currently under investigation.

Recent cross-linking studies have shown that antibodies raised against CD38 [23–25] or BST-1 [26] induce tyrosine phosphorylation of intracellular proteins. Thus these proteins could function as receptor molecules as part of a signal transduction pathway(s). In the case of CD38, a 120 kDa protein expressed predominantly on endothelial cells was identified as a CD38 ligand [27]. A putative BST-1 ligand was also postulated; however, the relationship between enzymic activity and other functions of BST-1 is still unclear, owing to the lack of a suitable method for analysing the enzyme activity.

In the present study, we developed a peptide inhibitor of ADP-ribosyl cyclase of BST-1. This inhibitor was identified in a phage display library screened by the purified soluble form of BST-1,

Abbreviations used: CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; NGD⁺, nicotinamide guanine dinucleotide; RA, rheumatoid arthritis; (s)BST-1, (soluble) bone marrow stromal cell antigen-1.

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The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers AB012598 and AB012599 for SN-1 and SN-16 respectively.

which was produced by a baculovirus system. Kinetic studies of the inhibition by the peptide were performed.

EXPERIMENTAL

Preparation of a soluble form of BST-1 (sBST-1)

A soluble form of human BST-1 was expressed by a baculovirus/insect cell system and purified to homogeneity. Detailed procedures will be described elsewhere (S. Yamamoto, A. Sato and H. Jingami, unpublished work). Briefly, recombinant transfer vector was constructed by ligating the 1.0 kbp *EcoRV/XbaI* fragment of pΔ63-BOS [10], which encoded a predicted soluble form of BST-1 (1–297 amino acids), into the *SmaI/XbaI* site of the baculovirus transfer vector, pVL1393 (PharMingen, San Diego, CA, U.S.A.). Recombinant baculovirus was produced by co-transfecting Sf9 insect cells with the recombinant transfer vector and linearized AcNPV DNA (PharMingen) using a lipofectin reagent. sBST-1 was generated by infecting Tn5 insect cells at a multiplicity of infection of 1 in monolayered culture. Post-infection (after 72 h), the culture supernatant was collected and the secreted sBST-1 was purified to homogeneity by ion-exchange chromatography (POROS HS gel matrix; PerSeptive Biosystems Cambridge, MA, U.S.A.) and dye ligand chromatography (HI-TRAP Blue). For Western blotting, the blot was probed with a rabbit anti-(BST-1) antibody [28] and then the bound antibody was detected with alkaline-phosphatase-conjugated anti-(rabbit IgG) as a secondary antibody. sBST-1 produced from Chinese hamster ovary (CHO) cells has been described previously [19].

Screening of sBST-1 binding phage in a 15-amino-acid random phage display library

A phage display library consisting of 15 random amino acids inserted in the minor coat protein pIII has been previously described [29]. For biopanning, 96-well microtitre wells (Nunc-Immuno Maxisorp, Nalge Nunc International, Naperville, IL, U.S.A.) were coated at 4 °C with 3 µg of monoclonal antibody (mAb) BEC7 [12,26], and then 5 µg of purified sBST-1 was conjugated with mAb BEC7 in 100 µl of 10 mM phosphate buffer (pH 7.0). Wells were blocked with 10 mM phosphate buffer (pH 7.0) containing 1% (w/v) BSA for 1 h at room temperature. An aliquot of a 15-amino-acid random phage display library (10^{12} virions) in dilution buffer [10 mM phosphate buffer (pH 7.0) containing 1% (w/v) BSA/1 mM ZnCl_2] was pretreated in mAb BEC7-coated wells for 1 h to eliminate background yields. The non-bound phage were incubated in sBST-1-coated wells for 1 h at room temperature, and subsequently removed by washing 10 times with washing buffer [10 mM phosphate buffer (pH 7.0)/0.05% (v/v) Tween 20] and the bound phage were then eluted with 0.1 M glycine/HCl (pH 2.2) containing 1 µg/ml BSA. The eluted phage were neutralized and amplified in *Escherichia coli* K91 kan. Biopanning was repeated three times; no NAD^+ was added during the biopanning step. Individual phage clones were lifted from agar plates and amplified. sBST-1-binding phage were identified using an ELISA method for phage [29]. The single-stranded DNA molecules were prepared using the standard protocol [30] and their sequences were determined using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, U.S.A.). The peptides used in the present study were chemically synthesized by standard automated methods on an Applied Biosystems Model 430A peptide synthesizer and purified by reverse-phase HPLC. The purity was routinely more than 95%.

Phage ELISA

Phage ELISA was carried out as described previously [29], except that sBST-1 was immobilized by mAb BEC7 in a microtitre well. When a soluble form of CD38 (sCD38) was used, 100 µl of 4 µg/ml sCD38 in 10 mM phosphate buffer (pH 7.0) was used for direct immobilization in a well. In competitive ELISA, each peptide at the indicated concentration was added to compete with phage during the phage-binding step.

Fluorimetric assay for ADP-ribosyl cyclase

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 cGDP-ribose [31], a product which is fluorescent. The purified sBST-1 or sCD38 (at the indicated concentrations) was incubated at 25 °C with various concentrations of NGD^+ in 500 µl buffer containing 50 mM Mes (pH 6.0) and 1 mM ZnCl_2 . The cyclization reaction was continuously monitored by measuring cGDP-ribose fluorescence using a Hitachi fluorescence spectrophotometer (F-4500) at excitation and emission wavelengths of 300 nm and 410 nm respectively. When the K_i value was determined, inhibition was evaluated by varying the concentrations of NGD^+ (280–800 µM) and fixing the concentration of sBST-1 at 1.5 µg/ml in the presence or absence of SNP-1. Data were calculated by the method of Dixon [32] using commercial software called 'Enzyme kinetics' (Trinity Software, Campton, NH, U.S.A.).

cADP-ribose hydrolase activity

cADP-ribose hydrolase activity was assayed by HPLC essentially as described previously [19]. Briefly, sBST-1 (50 µg/ml) was incubated at 37 °C with 20 µM cADP-ribose (Sigma) in a buffer containing 50 mM Mes (pH 6.0) and 1 mM ZnCl_2 in the presence or absence of SNP-1. The reaction products were separated on an anion-exchange column (PL-SAX 1000A; Polymer Laboratories, Amherst, MA, U.S.A.) with a flow rate of 0.5 ml/min, and were eluted with a linear gradient of 0.02–0.51 M NH_4HCO_3 for 15 min. Concentrations of cADP-ribose and ADP-ribose were represented as the % of their peak areas on HPLC by monitoring A_{260} .

Preparation of sCD38

Recombinant sCD38 was produced and purified from a CHO cell line producing human sCD38, as described previously [26].

RESULTS

sBST-1 was generated by a baculovirus system in insect cells. A transfer vector, shown schematically in Figure 1(A), was used for obtaining a recombinant baculovirus for sBST-1. From the medium of insect cells infected with the recombinant baculovirus, we purified the sBST-1. Figure 1(B) shows the purified sBST-1 revealed by Coomassie Brilliant Blue staining (left lane). This band was detected by Western blotting with a polyclonal antibody raised against BST-1 (right lane). A phage library was screened with the purified sBST-1; this 15-mer random peptide library contains 2×10^8 clones [29]. The purified sBST-1 was immobilized in microtitre wells. We compared three different conditions of immobilization of sBST-1: direct immobilization, and immobilization of BST-1 by mAb RF3 and mAb BEC7 [12]. After three

Figure 1 Expression of a soluble form of BST-1 (sBST-1) in insect cells

(A) Schematic representation of the construct used in the present study. A predicted soluble form of BST-1, which was prepared by introducing a stop codon at Thr²⁹⁸, was expressed under the control of polyhedrin promoter in insect cells. SP represents a native signal peptide of BST-1. (B) Analysis of purified sBST-1 by SDS/PAGE. Purified sBST-1 (1 µg) was subjected to SDS/PAGE (12% gels) under reducing conditions. Coomassie Brilliant Blue staining (centre panel) and immunoblotting analysis (right panel) are shown, with respect to standard molecular-mass markers (shown on the left).

Figure 2 Specific binding of phage SN-1 to sBST-1

After three rounds of biopanning, supernatants of individual clones were used directly for analysis by phage ELISA. One representative clone (SN-1) that specifically binds to sBST-1 is shown. Unrelated phage does not show any reactivity with sBST-1. Representative data obtained from two independent experiments are shown.

cycles of biopanning, potentially reactive phage were isolated only from the wells on to which sBST-1 was immobilized by mAb BEC7. Figure 2 shows the results of phage ELISA. The most potent clone, SN-1, specifically bound the sBST-1 produced from insect cells, as well as that from CHO cells, whereas

Table 1 BST-1-binding sequences isolated from a phage display library

Clone	Sequence	Frequency*
SN-1	HSQISGKYQRYLKDA	7/8
SN-16	DDVVYTNHKGWRRE	1/8

* Number of times each sequence was independently isolated.

Figure 3 Inhibition of selected phage binding to sBST-1 by synthetic peptides, SNP-1 and SNP-16

Phage SN-1 and SN-16 were incubated in sBST-1-coated wells, with or without the indicated peptides. Absorbances (OD) at 450 nm are shown. All of the data were calculated from four wells (means ± S.D.). Representative data obtained from two independent experiments are shown.

Figure 4 Inhibition of ADP-ribosyl cyclase activity of sBST-1 with a synthetic peptide, SNP-1

NGD⁺ (300 µM) was incubated with sBST-1 (4 µg/ml) in a buffer containing 50 mM Mes, pH 6.0, 1 mM ZnCl₂ at 25 °C, and the fluorescence at 410 nm (excited at 300 nm) was monitored. After incubation for 5 min, 20 µM control or SNP-1 peptide was added. The inset shows inhibition of ADP-ribosyl cyclase by 10 mM dithiothreitol (DTT).

Figure 5 Kinetics of ADP-ribosyl cyclase activity of sBST-1 in the presence of SNP-1

(A) sBST-1 (1.5 $\mu\text{g/ml}$) was incubated for 10 min with various concentrations of NGD⁺ in the absence of SNP-1 (■), or in the presence of 200 nM (○) or 400 nM (●) SNP-1. The product cGDP-ribose was monitored fluorimetrically with an excitation wavelength of 300 nm and an emission wavelength of 410 nm. (B) Dixon plot of the data in (A): $1/V$ versus $[I]$ at different fixed concentrations of NGD⁺. (C) Dixon plot of the data in (A): $[S]/V$ versus $[I]$ at different concentrations of NGD⁺. The data are representative of three independent experiments.

unrelated phage did not show any reactivity towards sBST-1. Although no reactive phage in the screening was obtained from the wells in which sBST-1 was directly immobilized, SN-1 was able to recognize sBST-1 directly immobilized on the surface of wells (Figure 2). The eight phagemids were purified and the DNA sequences of their randomized parts were determined. The DNA sequences of seven clones were identical, and a representative one was designated as SN-1; the remaining clone was designated as SN-16. As shown in Table 1, the deduced amino acid sequences of the two phage clones were completely different. The peptides corresponding to the identified amino acid sequences were synthesized, and the inhibitory effects of these peptides on the binding of phage to sBST-1 were examined

Figure 6 Inhibition of cADP-ribose hydrolase activity by SNP-1

sBST-1 (50 $\mu\text{g/ml}$) was incubated at 37 °C for 4 h with 20 μM cADP-ribose in the presence or absence of SNP-1. cADP-ribose and ADP-ribose were separated on an anion-exchange column. Concentrations of cADP-ribose and ADP-ribose are represented as the percentage of their peak area on HPLC (100% represents the total area of two peaks of cADP-ribose and ADP-ribose). Hydrolysed products during the incubation of cADP-ribose only were expressed as the background yield. The data shown are representative of three independent experiments.

(Figure 3). The synthetic peptide SNP-1 corresponding to the sequence of phage SN-1 was soluble, whereas the synthetic peptide SNP-16 corresponding to that of phage SN-16 was insoluble, and therefore this was dissolved in DMSO for subsequent use. SNP-1, at a concentration of 11 μM , completely inhibited the phage SN-1 binding to sBST-1, whereas SNP-16 partially inhibited the phage SN-16 binding to sBST-1 at the same concentration. The control peptide, which encodes a sequence in the reversed orientation to that of SNP-1, did not show any inhibition. Because SNP-1 also inhibited the phage SN-16 binding to sBST-1, and vice versa, the binding sites of the two peptides appeared to be either close or overlapping.

Next we examined the inhibition of the ADP-ribosyl cyclase activity by these peptides. A fluorimetric assay was performed using NGD⁺ as a substrate instead of NAD⁺. NGD⁺ is converted into cGDP-ribose by ADP-ribosyl cyclase. Because cGDP-ribose is resistant to hydrolysis and is also fluorescent, the cyclase reaction can be monitored. After addition of sBST-1 to the reaction mixture, the fluorescence increased linearly, as shown in Figure 4. Addition of 20 μM of SNP-1 at 5 min blunted the accumulation of the reaction product, cGDP-ribose. The specificity of this inhibition was supported by the failure of the control peptide to inhibit sBST-1 at the same concentration. In contrast, SNP-16 did not show any inhibitory effect at 20 μM (results not shown). The inset to Figure 4 shows that the addition of 10 mM dithiothreitol completely stopped the reaction. The cyclase activity of CD38 has been shown to be sensitive to reducing agents in a similar manner [20].

To evaluate the mode of inhibition of the ADP-ribosyl cyclase by SNP-1, we performed a kinetic study. Figure 5(A) shows the V versus $[S]$ plot in the fixed concentrations of the inhibitor peptide, $[I]$, where V , $[S]$ and $[I]$ represent the velocity of the enzyme reaction, the concentration of NGD⁺ and the concentration of SNP-1 respectively. The K_m value of ADP-ribosyl cyclase for NGD⁺ was $610 \pm 10 \mu\text{M}$ ($n = 3$). The reciprocal plot

Figure 7 No inhibitory effect of SNP-1 on ADP-ribosyl cyclase activity of sCD38

(A) The failure of phage SN-1 and SN-16 to cross-react with sCD38 is shown. Phage SN-1 and SN-16 were tested for their ability to bind to immobilized sCD38 by using phage ELISA. Absorbances at 450 nm are shown. As a control, phage SN-1 and SN-16 recognized immobilized sBST-1, and their binding was inhibited by the addition of corresponding synthetic peptides. The data represent means for experiments in duplicate wells. Representative data obtained in two independent experiments are shown. (B) and (C) The absence of any inhibitory effect of SNP-1 on ADP-ribosyl cyclase activity of sCD38 is shown. NGD⁺ (2 μM) was incubated with sCD38 (200 ng/ml) in a buffer containing 50 mM Mes, pH 6.0, 1 mM ZnCl₂ at 25 °C in the presence (B) or absence (C) of 20 μM SNP-1. The excitatory wavelength used was 300 nm and fluorescence was measured at 410 nm.

of $1/V$ versus $[I]$, shown in Figure 5(B), gave rise to linear and parallel lines at four fixed concentrations of substrate. These data indicate that SNP-1 inhibits the ADP-ribosyl cyclase of sBST-1 uncompetitively. When data were replotted using $[S]/V$ versus $[I]$, as shown in Figure 5(C), the lines converged at a common point. The x -axis value corresponding to the point of convergence yielded a K_i value of 180 ± 40 nM (mean $\pm$ S.E.M., $n = 3$).

The inhibition of cADP-ribose hydrolase activity by SNP-1

was also examined, as shown in Figure 6. Both cADP-ribose and ADP-ribose were separated on an anion-exchange column, as described in the Experimental section. SNP-1 inhibited the conversion of cADP-ribose into ADP-ribose dose-dependently. The inhibition at 20 μM was 60 % of the control value. Control peptide (20 μM) did not show any inhibitory effect on the conversion. The effect of SNP-16 on the cADP-ribose hydrolase activity was not tested because of its insolubility.

Figure 7(A) shows the reactivity of phage SN-1 and SN-16 with sCD38. Recombinant phage displaying neither SN-1 nor SN-16 reacted with sCD38 immobilized in microwells. ADP-ribosyl cyclase activity of sCD38 was not influenced by the presence of 20 μM SNP-1 (Figure 7B), as confirmed by the data reflecting the fluorescence increase in the absence of SNP-1 to be the same (Figure 7C); thus the inhibition of SNP-1 is specific to BST-1.

DISCUSSION

In most of the experiments involving cADP-ribose, permeabilized cells or isolated intracellular membrane preparations have been usually used. Alternatively, cADP-ribose or its antagonist 8-amino derivative (8-amino-cADP ribose) [33] has been injected intracellularly. Our initial idea was whether or not intracellular effects of cADP-ribose could be modified at the plasma membrane, where the cADP-ribose-synthesizing enzymes reside. We intended to obtain peptides that interacted with the catalytic domain of the mammalian ADP-ribosyl cyclase. The peptides were expected to inhibit the enzyme activity and/or mimic the putative ligand molecule. Possible mechanisms are proposed for the entry of cADP-ribose into cells, e.g. there might be either a receptor or transporter for cADP-ribose in the plasma membrane, or GPI-anchored protein either by itself or in association with other protein(s) might be internalized. Recently, the GPI-anchored proteins, CD59 and CD48, have been reported to be co-immunoprecipitated with trimeric G-proteins [34]. Furthermore, muscarinic acetylcholine receptor is known to couple to ADP-ribosyl cyclase via G-protein [35]. In the case of CD38, substrate- or ligand-induced internalization has been reported [36,37], suggesting that the enzymic activity of CD38 is in the cytoplasmic compartment. Therefore it was of interest to investigate whether the inhibitory peptide SNP-1 could prevent BST-1 from binding to its putative ligand, if this indeed exists, or whether it could interfere with BST-1's ability to transmit signals into the cells.

BST-1 was identified as a cell-surface molecule by expression cloning with mAb, which was raised against rheumatoid arthritis (RA)-derived bone marrow stromal cell lines. Bone marrow stromal cells not only produce soluble factors, but also express surface molecules acting on B-lineage cell growth and development. In sera of patients with severe RA, significantly high levels of a soluble form of BST-1 have been detected [28]. Thus the peptide inhibitor, SNP-1, might provide an insight into the relationship between ecto-enzyme activities and the pathogenesis of RA, although so far we have not obtained obvious effects of SNP-1 on growth in cells that express BST-1 (results not shown).

Phage display, which we used in the present study, is a very powerful method and has been applied to various aspects of biological experiments, including a search for natural ligands for uncharacterized receptor molecules. A homology search of the two peptide sequences has not disclosed any known similar sequences. As the inhibitor peptide that we obtained is specific for the ADP-ribosyl cyclase of BST-1, this peptide can be used for discrimination of BST-1 cyclase activity from other cyclase activities. The same kind of phage display approach could be

applied to other ADP-ribosyl cyclases to obtain their peptide inhibitors. In order to generate a more potent inhibitor, an experiment to delineate the residues in SNP-1 essential for the inhibition is now in progress.

Though SNP-1 binds to BST-1 in the absence of the substrate, it has been found to inhibit the enzyme uncompetitively. A classical uncompetitive inhibitor is defined as a compound that binds reversibly to the enzyme-substrate complex, ES. It is possible that the mode of binding of SNP-1 to ES is different from that to free enzyme, E. A relevant conformation similar to ES might be produced by the binding of E to the antibody in the screening. Uncompetitive inhibition is often observed in reactions that involve multiple substrates. Because BST-1 performs two consecutive enzymic reactions, the bifunctionality of this enzyme might account for the observed uncompetitive inhibition. Development of a more specific inhibitor peptide that can recognize a difference between ADP-ribosyl cyclase and cADP-ribose hydrolase in BST-1 will provide insights into the mechanism of the dual enzymic reaction.

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