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ADP ribosyl cyclase activity of a novel bone marrow stromal cell surface molecule, BST-1

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Abstract Human BST-1, a bone marrow stromal cell surface molecule, is a GPI-anchored protein that facilitates the growth of pre-B cells. The deduced amino acid sequences of human and mouse BST-1 show around 30% homology with those of CD38 and *Aplysia* ADP ribosyl cyclase. Therefore, like CD38, BST-1 might possess ADP ribosyl cyclase activity. Here, we report the establishment of a stable transformant CHO cell line, which secretes truncated human soluble BST-1, and show that purified soluble BST-1 displays both ADP ribosyl cyclase and cADPR hydrolase activities.

Key words: BST-1; CD38; ADP ribosyl cyclase; cADPR hydrolase

1. Introduction

We have recently isolated the cDNA for a novel surface molecule BST-1 (bone marrow stromal cell antigen 1) expressed on human bone marrow stromal cell lines, and showed that BST-1 is a glycosyl-phosphatidylinositol (GPI)-anchored protein that facilitates the stroma cell-dependent growth of pre-B cell line [1]. The expression of BST-1 was enhanced in rheumatoid arthritis-derived bone marrow stromal cell lines compared with healthy donor-derived ones. In addition, BST-1 mRNA was detected in various tissues including placenta, lung, liver and kidney, where its physiological roles, however, are still unclear. The deduced amino acid sequence of both human and murine BST-1 showed around 30% homology with those of *Aplysia* ADP ribosyl cyclase and CD38 [1,2]. The molecular cloning of murine BST-1 [2] and murine BP-3 [3] showed that BST-1 is identical with BP-3 which was originally identified as a molecule expressed on pre-B cells transformed either by Abelson murine leukemia virus or an *erb* B oncogene construct [4].

CD38, which is expressed in lymphocytes and various tissues, bears a 31% homology to *Aplysia* ADP ribosyl cyclase [5], and possesses both ADP ribosyl cyclase and cyclic ADP ribose (cADPR) hydrolase activities [6–12]. Anti-CD38 antibody induces an increase of intracellular Ca^{2+} and an immunoglobulin-independent activation and proliferation of B cells [12,13]. It has been suggested that this activation is partially mediated by the ADP ribosyl cyclase activity of CD38 that catalyzes the synthesis of cADPR from NAD, since cADPR but not ADP ribose (ADPR) augmented the stimulatory effect of anti-CD38 mAb on B cells [12]. Cyclic ADPR is known to serve as an endogenous second messenger that regulates the mobilization of intracellular calcium [14–16], but the mechanism by which extracellular cADPR stimulates B cells remains to be clarified.

In this study, we first designed and purified a recombinant human soluble BST-1 molecule (sBST-1) expressed in CHO cells, and then evaluated its enzymic activity as ADP ribosyl cyclase.

2. Materials and methods

2.1. Preparation of soluble BST-1

In order to express human BST-1 in its soluble form, the cDNA encoding amino acids 1–297 was amplified by polymerase chain reaction (PCR) using two primers, one sense (5-'CCGAATTCCACCATGGCGGCCCAGGGGTGCGCGGCA-3') and one antisense (5'-GGTGATCATTAATAAAGACTTGGGGCTTTTCTTT-3'), containing respectively a Kozak consensus sequence [17] and a termination codon. After digestion by *Eco*RI and *Bcl*I, the PCR product was placed under the control of the EF-1 α-promoter in a pCHO-1 plasmid containing dihydrofolate reductase (DHFR) cDNA as a selective marker, thus yielding pCHO-BST1EC. The expression vector (40 μg) was linearized by *Pvu*I and introduced into CHO cells (7×10^6 cells, DHFR-) by electroporation (1.6 kV, 25 μF) using a Gene-Pulser (Bio-Rad). The sBST-1 secreted by transformants was analyzed by a Biosensor (BIAcore, Pharmacia), in which RF3 (3 μg) [1], a mouse anti-human BST-1 monoclonal antibody, was immobilized on a sensor chip. After sequential gene amplification in the presence of 20 nM and 100 nM methotrexate, a CHO clone (4–9) highly expressing sBST-1 was established, and cultured in ASF104 serum-free medium (Ajinomoto). After 3 or 4 days of incubation, the culture supernatant was collected, concentrated, and exchanged for binding buffer (10 mM Tris-HCl, pH 8.6) using an Ultra-Filtration miniset Omega 3K (Filtron). The solution was then applied onto a DE52 column (Whatman) equilibrated with the binding buffer. The column was washed with 5 bed volumes of the same buffer, and eluted with 0.1 M NaCl in 10 mM Tris, pH 8.6. The elution fraction corresponding to the largest absorption peak at 280 nm was collected, concentrated, and the buffer exchanged for RCA buffer (10 mM Tris-HCl, pH 7.6) using a Centriprep-30 (Grace). The fraction was then applied onto a RCA120 column (Hohnen) equilibrated with the RCA buffer. The column was washed with 10 bed volumes of RCA buffer, and eluted with RCA buffer containing 200 mM galactose. The fraction corresponding to the largest absorption peak was collected, and concentrated. The protein sample was finally analyzed by SDS-PAGE, and its N-terminal amino acid sequence determined by a Model 476A protein sequencer (Applied Biosystems). Glycosylation analysis was performed with *N*-glycanase (Genzyme) according to the manufacturers procedure.

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Abbreviations: BST-1, bone marrow stromal cell antigen 1; GPI, glycosyl-phosphatidylinositol; ADPR, adenosine diphosphate ribose; cADPR, cyclic adenosine diphosphate ribose; DHFR, dihydrofolate reductase; IP3, inositol 1,4,5-triphosphate.

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2.2. Detection of ADP ribosyl cyclase activity

ADP ribosyl cyclase and cADPR hydrolase activities were measured through the production of cADPR from NAD and of ADPR from cADPR, respectively. Assays for these activities were essentially performed under the following conditions. First, sBST-1 (10 μg/ml) was incubated at 37°C with 0.2 mM NAD (or 20 μM cADPR) in 50 mM MES buffer, pH 6.0, containing 1 mM $ZnCl_2$. Aliquots were collected at successive time intervals for time course analysis, and after 2 h for the studies on the effects of pH or metal ions. Soluble BST-1 inactivated by heating at 96°C for 2 h was used as a negative control. NAD, cADPR and ADPR present in the samples were then separated using a PL-SAX 1000A HPLC column (50 × 4.6 mm; Polymer Laboratories) with a linear gradient of 0.02 M to 0.51 M NH_4CO_3 for 15 min, and a flow rate of 0.5 ml/min. Standard samples were purchased from Sigma (NAD and ADPR) and Amersham (cADPR), and applied under the same conditions. Concentrations of each product were represented as the integrated percentage of their peak areas on HPLC by monitoring their absorption at 260 nm.

3. Results

3.1. Characterization of sBST-1

By subcloning BST-1 cDNA into a pCHO-1 plasmid and transfecting it into CHO cells, we established a CHO cell line that produces sBST-1 at a concentration of approximately 30 μg/ml. Using two-step column chromatography, successively on anion-exchange and lectin columns, sBST-1 was purified as a broad band with an apparent molecular weight of 43 kDa after SDS-PAGE and Coomassie brilliant blue staining (Fig. 1, lane 1). The molecular weight was not consistent with the predicted molecular weight of 30.2 kDa based on the deduced amino acid sequence, suggesting the presence of posttranslational modifications. Indeed, the presence of four potential N-linked glycosylation sites on this sequence suggested that sBST-1 is heavily glycosylated. This was confirmed by *N*-glycanase treatment of sBST-1 which produced a sharp single band at approximately 31 kDa (Fig. 1, lane 2).

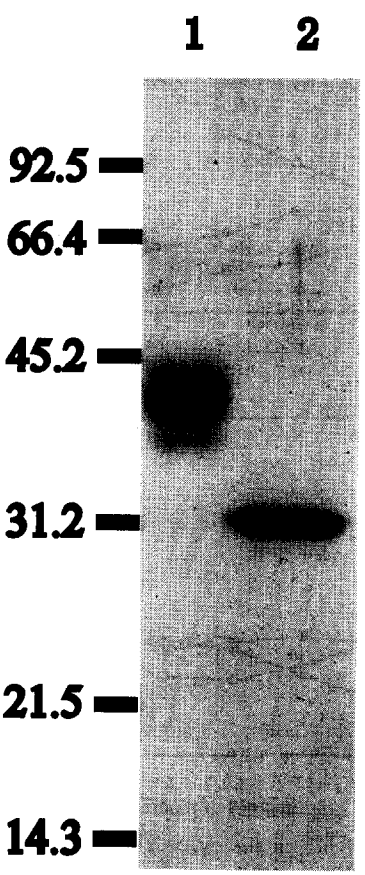

Fig. 1. Analysis of N-linked glycosylation. Purified sBST-1 (lane 1) and *N*-glycanase treated sBST-1 (lane 2) were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Protein size markers are indicated on the left side.

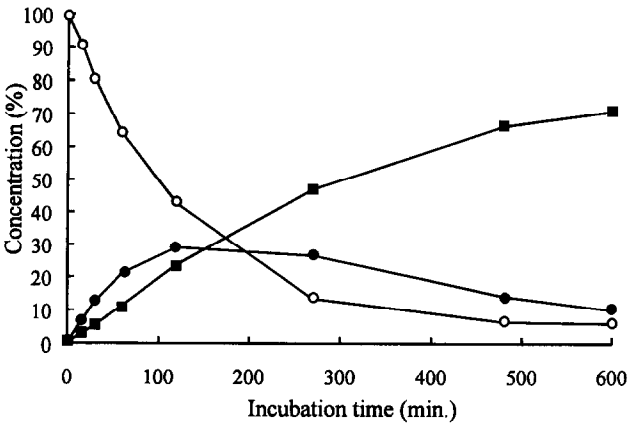

Fig. 2. Time course analysis of NAD metabolization by sBST-1. Purified sBST-1 (50 μg/ml) was incubated with 0.2 mM NAD in 50 mM MES buffer at pH 6.0 containing 1 mM $ZnCl_2$ at 37°C. Aliquots (200 μl) were collected at the indicated times, and analyzed by anion-exchange HPLC as described in section 2. Concentrations of NAD (○), cADPR (●) and ADPR (■) are represented as the percentage of their peak areas on HPLC. 100% represents the total area of 3 peaks.

We also identified the first eleven amino acids of BST-1 N-terminal sequence as Arg-Trp-Arg-Ala-Glu-Gly-Thr-Ser-Ala-His-Leu, which were completely identical to the Arg^{33}-Leu^{43} deduced amino acid sequence of BST-1. This sequence analysis revealed that the sBST-1 precursor peptide is cleaved between Ala^{32} and Arg^{33}, which corresponds well to the potential cleavage site (second candidate site) determined by computer analysis [18].

3.2. Enzymic activity

Soluble BST-1 was incubated with NAD or cADPR, and its enzymic activities were determined by HPLC by measuring the reaction products, cADPR or ADPR. Time course experiments showed that sBST-1 produced both cADPR and ADPR from NAD, suggesting that sBST-1 possesses two enzymic activities, ADP ribosyl cyclase and cADPR hydrolase (Fig. 2). Evidence for each enzymic activity was further supported by comparison with heat-inactivated sBST-1 (Fig. 3). Native sBST-1 was able to generate cADPR from NAD, and ADPR from cADPR, whereas heat-inactivated sBST-1 did not show any enzymic activity.

The effect of pH on the ADP ribosyl cyclase activity of sBST-1 was examined next. Enzymic activity of sBST-1 in the presence of EDTA was maximum at pH 4.0 and decreased at higher pH (Fig. 4a). By contrast, in the presence of Zn^{2+}, sBST-1 showed an almost equal enzymic activity between pH 4.0 and pH 6.5 (Fig. 4b), suggesting that sBST-1 may require a metal ion for its enzymic activity.

The effect of metal ions on sBST-1 enzymic activity was further examined. Zn^{2+} and Mn^{2+} remarkably increased the ADP ribosyl cyclase activity, up to 5-fold compared to the control (Fig. 5a). Cyclic ADPR hydrolase activity was also

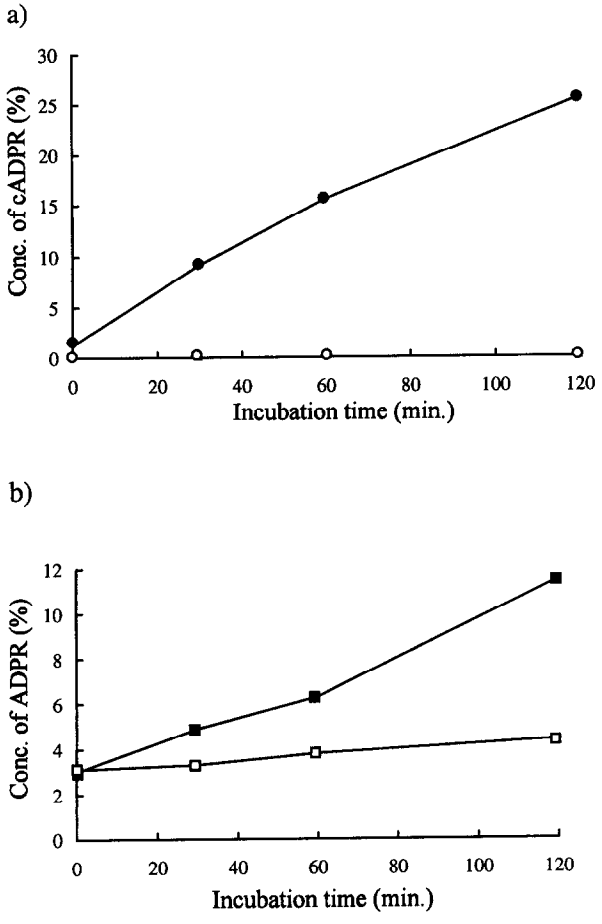

Fig. 3. Analysis of ADP ribosyl cyclase and cADPR hydrolase activities. Native sBST-1 (10 μg/ml) (●, ■) was incubated with 0.2 mM NAD (a) or 20 μM cADPR (b) in 50 mM MES buffer, pH 6.0, containing 1 mM $ZnCl_2$ at 37°C. Heat-inactivated sBST-1 (○ □) used as negative control was tested in parallel. Aliquots (200 μl) were collected at time 0 and after 30, 60 and 120 min, and analyzed by anion-exchange HPLC as described in section 2. 100% represents the total area of 3 peaks of NAD, cADPR and ADPR in (a) and 2 peaks of cADPR and ADPR in (b).

increased by Zn^{2+} and Mn^{2+} (Fig. 5a,b). Inversely, Cu^{2+} showed an inhibitory effect on both cyclase activity and hydrolase activity, while Mg^{2+} and Ca^{2+} did not have any significant influence.

4. Discussion

4.1. Characterization of sBST-1

We have established a CHO cell line that produces large quantity of human sBST-1, and successfully obtained sufficiently pure sBST-1 to study its biological activities. Since the ratio of sBST-1 in serum-free conditioned medium was very high (approximately 50% to 60% of the total proteins, data not shown), purification was relatively easy. Purified sBST-1 gave a broad band with an apparent molecular weight of 43 kDa on SDS-PAGE, whereas sBST-1 treated with *N*-glycanase gave a sharp single band at approximately 31 kDa, indicating that sBST-1 is a glycosylated protein. Since the difference in molecular weight is about 12 kDa, all four potential *N*-linked glycosylation sites can be glycosylated. In addition, the N-terminal sequence analysis revealed that the sBST-1 precursor peptide is cleaved between Ala^{32} and Arg^{33}, and no other minor cleavage site was detected. The broad 43 kDa band must thus be due to the heterogeneity of N-linked carbohydrates, and not to that of signal peptide cleavage sites. We previously reported that the putative cleavage site was between Gly^{28} and Gly^{29}, and predicted that the mature membrane form of BST-1 consists of 290 amino acids [1]. However, the N-terminal sequence analysis suggests rather that this form of BST-1 contains only 286 amino acids.

4.2. Enzymic activity of sBST-1

As expected from the sequence homology with CD38 and *Aplysia* ADP ribosyl cyclase, sBST-1 possessed ADP ribosyl cyclase and cADPR hydrolase activities. The combined effect of these two enzymic activities would thus result in conversion of NAD to cADPR, and then hydrolysis of cADPR to ADPR.

Previous studies showed that the optimum pH for CD38 enzymic activities was 6.5 and that the cyclase activity of CD38 on the erythrocyte membrane was increased by the addition of Zn^{2+} or Cu^{2+} [7]. Most recently, amino acid residues 119 and 201 of human CD38 have been demonstrated to play a crucial role in the cADPR synthesis and hydrolysis [19]. The corresponding amino acid residues of Cys^{119} and Cys^{201} in the human CD38 are Arg and Ser, respectively, in both human (Arg^{103} and

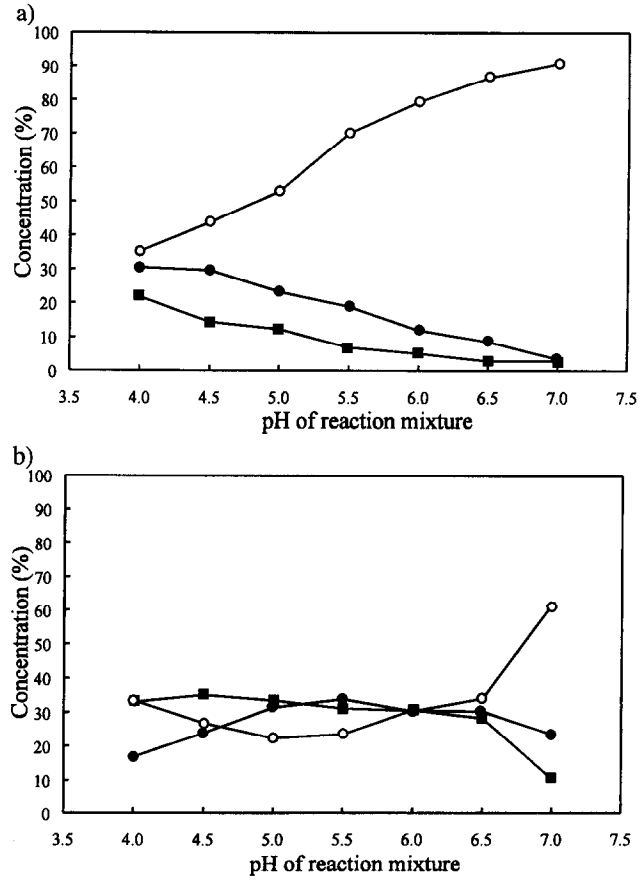

Fig. 4. Effect of pH on the catalytic activities of sBST-1. Purified sBST-1 (50 μg/ml) was incubated with 0.2 mM NAD in 50 mM MES buffer adjusted to the appropriate pH, in the presence of 1 mM EDTA (a) or 1 mM $ZnCl_2$ (b) for 2 h at 37 °C. Concentrations of NAD (○), cADPR (●) and ADPR (■) are represented as the percentage of their peak area on HPLC. 100% represents the total area of 3 peaks.

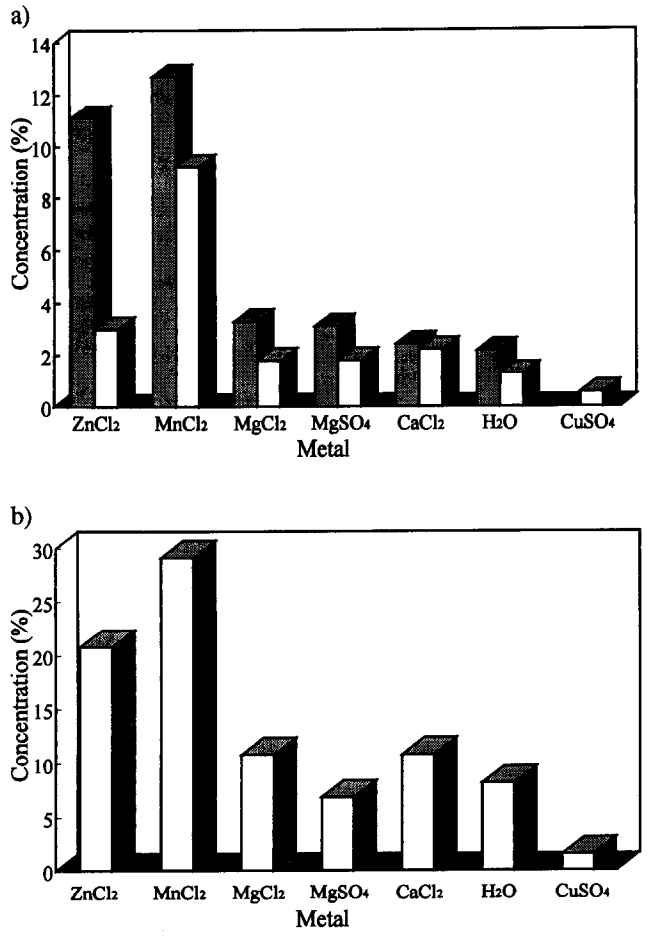

Fig. 5. Effect of various metal ions on ADP ribosyl cyclase and cADPR hydrolase activities. Purified sBST-1(20 μg/ml) was incubated with 0.2 mM NAD (a) or 20 μM cADPR (b) in 50 mM MES buffer pH 7.0 containing 1 mM of various metal ions as indicated, for 2 h at 37 °C. Concentrations of generated cADPR (gray boxes) and ADPR (open boxes) are represented as the percentage of their peak area on HPLC.

Ser^{184}) and mouse (Arg^{96} and Ser^{177}) BST-1 [1,2]. In the present study, the cyclase activity of sBST-1 in the absence of metal ions was scarcely detected around neutral pH (Fig. 4a), in agreement with Dong et al. [3] who did not detect any enzymic activity in BP-3 expressed on cell surface. This is consistent with the results that a BST-1 type mutant CD38 (Cys^{119} to Arg) showed neither ADP ribosyl cyclase nor cADPR hydrolase activity [19]. The pH dependent changes in the enzymic activity of sBST-1 (Fig. 4a) may account for the amino acid residues of this type of proteins including Arg^{103} and/or Ser^{184} in sBST-1. In the presence of Zn^{2+}, sBST-1 showed no pH dependency between pH 4.0 and pH 6.5, in which range the optimal enzymic activity was retained (Fig. 4b). In the extracellular region, ten Cys, one Glu, one His and one Asp residues were completely conserved among *Aplysia* ADP ribosyl cyclase [20,21], human CD38 [22], mouse CD38 [23], rat CD38 [24], human BST-1 [1] and mouse BST-1/BP-3 [2,3] which may constitute a new family. All members of this family may physiologically contain a metal ion in a common manner, since Cys, His, Glu and Asp can readily have coordinated binding to cations.

The addition of Cu^{2+} inhibited both the cyclase and hydrolase activities of sBST-1, whereas it increased the cyclase activity of CD38 on the erythrocyte membrane [7]. This discrepancy may prove to be useful in discriminating between BST-1 and CD38 activities.

4.3. The enzymic and biological activities of BST-1

BST-1, a GPI-anchored membrane protein, is expressed in stroma cells, whereas CD38, a type II transmembrane protein, is expressed in immature and activated lymphocytes, and both are thought to be involved in the growth and differentiation of lymphocytes [1,10,12,13]. It has been suggested that the physiological role of CD38 on B lymphocytes is at least in part due to the cyclase activity of CD38 [12].

It is known that cADPR mobilizes internally stored Ca^{2+} as effectively as inositol 1,4,5-triphosphate (IP3). The Ca^{2+} release mechanism by cADPR, however, is totally distinct from the IP3 pathway and related to the Ca^{2+}-induced Ca^{2+} release system [14–16]. Nevertheless, the function of ADPR still remains elusive. If BST-1 and CD38 act on the cell surface of lymphocytes through their enzymic activities, extracellularly produced cADPR requires the presence of specific cellular receptors to enter into the cells [25,26], although such receptors have not yet been identified.

At the moment, it is completely unknown whether the enzymic activity of BST-1 is involved in its support of pre-B cell growth. Alternatively, the enzymic activity of BST-1 may not be related to its effects on pre-B cell growth, but may be involved in other biological activities of cADPR, such as insulin release from pancreatic β-cells, which has been demonstrated previously [15]. Whether the cyclase and hydrolase activities of BST-1 are related to the physiological function deserves further investigation.

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