Cloning and heterologous expression of the gene for BLIP-II, a β -lactamase-inhibitory protein from *Streptomyces exfoliatus* SMF19

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A β -lactamase-inhibitory protein (BLIP-II) was purified from the culture filtrate of *Streptomyces exfoliatus* SMF19 and its N-terminal amino acid sequence was determined. A clone containing the gene encoding BLIP-II (*bliB*) was selected from a cosmid library by colony hybridization using an oligonucleotide probe based on the N-terminal amino acid sequence of BLIP-II. The *bliB* gene was isolated and sequenced. Analysis of the nucleotide sequence revealed that the gene consists of 1116 bp and encodes a mature protein of 332 amino acids preceded by a 40 amino acid signal sequence. *bliB*, expressed under the control of the T7 promoter in *Escherichia coli*, was accumulated in an inactive form in inclusion bodies, but β -lactamase-inhibitory activity was recovered after refolding. In addition, *bliB* was heterologously expressed in *Streptomyces lividans* TK24 using the *melC1* promoter. The BLIP-II protein produced in recombinant strains of *S. lividans* was secreted into the culture supernatant in a biologically active form.

Keywords: *Streptomyces exfoliatus*, β -lactamase-inhibitory protein, cloning, heterologous expression

INTRODUCTION

Widespread clinical use of β -lactam antibiotics has caused a continuous increase in the incidence of infections due to pathogenic bacteria resistant to these antibiotics (Neu, 1992). β -Lactamases showing various substrate specificities have been identified from the resistant isolates (Abraham, 1983). β -Lactamase inhibitors of microbial origin have also been identified and some of them, for example clavulanic acid (Brown *et al.*, 1976), olivanic acid (Butterworth *et al.*, 1979) and thienamycin (Kahan *et al.*, 1979) have been used clinically. A protein that inhibited the β -lactamase of *Staphylococcus aureus* was first isolated from *Streptomyces gedaensis* (Hata *et al.*, 1972). Another protein (BLIP) inhibiting various β -lactamases was characterized from the culture filtrate of *Streptomyces clavuligerus*, and showed K_i values in the nanomolar to picomolar range (Doran *et al.*, 1990; Strynadka *et al.*, 1994). The interaction between purified BLIP and the β -lactamase

of *Escherichia coli* (pUC) was noncovalent, with an estimated 1:1 molar stoichiometry (Doran *et al.*, 1990). The corresponding gene, *bli*, encoded a mature protein with a deduced sequence of 165 amino acids (M_r 17 523) and a 36 amino acid leader peptide.

X-ray crystallographic analysis revealed that BLIP was a relatively flat molecule with a unique fold, comprising tandemly repeated 76 amino acid domains, each containing a helix-loop-helix motif. The molecular interaction between β -lactamase RTEM-1 and BLIP was determined by establishing the structure of the complex, and a mechanism to explain the inhibiting effect of BLIP was proposed (Strynadka *et al.*, 1996).

Recently, β -lactamase-inhibitory proteins (BLIP-I and BLIP-II) were found in culture supernatants of *Streptomyces exfoliatus* SMF19 (Kim *et al.*, 1991; Kim & Lee, 1994). Inhibition by BLIP-I (48 kDa) was uncompetitive, whilst that of BLIP-II (33 kDa) was noncompetitive; the inhibition constants were 0.62×10^{-4} and 2.74×10^{-1} μ mol, respectively. Here, we report the cloning and analysis of a gene encoding BLIP-II, and heterologous expression of the gene in *E. coli* and *Streptomyces lividans*.

Abbreviation: BLIP, β -lactamase-inhibitory protein.

The GenBank accession number for the sequence determined in this work is U97057.

METHODS

Bacterial strains, cosmid and plasmids. *S. exfoliatus* SMF19 (Kim *et al.*, 1991; Kim & Lee, 1994) was the source of genomic DNA from this species. *S. lividans* TK24 (Hopwood *et al.*, 1985) was used as a host strain for the plasmid pIJ702 (Katz *et al.*, 1983) and for expression of the *bliB* gene. *E. coli* DH5 α (Hanahan, 1983) was used to propagate recombinant cosmids and plasmids. *E. coli* Top10F' (Invitrogen) was used as host for pZero-2 (Invitrogen). pWE15 (Wahl *et al.*, 1987) was used to construct a genomic library of *S. exfoliatus* SMF19.

For subcloning, the vectors pBluescript II KS(+) (Stratagene), pZero-2 and pUC18 (Yanisch-Perron *et al.*, 1985) were used. *E. coli* BL21 (DE3), a lysogen bearing the bacteriophage T7 polymerase gene under the control of the *lacUV5* promoter, was used for heterologous expression of *bliB* cloned in pET3a (Studier & Moffatt, 1986); *S. lividans* was used for heterologous expression of *bliB* in pIJ702.

Media and culture conditions. Strains of *Streptomyces* spp. were maintained on Bennett agar (Jones, 1949) and spore suspensions were stored in 20% (v/v) glycerol at -20°C . YEME and R2YE media (Hopwood *et al.*, 1985) were used to grow streptomycetes for DNA manipulation. Strains of *E. coli* were grown in LB medium with ampicillin ($60\text{ }\mu\text{g ml}^{-1}$) or kanamycin ($50\text{ }\mu\text{g ml}^{-1}$) for plasmid or cosmid maintenance (Sambrook *et al.*, 1989). To prepare seed cultures of *Streptomyces* spp., seed medium was inoculated with spore stocks in 20% glycerol and cultured at 30°C on a rotary shaker (150 r.p.m.). Seed medium contained (w/v): 2% glucose, 1.6% soytone, 0.3% peptone and 0.1% CaCO_3 . The seed culture was used to inoculate BLI production medium containing (w/v): 1.5% glucose, 1.5% soytone, 0.2% yeast extract, 0.12% K_2HPO_4 , 0.06% CaCO_3 , 0.001% CoCl_2 and 0.0025% FeSO_4 . Production cultures were grown in a glass jar fermenter (model KF-5L; Korea Fermenter) at 30°C for 50 h; agitation and aeration were 300 r.p.m. and $1\text{ vol. vol.}^{-1}\text{ min}^{-1}$, respectively (Kim & Lee, 1994).

Measurement of cell growth. Biomass was estimated periodically as dried cell weight determined as follows: cells collected by vacuum filtration (Whatman filter paper GF/C) were washed with 2 vols distilled water and dried at 80°C for 24 h.

Measurement of glucose and protein concentration. Glucose was determined by the dinitrosalicylic acid method (Miller, 1959). Protein was determined by the dye-binding method (Bradford, 1976).

β -Lactamase and β -lactamase inhibitor assays. β -Lactamase activity was estimated at pH 7.0 and 37°C by a modified iodometric method (Sawai *et al.*, 1978). β -Lactamase-inhibitory activity (%) was calculated as $[(A-B)/A] \times 100$, where A is the β -lactamase activity without inhibitor and B is the β -lactamase activity with inhibitor. One unit of β -lactamase-inhibitory activity was defined as the amount of inhibitor needed for 50% inhibition of $2 \times 10^4\text{ U}$ β -lactamase (Bactopenase; Difco). β -Lactamase-inhibitory activity in solid culture was estimated using a bioassay procedure as described elsewhere (Jensen *et al.*, 1982).

Protein purification and N-terminal amino acid sequence determination. BLIP-II from the culture supernatant of *S. exfoliatus* SMF19 was purified as described previously (Kim & Lee, 1994). SDS-PAGE was carried out with 12% polyacrylamide gels as described by Laemmli (1970). The N-terminal amino acid sequence of BLIP-II was determined by a modified phenylthiohydantoin method using a Milligen/Bioscience 6600 Prosequencer (Millipore) (Matsudaira, 1987).

DNA manipulation. General methods for DNA manipulations in *Streptomyces* spp. were performed as described by Hopwood *et al.* (1985). For large-scale purification of cosmid and plasmid DNA, an alkaline lysis method was used (Sambrook *et al.*, 1989). Small-scale preparation of plasmid DNA, transformation of *E. coli* and construction of recombinant cosmids and plasmids also followed the procedures described by Sambrook *et al.* (1989). To construct a library of *S. exfoliatus* SMF19 DNA in the cosmid vector pWE15, $100\text{ }\mu\text{g}$ of *S. exfoliatus* SMF19 chromosomal DNA was first partially digested with *Sau3AI* (Poscochem). The digestion mixture was fractionated on a 10–40% sucrose density gradient. Fractions containing DNA fragments of 40–60 kb were pooled and the DNA was precipitated with ethanol. A sample (500 ng) of this DNA was then ligated with $1.4\text{ }\mu\text{g}$ *Bam*HI-digested pWE15 that had been treated with calf intestinal alkaline phosphatase (Poscochem). After incubation at 16°C for 12 h, DNA from the ligation mixture was packaged in λ -particles using a Giga Pack II kit (Stratagene) and was transduced into *E. coli* DH5 α .

E. coli colonies containing recombinant cosmids were screened for hybridization to a degenerate 36-mer oligonucleotide probe based on the N-terminal amino acid sequence of BLIP-II (Genosys). The probe was labelled with the ECL 3'-oligolabelling kit (Amersham). Southern hybridization, colony hybridization and physical mapping of the selected recombinant plasmid were performed using standard techniques (Sambrook *et al.*, 1989).

DNA sequence and analysis. Both DNA strands were sequenced by the dideoxy method of Sanger *et al.* (1977), using Sequenase 2 and 7-deaza-GTP in place of dGTP to relieve compressions. The DNA sequence of each fragment was determined repeatedly to provide unequivocal results in regions of high G + C content. DNA fragments of various sizes were generated with an exonuclease III deletion kit (Promega), and the sequences of those fragments were assembled into a complete sequence using the DNASIS program (Hitachi).

The nucleotide sequence data were also analysed with the DNASIS program to identify restriction sites and regions of dyad symmetry; analysis of codon usage was used to detect ORFs. Protein sequence was deduced from the DNA sequence and examined using FASTP (Pearson & Lipman, 1988) and the available protein sequence databases.

PCR amplification. Reaction mixtures contained 500 nM each of the forward and reverse primers, 0.2 mM dNTPs, 10% (v/v) DMSO, $1 \times$ *Taq* polymerase reaction buffer (Poscochem), less than 100 ng of template DNA and 0.5 U *Taq* polymerase (Poscochem) per $100\text{ }\mu\text{l}$ at various MgCl_2 concentrations (0–3.0 mM). The mixture was subjected to 30 cycles of 1 min denaturation at 94°C , 1 min annealing at 37°C or 42°C and 1.5 min extension at 72°C . Reaction mixtures (5–10 μl) were analysed by electrophoresis on 1.2% agarose gels.

Induction of *bliB* and refolding of the recombinant BLIP-II protein overexpressed in *E. coli*. Synthesis of *bliB* under the control of the T7 promoter was induced as described elsewhere (Sambrook *et al.*, 1989). The BLIP-II protein was overexpressed in *E. coli* and refolded as follows. After induction with IPTG, cells were harvested by centrifugation and washed once with washing buffer (50 mM Tris/HCl pH 8.0, 2 mM EDTA) in a volume of one half that of the starting culture. The cells were frozen at -50°C , thawed in 3 ml washing buffer and disrupted by sonication with 5 cycles of 10 s pulses followed by 10 s pauses. Sonicates were centrifuged at 4°C and 10000 g, and the supernatant was used as the soluble fraction. The pellet was washed with 3 ml lysis buffer (50 mM

Tris/HCl pH 8.0, 10 mM EDTA, 0.5% Triton X-100). After centrifugation, the pellet was used as the insoluble fraction and solubilized for 30 min at 4 °C with a buffer (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) supplemented with 4 M urea. It was then centrifuged. The supernatant was collected and concentrated with Centrplus-10 (Amicon). The concentration of urea was decreased twofold by adding an equal volume of 5 mM potassium phosphate buffer pH 7.0 and the solution was again concentrated with Centrplus-10. This step was repeated four times and then the sample was dialysed against 5 mM phosphate buffer pH 7.0. The dialysate was analysed by SDS-PAGE and assayed for β -lactamase inhibition.

RESULTS

Cloning the gene encoding BLIP-II

BLIP-II produced in cultures of *S. exfoliatus* SMF19 was purified by sequential chromatography on hydroxylapatite, anion exchange and gel permeation columns (Kim & Lee, 1994). SDS-PAGE of the purified BLIP-II is shown in Fig. 1. The N-terminal amino acid sequence was determined to be NH₂-Ala-Thr-Ser-Val-Val-Ala-Trp-Gly-Gly-Asn-Asn-Asp. A degenerate 36-mer oligonucleotide [5' GC(G/C)AC(G/C)TC(G/C)GT(G/C)-GTCGCGTGGGGCGGCAACAACGAC-3'] based on the N-terminal 12 amino acid residues was synthesized and used as a probe in Southern and colony hybridization procedures. *Sau*3A1-digested fragments of *S. exfoliatus* SMF19 chromosomal DNA were ligated into a cosmid vector (pWE15). The ligated DNA was packaged in λ -particles using a Giga Pack II kit (Stratagene) and was transduced into *E. coli* DH5 α . About 8000 colonies were picked as a genomic library of *S. exfoliatus* SMF19. Fourteen of them hybridized strongly with the DNA probe in colony hybridizations. Recombinant cosmid DNAs purified from the selected colonies were digested with *Apa*I and a 4 kb DNA fragment that hybridized to the probe was subcloned into a plasmid (pZero-2) to give the recombinant plasmid pSMF251.

Fig. 1. SDS-PAGE of the BLIP-II protein. Lanes; 1, molecular mass markers; 2, BLIP-II after anion-exchange chromatography; lane 3, BLIP-II after gel permeation chromatography.

Fig. 2. Physical map of the 4 kb *Apa*I fragment containing *bliB* from *S. exfoliatus* SMF19. The fragment was ligated into the *Apa*I site of pZero-2 to yield the plasmid pSMF251. The DNA probe binding site (PBS) is indicated by the black box. The position and orientation of the *bliB* gene are indicated by the arrow. A, *Apa*I; B, *Bam*HI; S, *Sac*II; SL, *Sal*I.

Nucleotide sequence and amino acid sequence analyses

A restriction map of the *Apa*I DNA fragment and the location of *bliB* are presented in Fig. 2. The nucleotide sequence of *bliB* was determined and is available from GenBank (accession number U97057). The correct reading frame was initially recognized by the presence of a DNA sequence which corresponded precisely to the N-terminal amino acid sequence of BLIP-II. By analysis using the DNASIS program, three nested ORFs were identified: the first started with an ATG codon at nt 476, the second with GTG at nt 521 and the third with ATG at nt 524. The ATG codon at nt 476 was chosen as the likely translation initiation site since it is preceded by a potential RBS (GAGG; Bibb & Cohen, 1982) and followed by a typical leader-peptide-encoding sequence (Table 1) observed in other secreted proteins of *Streptomyces* spp. (Doran *et al.*, 1990; Garcia-Gonzalez *et al.*, 1991). The ORF was terminated by TAG at nt 1592. Its overall G+C content was 73 mol%; the values for codon positions 1, 2 and 3 were 68, 59 and 89 mol%, respectively.

With these assumptions, *bliB* (1116 bp) encodes 372 amino acids, of which 8.2% are acidic, 8.2% are basic and 62.8% are hydrophobic. The deduced precursor protein has a 40 amino acid leader peptide and gives a mature protein of 332 amino acids with a calculated molecular mass of 33335.9 Da. This corresponds well to the molecular mass (33 kDa) determined by SDS-PAGE of the protein. The leader peptide contained three positively charged amino acids (two arginines at positions 7 and 10 and a histidine at position 9), followed by a long hydrophobic stretch from position 11 to 32 (Table 1). Analysis of the deduced amino acid sequence of the ORF showed six tandem repeats of a domain of 40 amino acids that were highly conserved (Fig. 3).

Expression of *bliB* in *E. coli*

bliB was amplified by PCR using the forward primer (FP1) 5' GTTGACATATGCCGAACGGAATCGTC 3' and the reverse primer (RP) 5' GGTAAGATCTCT-ACGCCGCCATCTGGA 3'. As shown in Fig. 4, the FP1 primer was designed to include an *Nde*I site

Table 1. Signal sequences of *Streptomyces* proteins

Protein	Signal sequence*	Length	Source	Reference
BLIP-II	fM P N G I V R L H R F G A A L V M S G L L S A L S A A P G V A A E P S S V S V A	40	<i>S. exfoliatus</i> SMF19	This study
BLIP	fM R T V G I G A G V R R L G R A V V M A A A V G G L V L G S A G A S N A	36	<i>S. clavuligerus</i>	Doran <i>et al.</i> (1990)
Streptavidin	fM R K I V V A A I A V S L T T V S I T A S A S A	24	<i>S. avidinii</i>	Argarana <i>et al.</i> (1986)
α -Amylase	fM Q Q R S R V L G G T L A G I V A A A A A T V A P W P S Q A	30	<i>S. hygroscopicus</i>	Hoshiko <i>et al.</i> (1987)
α -Amylase inhibitor (Tendamistat)	fM R V R A L R L A A L V G A G A A L A L S P L A A G P A S A	30	<i>S. tendae</i>	Koller & Riess (1987)
Tyrosinase (<i>melC1</i>)	fM P E L T R R R A L G A A A V V A A G V P L V A L P A A R A	30	<i>S. antibioticus</i>	Bernan <i>et al.</i> (1985)
Agarase (<i>dagA</i>)	fM V N R R D L I K W S A V A L G A G A G L A G P A P A A H A	30	<i>S. coelicolor</i> A3(2)	Buttner <i>et al.</i> (1987)
α -Amylase inhibitor (Haim II)	fM K R Y V C S T F V A C V M V L C V I P A S G A A A H E A V A E D A G N R	31	<i>S. griseosporus</i>	Nagaso <i>et al.</i> (1988)
DD-Peptidase	fM V S G T V G R G T A L G A V L L A L L A V P A N A G T A A A	31	<i>Streptomyces</i> R61	Deuz <i>et al.</i> (1987)
Protease A (<i>sprA</i>)	fM T F K R F S P L S S T S R Y A R L L A V A S G L V A A A A L A T P S A V A	38	<i>S. griseus</i>	Henderson <i>et al.</i> (1987)
Cholesterol oxidase	fV T A N N H L S R R R M L G M A A F G A A A L A G G T T I A A P R A A A A A K S A A	42	<i>Streptomyces</i> SA-COO	Ishizaki <i>et al.</i> (1989)

* Positively charged residues are shown in bold and each series of hydrophobic residues is indicated by underlining.

Fig. 3. Alignment of six tandem repeats of a domain of 40 amino acids. The conserved amino acid residues are shown on a black background. Dashes indicate gaps introduced to maximize alignment.

(CATATG) and the RP primer a *Bgl*II site (AGATCT). The PCR product was digested with *Nde*I and *Bgl*II and subcloned into the expression vector pET3a at the *Nde*I and *Bam*HI sites. The recombinant plasmid (pSMF268) was used to transform *E. coli* BL21 (DE3).

A transformant harbouring pSMF268 was cultured in LB medium supplemented with IPTG. From the SDS-PAGE patterns of clarified cell sonicates (Fig. 5), it was apparent that a 35 kDa protein was produced in large amounts (about 25% of total cell protein) only in the culture of pSMF268 with IPTG present (lane 4). The protein was detected in the insoluble fraction, indicating that the protein was induced and accumulated in inclusion bodies. This protein was extracted with urea

and refolded by step-wise removal of the urea. The refolded protein showed β -lactamase-inhibitory activity in both the iodometric assay (data not shown) and the agar diffusion bioassay (Fig. 6).

Expression of *bliB* in *S. lividans*

To express *bliB* under the control of the *melC1* promoter located in pIJ702, the gene was amplified by PCR using the forward primer (FP2) 5' TTGATGCATGCCGA-ACGGAATCGTCCGA 3' and the same reverse primer (RP) as used to construct pSMF268. The FP2 primer was designed to introduce an *Sph*I site (GCATGC) into the PCR product. The PCR product was digested with *Sph*I and *Bgl*II and subcloned into pIJ702 at the *Sph*I and

Fig. 4. Construction of pSMF268. *bliB* was inserted downstream of the T7 promoter (P_{T7}) located in pET3a. The putative start codon (ATG) was part of an *Nde*I site. *bliB* is indicated by the solid bar which immediately follows the ATG start codon. The primers used for PCR amplification are denoted by the letters FP1 and RP, and arrows represent their orientations.

Fig. 5. SDS-PAGE of recombinant BLIP-II protein from *E. coli* BL21 (DE3) containing pSMF268. Lanes: 1, molecular mass markers; 2, pET3a; 3, pSMF268, uninduced; 4, pSMF268, induced; 5, pSMF268, induced, soluble fraction; 6, pSMF268, induced, insoluble fraction.

*Bgl*II sites. The recombinant plasmid pSMF267 was then used to transform *S. lividans* TK24.

To demonstrate heterologous expression of *bliB*, *S. lividans* TK24 (pSMF267) was grown in BLI production

Fig. 6. β -Lactamase-inhibitory activity of the BLIP-II protein refolded from inclusion bodies of *E. coli* BL21 (DE3). Units of recombinant BLIP-II protein were determined by an iodometric assay. Isolated inclusion bodies were solubilized by treatment with urea and proteins were renatured by stepwise removal of urea.

medium. For comparison, *S. lividans* TK24 was grown in the same medium. Changes in glucose concentration, β -lactamase-inhibitory activity and biomass formation in the two cultures are compared in Fig. 7. Since BLIP-II activity was detected only in the recombinant strain, it was concluded that *bliB* encoded BLIP-II and that the gene was expressed in both *E. coli* and *S. lividans*.

DISCUSSION

The molecular mass and amino acid sequence of BLIP-II were not similar to those of BLIP produced by *S. clavuligerus* (Doran *et al.*, 1990), suggesting that BLIP-II is a different protein. However, the length and position of the positively charged and hydrophobic residues of the leader peptide of BLIP-II resemble those of the leader peptides of BLIP and of other secreted proteins from *Streptomyces* spp. (Doran *et al.*, 1990; Garcia-Gonzalez *et al.*, 1991).

Pairwise comparison (Pearson & Lipman, 1988) of the deduced amino acid sequence of BLIP-II with sequences in the SWISS-PROT database showed similarities between BLIP-II and two eukaryotic proteins. With the PIM1 protein of *Schizosaccharomyces pombe* it showed 29.9% identity and with the RCC1 protein in *Candida albicans* 28.9% identity. Both eukaryotic proteins are regulators of chromosome condensation involved in the control of mitosis (Matsumoto & Beach, 1991; Sazer & Nurse, 1994). BLIP-II also contains a domain that is repeated six times within the polypeptide (Fig. 3). These repetitions are also found in PIM1 and RCC1 (Matsumoto & Beach, 1991; Dasso, 1993). However, the functional implication of the 40 amino acid repeats is unclear.

Several biological roles have been proposed for BLIP of *S. clavuligerus* (Doran *et al.*, 1990): one was that BLIP

Fig. 7. Growth (○), production of BLIP-II (●) and glucose utilization (□) in batch cultures of *S. lividans* TK24 (a) and *S. lividans*(pSMF267) (b).

functions as a protector of autogenous β -lactam antibiotics from β -lactamase produced by other organisms in the soil microenvironment; another was that it functions as a regulator of cell growth or morphogenesis. These are reasonable hypotheses for *S. clavuligerus*, which produces β -lactam antibiotics, including penicillin N and cephamycin C, and the small-molecule β -lactamase inhibitor clavulanic acid. However, no β -lactam antibiotic was detected in the culture filtrate of *S. exfoliatus* SMF19, so the biological role of BLIP-II is unlikely to be the protection of autogenous antibiotics; a role in regulating cell growth or morphogenesis remains a possibility. Further experiments, including

gene disruption, are required to understand the biological role of BLIP-II.

When the BLIP-II gene was expressed in *E. coli*, BLIP-II with its own leader peptide was not secreted into the periplasmic space but accumulated in inclusion bodies, the activity of which was restored after extraction and refolding. This result indicated either that the presence of the leader peptide in BLIP-II played no role in translocation of the precursor protein, or that the precursor protein was not post-translationally processed.

When BLIP-II was expressed in *S. lividans*(pSMF267), production was maximal early in exponential growth and then decreased drastically. The mechanisms responsible for this instability are unclear. Previous studies have shown that the decrease in heterologous expression of genes encoding β -lactamase (*bla*) and tyrosinase (*mel*) that occurred during exponential growth in *S. lividans* was caused by changes in cultural conditions (Epicum *et al.*, 1989; Gardner & Cadman, 1989), so similar effects may be occurring here.

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