Volume 318, number 3, 242–244 FEBS 12166 March 1993

Expression of native dimers of bovine seminal ribonuclease in a eukaryotic cell system

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Received 7 January 1993

Bovine seminal ribonuclease, a uniquely dimeric pancreatic-like RNase, with its dimeric structure stabilized by two intersubunit disulfides, and endowed with *special*, i.e. non-catalytic, actions (antitumor, immunosuppressive, antispermatogenic), was stably expressed in Chinese hamster ovary cells. The recombinant protein, secreted in the culture medium as a correctly folded dimeric enzyme, was purified to homogeneity and found to be fully active both in its catalytic and antitumor activities.

Ribonuclease; Seminal RNase; Expression

1. INTRODUCTION

The interest in bovine seminal ribonuclease (BS-RNase) arises from its very characteristic structure, and its catalytic and *special* (i.e. non-catalytic) biological actions ([1] and references cited therein). BS-RNase is in fact the only dimeric member of the pancreatic-like RNase superfamily [2], with a dimeric structure maintained also by intersubunit disulfides. As for its functional properties, it is the only RNase with allosteric properties, and it is also an antitumor, an antispermatogenic and an immunosuppressive factor [1]. The many questions on the structural and functional features of BS-RNase, such as the molecular basis of its allosteric properties, and of its multiform non-catalytic actions, have been addressed so far by classical protein chemistry methodologies [3–5]. A different, potentially much more productive approach, would be that of engineering BS-RNase through appropriate site-directed or regional mutagenesis of the DNA coding for the protein chain. This approach may shed light on the structural determinants underlying the biological actions of the enzyme, and may direct towards the preparation of biotechnologically interesting protein derivatives.

An essential and crucial step in protein engineering is the expression in suitable systems of the DNA coding for the protein. This step, already proven to be difficult for ribonucleases in general, probably due to the cellular toxicity of these enzymes, can be even more exacting in the case of BS-RNase, due to some of the structural features of the enzyme, such as the disulfide-maintained dimeric structure.

We report here the expression in a eukaryotic system, consisting of Chinese hamster ovary cells, of BS-RNase as a dimeric protein, fully active in its catalytic and antitumor activities.

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2. MATERIALS AND METHODS

2.1. *General procedures*

Bacterial cultures, plasmid purification, restriction endonuclease digestions, DNA ligations and bacterial transformations were performed according to Sambrook et al. [6]. Vectors were propagated in *Escherichia coli* strain JM 101 (Boehringer). DNA fragments were eluted from agarose gels with the Gene-Clean Kit (Bio 101). Double-strand DNA was sequenced using the Sequenase Sequencing Kit (US Biochemicals Corporation). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was carried out according to Laemmli [7]. After electrophoresis, the gels were stained for protein detection with Coomassie blue, or stained for RNase activity [8], or blotted on nitrocellulose for immunostaining, carried out as previously described [9]. RNase activity was assayed with minor modifications of a previously reported method [10]. Amino acid sequence determinations were carried out on the Applied Biosystems sequencer 473A.

2.2. *Construction of the expression vector*

The strategy employed for constructing the expression vector is illustrated in Fig. 1. The semi-synthetic cDNA coding for BS-RNase subunit chain, cloned in plasmid pMC-P-BS (de Nigris et al., manuscript in preparation), was removed from the vector by digestion with *Eco*RI and *Sal*I (Promega Biotech), and extended at its 5′ end by addition of 94 bp of synthetic DNA. This extension coded for the signal sequence (residue −26 to −1) as deduced from the cDNA sequence of the BS-RNase precursor [11], and contained an upstream *Bam*HI site. The addition was performed by a novel procedure based on the polymerase chain reaction (manuscript in preparation).

The expression vector was constructed from the pLEN-ACEVII vector, kindly provided by M.R.W. Ehlers (Harvard Medical School, Boston, MA, USA), and based on the eukaryotic expression vector pMThGHSV402, equipped with the inducible metallothionein IIA

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promoter, as prepared by Friedman et al. [12]. pLEN-ACEVII contained the SV40 splice sites and polyadenylation signals, and the coding sequence of the angiotensin-converting enzyme [13]. The latter was removed through digestion with *Bam*HI/*Sal*I and replaced with the extended cDNA described above, coding for pre-BS-RNase, and trimmed by digestion with *Bam*HI and *Sal*I. Sequence analyses of the resulting recombinant vector, denominated pMC-E-BS, revealed that it contained the expected sequence of BS-RNase cDNA, including the upstream leader sequence.

2.3. *Transfection and cell cultures*

The recipient cells were Chinese hamster ovary cells, CHO K1, provided by ATCC, Rockville, MD, USA. They were propagated and cotransfected as described [13] with the pMC-E-BS vector (16 μg per 100 mm plate) as well as with the pSV2NEO vector (2 μg per plate), inducing resistance to the neomycin analog G418 (Gibco). After selection for resistance to G418, stable transformants were individually retrieved, subcultured in 24-well plates, and induced with 40 μM zinc sulphate in the presence of 0.5% serum. Culture medium from each clone was then tested by an ELISA assay [14] using anti-BS-RNase polyclonal antibodies prepared as described [9]. Preparative inductions of selected clones were performed in 150 mm plates, replacing the medium every 2–3 days.

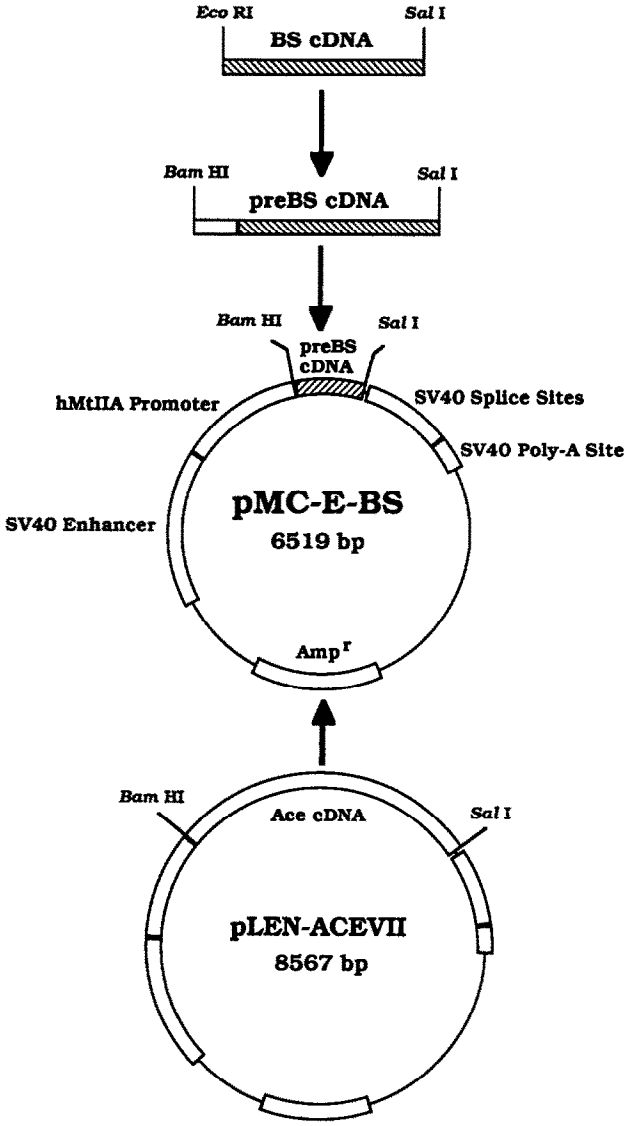

Fig. 1. Strategy for the construction of vector pMC-E-BS for expression of recombinant bovine seminal RNase.

3. RESULTS AND DISCUSSION

The clone expressing the highest level of BS-RNase-like immunoreactivity (0.4 μg/ml on the basis of ELISA assays) was propagated and found to be stable through ten population doublings in the absence of the selecting agent G418.

Medium from induced cultures (2 l) was concentrated 10-fold by ultrafiltration on an Amicon concentrator equipped with YM3 membranes. The concentrated material was dialyzed against 40 mM Tris-Cl, pH 7.4, clarified by centrifugation, and loaded on a heparin-agarose column (1.4 × 4 cm) equilibrated in the same buffer. The column, washed with the equilibrating buffer containing 50 mM NaCl, was eluted with 0.1 M Tris-Cl, pH 8, containing 0.4 M NaCl. The eluate was diluted 1:2 with NaCl-free buffer and loaded on a Mono-S column equilibrated with the same buffer containing 0.2 M NaCl. After a 30-min wash with this buffer, the NaCl concentration was raised to 0.24 M, and after 35 min to 0.4 M. This eluted a single peak of RNase activity, with a 32% final yield with respect to the original culture medium. The protein, desalted and concentrated by centrifugation on a Centricon concentrator, was found to have a specific activity identical to that of BS-RNase isolated from bovine seminal vesicles.

As shown in Fig. 2, upon SDS-PAGE under non-reducing conditions a single band was detected, comigrating with BS-RNase isolated from seminal vesicles. On gels run in parallel the protein band was found to stain for RNase activity, and after blotting on a nitrocellulose membrane, to react with anti-BS-RNase antibodies. When SDS-PAGE was performed under reducing conditions in 0.1% 2-mercaptoethanol, the recombinant protein was found to run with the molecular size of BS-RNase monomers (see Fig. 2).

These results indicated that the recombinant RNase:

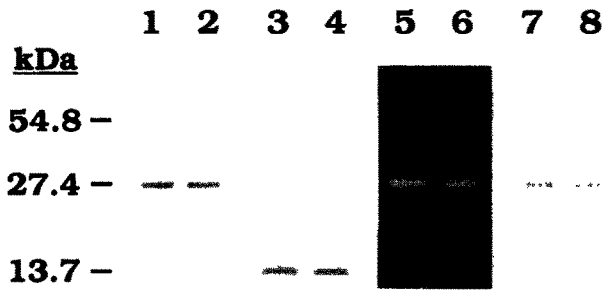

Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate in the presence (lanes 3 and 4), and in the absence (all other lanes) of 2-mercaptoethanol. Lanes 1, 3, 5 and 7: BS-RNase isolated from seminal vesicles; lanes 2, 4, 6 and 8: recombinant BS-RNase. Lanes 1 through 4 were stained with Coomassie blue; lanes 5 and 6 were stained for RNase activity; lanes 7 and 8 were from an immunoblot treated with anti-BS-RNase antibodies.

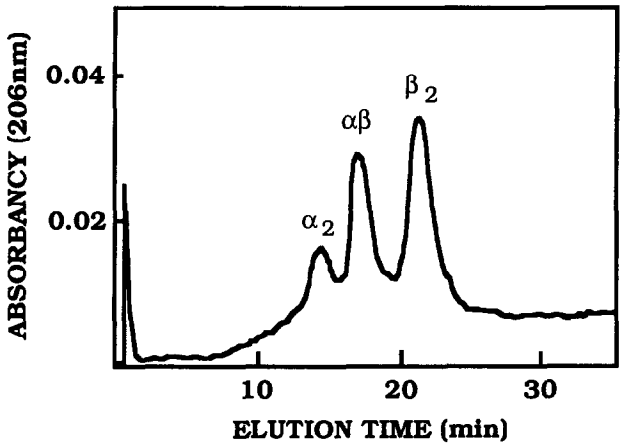

Fig. 3. Ion exchange chromatography of recombinant BS-RNase on a Mono S column eluted with a salt gradient for detection of isoenzymic subforms α_2, $\alpha\beta$ and β_2.

(i) was immunologically indistinguishable from BS-RNase; (ii) had the same molecular size as BS-RNase, and (iii) was a dimeric protein, with a dimeric structure stabilized by intersubunit disulfides, as in natural BS-RNase.

Sequence analyses of the recombinant protein revealed that its N-terminal sequence (residues 1–18) was identical to the sequence of BS-RNase [15], indicating a correct proteolytic cleavage of the signal sequence. Furthermore, when analyzed by ion exchange chromatography for the detection of isoenzymic forms [16], the protein separated into three components (see Fig. 3), presenting the characteristic pattern of native BS-RNase. It has been established that the three components correspond to isozymes α_2, $\alpha\beta$ and β_2, as generated from the nascent β_2 form by selective deamidation of Asn^{67} in one or both β subunits [16]. This event occurs in vivo during the storage of the protein in seminal vesicles [17]; apparently, rBS-RNase undergoes in the culture medium the same selective deamidation processes which occur to BS-RNase stored in the secretion of seminal vesicles.

Finally, rBS-RNase was tested as previously described [3] for one of the characteristic non-catalytic biological actions of BS-RNase, its antitumor action. Mouse 3T3 fibroblasts, transformed with SV40 virus, and the non-transformed parental line, were cultured for 48 h in the presence of 50 μg/ml recombinant enzyme, rBS-RNase was found to inhibit the growth of transformed cells by more than 70%, just like natural BS-RNase, whereas the non-transformed cells were unaffected by the enzyme treatment.

In conclusion, the data reported above show that CHO cells transfected with pMC-E-BS expression vector are an effective and convenient system for the production and secretion in the culture medium of a correctly processed rBS-RNase. The secreted enzyme can be purified with a simple 2-step procedure, and is indistinguishable in its structure and activity from the enzyme as isolated from its natural source.

Acknowledgements: The authors are very grateful to Dr. M.R.W. Ehlers for providing the pLEN-ACEVII vector, to Dr. A. Parente for the sequence analyses, and to Ms. D. Melck for testing the antitumor activity of rBS-RNase. This work was supported by grants from the National Research Council (PF-BTBS 70/9101161), and the Ministry of University and Research, Italy.

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