Biochemical and Biophysical Research Communications 370 (2008) 77–81

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Activity-based selection of a proteolytic species using ribosome display

Derek J. Quinn, Stephen Cunningham, Brian Walker, Christopher J. Scott *

Molecular Therapeutics, School of Pharmacy, Queen's University of Belfast, 97 Lisburn Road, Belfast, Antrim BT9 7BL, UK

ARTICLE INFO

Article history:
Received 28 February 2008
Available online 14 March 2008

Keywords:
Ribosome display
Protease
Activity-based selection

ABSTRACT

We have examined the potential of displaying a protease species *in vitro* using ribosome display and demonstrate specific capture on the basis of its catalytic activity. Using a model bacterial cysteine protease, sortase A (SrtA), we show that this enzyme can be functionally expressed *in vitro*. By overlap PCR we constructed ribosome display templates with the SrtA open reading frame fused to a C terminal glycine–serine rich flexible linker and a tether derived from eGFP. Using the broad range cysteine protease irreversible inhibitor E-64 linked to acrylic beads, we show that we can isolate SrtA ribosome display ternary complexes, and recover their encoding mRNA by RT-PCR. This recovery was lost when applied to a SrtA catalytically inactive mutant, or could be alleviated by competition with free inhibitor. This sensitive technique could be further developed to allow the screening of proteases against putative inhibitors and/or the identification of novel proteolytic species.

Proteases represent a broad range of hydrolytic enzymes that are involved in a myriad of crucial cellular processes such as protein turnover, cell signaling, cell cycle control, and apoptosis. It has been estimated that 2% of all genes in the human genome encode proteolytic species [1,2]. The aberrant expression or activity of proteases can underlie a range of pathological conditions including cancer, rheumatoid arthritis, neurodegenerative disorders, and infectious disease [3]. Methodologies that can elucidate and characterize protease species are keenly sought after, particularly in light of their focus as druggable targets for the treatment of these disease states [4,5].

Protein display technologies have become useful tools for the selection, isolation and identification of proteins on the basis of their biological activity. These techniques involve the use of a 'bait' to allow selective capture of 'prey' binders. The approaches for characterizing interactions with bait diversify in the approach used for the presentation of the library of prey, followed by its selection and characterization. Two such techniques, ribosome and mRNA display are performed using *in vitro* transcription–translation systems where the principle is to couple together the nascent proteins or peptides to its individual encoding mRNA molecule. In the case of ribosome display, the mRNA template contains no stop codon and under appropriate conditions, as the ribosome moves along to the end of the template it is unable to dissociate and it stalls, thereby generating a stable ternary complex providing a link between the phenotype (protein or peptide) and its genotype (mRNA molecule). These ternary complexes can then be incubated against an appropriate 'bait' and proteins which bind identified rapidly and sensitively by means of their mRNA by RT-PCR [6,7].

Ribosome display has been used for the evolution of function in proteins, namely the selection and maturation of antibodies based on their affinity with cognate antigen [8–10]. Here we have examined the ability to use this technique in the display of a protease, sortase A (SrtA). SrtA is a cysteine protease–transpeptidase found in the cell envelope of *Staphylococcus aureus* where it is responsible for the cleavage and covalent linkage of surface proteins containing the conserved -Leu-Pro-Xaa-Thr-Gly- sequence motif [11,12]. Many of the proteins anchored to the cell wall by this reaction are involved in interaction with host tissues enabling colonization and infection and therefore SrtA has been proposed as a target for the development of novel anti-microbial agents [13]. In this study we generated ribosome display ternary complexes *in vitro* to display SrtA and examined the capture and retrieval of this enzyme based on its catalytic activity using the general cysteine protease inhibitor E-64 (schematically demonstrated in Fig. 1).

Materials and methods

Generation of in vitro templates. The open reading frame for SrtA, devoid of its N terminal transmembrane domain was amplified from *S. aureus* (ATCC 9144) using SrtAF and SrtAR (see Table 1) which contain a BamHI and SalI restriction site, respectively, and cloned into pQE30 (Qiagen) restricted with BamHI and SalI resulting in the generation of pSRT-WT. An inactive mutant was generated from pSRT-WT by site-directed mutagenesis using primers mutF and mutR (Table 1). This mutation results in a generation of a C184S mutation, thus rendering the active site of the protease inactive. The site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer's instructions, resulting in the generation of pSRT-CΔS. The pSRT-WT and pSRT-CΔS vectors were used as templates for subsequent PCR amplifications.

* Corresponding author. Fax: +44 2890247794.
E-mail address: c.scott@qub.ac.uk (C.J. Scott).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.03.013

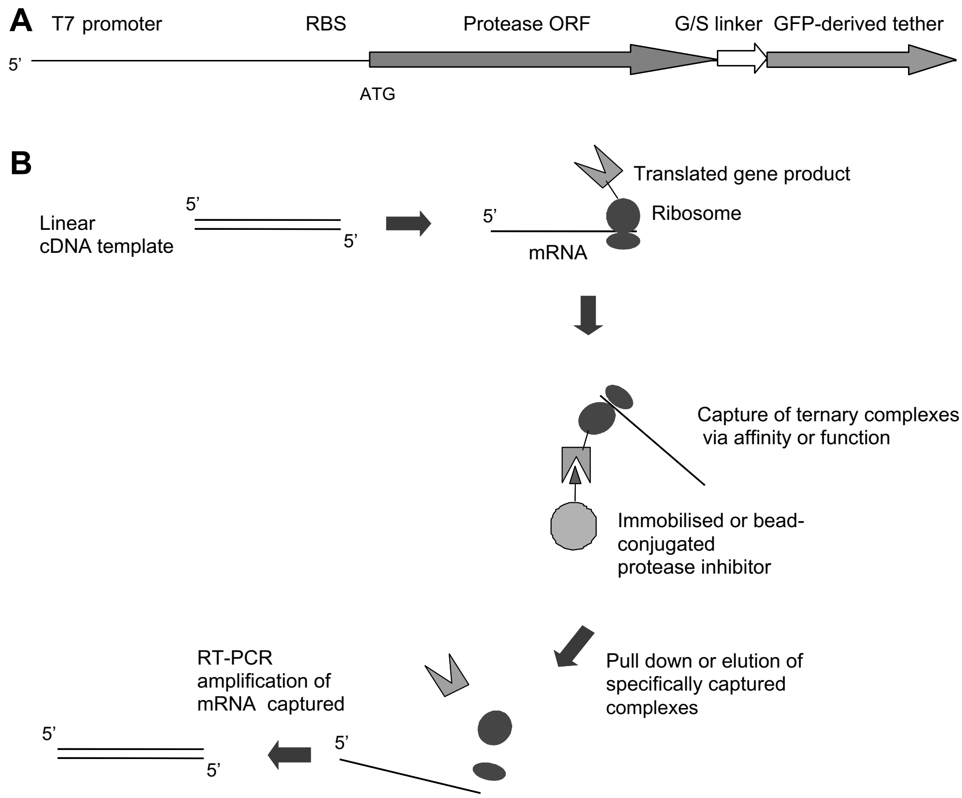

Fig. 1. Diagrammatic overview of selection of proteolytic species using ribosome display. Linear templates (A) are prepared by PCR to include all elements required for ribosome display including a 5′ T7 promoter, a Shine–Dalgarno ribosome binding site (RBS), and translation initiation methionine ATG codon. Crucially, no stop codon is present in this template to allow stalling of the ribosome at the 3′ end of resulting mRNA transcripts. Coupled transcription–translation resulting from this template results in the generation of the protease followed directly by a glycine–serine rich flexible linker (G/S linker) and a ribosome tunnel tether region derived from eGFP as a fusion protein. Upon addition of this template to *E. coli* transcription/translation lysates, ternary complexes are formed whereby the ribosome provides a physical link between the encoding mRNA and resultant nascent protein (B). These complexes are then incubated with immobilised protease inhibitor to isolate complexes on the basis of functionality. Successful capture is then determined by recovery of bound mRNA by RT-PCR.

Table 1
Details of oligonucleotide primers employed in this study

Primer name	Primer sequence
SrtAF	5′TTTTTTGGATCCAAACCACATATCGATAATTATTCACG
SrtAR	5′TTTTTTGTCGACTCATTTGACTTCTGTAGCTACAAATTTTACG
mutF	5′GATAAACAATTAACATTAATTACTTCTGATGATTACAATGAAAAGA CAGGCG
mutR	5′ CGCCTGTCTTTTCATTGTAATCATCAGAAGTAATTAATGTTAATTGT TTATC
SrtRiboF	5′AGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATATCCATGAAACCACATATCGATAATTA
SrtRiboR	5′GCCTCCAGAGCCACCTCCGCTGCCACCTCCTTTGACTTCTGTAGCTAC AAAGATTTTACG
gfpF	5′GGAGGTGGCAGCGGAGGTGGCTCTGGAGGCGAGTACAACTACAAC AGCCACAACGTC
gfpR	5′ CCGCACACCAGTAAGGTGTGCGGTGGCGGTCACGAACTCCAGCAG
ssrA	5′ TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGC
Int-srt-F	5′GTATATCCAGGACCAGCAACACC
Int-srt-R	5′CTAGAACTCCTACATCTGTAGGC

In vitro translation and labeling. The plasmids pSRT-WT was used to generate a linear PCR template for the expression of the proteolytic species in *Escherichia coli* lysates using SrtRiboF and SrtAR primers. The forward primer introduces an upstream T7 promoter and Shine–Dalgarno sequence to facilitate efficient transcription and translation in T7 RNA polymerase supplemented RTS 100 *E. coli* HY kit (Roche) *E. coli* extracts. PCR reactions were prepared using 150 nM of each primer and 10 ng vector template in a final reaction volume of 50 μl. After an initial denaturation step at 96 °C for 5 min, 32 cycles of 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C were carried out followed by a final extension of 10 min. After amplification, samples were analyzed by agarose gel electrophoresis. Neat, un-purified PCR products (50 ng) were added to *E. coli* extract (100 μl, Roche) and incubated for 30 min at 30 °C. To an aliquot of this translation product (30 μl), the affinity binding probe Biotin-Ahx (aminohexanoyl)-Leu-Pro-Ala-Thr-CHN_2 (Bio-LPAT-DK, 50 μM), previously shown to specifically and irreversibly label SrtA [13], was added and incubated for a further 30 min at 37 °C. Samples (10 μl) were separated by SDS–PAGE analysis (4–12% density gradient gel, Invitrogen), and blotted onto a nitrocellulose membrane (Amersham). The membrane was blocked with 3% BSA (Sigma) and the detection of incorporated biotinylated affinity probe was disclosed with a streptavidin–horseradish peroxidase conjugate at a concentration of 1 in 5000 (Vector Laboratories) by chemolluminence (ECL, GE Healthcare).

Construction of ribosome display overlap linker region and ribosome tether. To generate ribosome display SrtA WT and SrtA CΔS templates it is necessary to have both a linker and tether region fused to the C terminus of the nascent protease species (Fig. 1A). In this investigation the tether chosen consisted of a fragment of enhanced Green Fluorescent Protein (eGFP) comprised of residues (143–227) and an additional upstream flexible glycine–serine rich linker (GGGSGGGSGG). This construct was produced by PCR using the following primers gfpF and gfpR which facilitated incorporation of the linker region in the forward primer as shown in Table 1. Amplification reactions (25 μl) were performed using 1× BioMix Taq polymerase (BioLine), 150 nM of each primer and 1 ng of the pEGFP-N1 plasmid as template (Clontech) amplification conditions were as described above. After amplification, samples were subjected to agarose gel electrophoresis and purification to isolate the desired amplification product (Qiaquick Minielute, Qiagen).

Construction of ribosome display SrtA wt and SrtA mut with overlap linker region. The linear templates for SrtA-WT and CΔS and mutant templates for overlap PCR with the linker and tether region were amplified using SrtRiboF and SrtRiboR (Table 1). SrtRiboR primer amplifies the C terminus of SrtA devoid of its stop codon and introduces the linker region for subsequent overlap PCR with the tether region. Amplification reactions were performed using 1× BioMix Taq polymerase (BioLine), 150 nM of each primer and 1 ng of either pSRT WT or pSRT CΔS plasmids as template in a 25 μl reaction volume. Amplification conditions were the same as described before with the exception that the cycled 72 °C extension step was elongated to 1 min. After amplification samples were subjected to gel electrophoresis and purification to isolate the desired amplification product as before.

Generation of ribosome display template by overlap PCR. The previously amplified and gel purified SrtA linear PCR templates with 3′ linker introduced and the tether region (eGFP fragment) were fused together by the complementary linker region using overlap PCR with the following primer pair SrtRiboF and gfpR.