Research Article

Expression by Streptomyces lividans of the Rat α Integrin CD11b A-Domain as a Secreted and Soluble Recombinant Protein

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We already reported the use of a long synthetic signal peptide (LSSP) to secrete the Streptomyces sp. TO1 amylase by Streptomyces lividans strain. We herein report the expression and secretion of the rat CD11b A-domain using the same LSSP and S. lividans as host strain. We have used the Escherichia coli/Streptomyces shuttle vector pIJ699 for the cloning of the A-domain DNA sequence downstream of LSSP and under the control of the constitutive ermE-up promoter of Streptomyces erythraeus. Using this construct and S. lividans as a host strain, we achieved the expression of 8 mg/L of soluble secreted recombinant form of the A-domain of the rat leukocyte β2 integrin CD11/CD18 alpha M subunit (CD11b). This secreted recombinant CD11b A-domain reacted with a function blocking antibody showing that this protein is properly folded and probably functional. These data support the capability of Streptomyces to produce heterologous recombinant proteins as soluble secreted form using the “LSSP” synthetic signal peptide.

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1. INTRODUCTION

Streptomyces species are saprophytic gram-positive soil bacteria that possess a large range of extracellular hydrolytic enzymes such as α-amylases, agarases, cellulases, xylanases, nucleases, and lipases [1]. The potential of S. lividans, as a host for the expression of heterologous recombinant bacterial and eukaryotic proteins, has extensively been investigated [2]. Indeed, this microorganism has several features that make it a suitable host for efficient recombinant protein expression among which are the well-established genetic manipulation procedures [3] and the absence of extensive restriction modification systems that are generally present in other Streptomyces species. Furthermore, S. lividans has very low endogenous extracellular proteolytic activity when compared to other Streptomyces species [4] and does not elicit the formation of inclusion body of recombinant protein in the cytoplasm, a problem encountered in the majority of the expression systems used for eukaryotic and prokaryotic proteins in E. coli. Indeed, overproduction of some proteins in E. coli led to their incorporation into insoluble inclusions bodies. While it is possible to solubilize the inclusions bodies, there is no guarantee that attempted renaturation will lead to significant level of active protein. The high level of solubility is a general characteristic of proteins made in Streptomyces [3]. For these diverse reasons, S. lividans is the host of choice for the secretary production of heterologous proteins and a very attractive biotechnology platform.

Over the past two decades, a growing number of studies have demonstrated that S. lividans is a model host for the production of secreted heterologous recombinant proteins [5–12]. In most cases, genes of interest were fused to well-characterized signal peptide sequences of naturally highly secreted Streptomyces proteins [11, 13, 14]. Alternatively, synthetic signal peptides were used such as the 35 amino acids long synthetic signal peptide (LSSP) that we have previously used successfully for the secretion of the Streptomyces sp. TO1 amylase [10] which is a naturally secreted protein. This signal peptide was deduced by alignment of several Streptomyces signal peptides and carried an additional ribosome binding site as well as a second initiation codon. The addition of a second ribosome binding site and a second initiation codon
increased the yield of secreted protein according to Pagé et al. [15].

Several Streptomyces secretion systems have successfully been developed for the production of eukaryotic proteins especially those of medical interest [9]. For examples, we can mention the efficient production in S. lividans of the murine tumor necrosis factor (TNF) alpha fused to the secretory signal peptide of the subtilisin inhibitor protein from S venezue-

lae [8, 16]. Expression of biologically active human interferon alpha 2 was also achieved in S. lividans [17].

The β2 integrins (CD11/CD18) are surface heterodimeric glycoproteins and represent one of the most important families of the leucocytes adhesion molecules. This family is composed of 4 glycoprotein complexes each one consists in an α/β heterodimer containing two distinct chains: the alpha (CD11a, CD11b, CD11c, and CD11d) and the beta (CD18) subunits. They have a widespread vital function in development, embryogenesis, tissue organization, wound healing and immune response [18]. The βα subunits display an A-domain in their amine termini that mediates proinflammatory functions [19]. These are mainly the adhesive interaction with vascular endothelial cells that lead to leukocyte transmigration and diapedesis [20]. The A-domain of the αM subunit (CD11b) of the rat leukocyte β2 integrin CD11/CD18, which was used for this work, was already cloned and expressed in E. coli in the intracellular compartment as a soluble recombinant fusion protein with GST. Recombinant CD11b A-domain was released from the fusion protein by thrombin cut. This recombinant domain retained its adhesive function as shown by its binding in vitro with members of the immunoglobulin superfamily [20]. Furthermore it was used to demonstrate that it is capable to protect the skeletal muscle from inflammatory injury in vivo using a rat model of crush syndrome [21]. In this paper, we report for the first time the heterologous expression and efficient secretion, by S. lividans, of the soluble recombinant rat CD11b A-domain; using the constitutive ertmE-up promoter (PermE) from Streptomyces erythraeus [22] and the long synthetic signal peptide (LSSP) [10].

2. MATERIAL AND METHODS

2.1. Bacterial strains and media

S. lividans 1326 was used as a host cell for transformation. Protoplasts preparation and transformation procedures were performed as described by Hopwood et al. [23]. The R2YE medium was used for protoplasts regeneration and thiostrepton (the antibiotic selection marker carried by the E. coli/Streptomyces shuttle vector pIJ699 [24]) was added at 50 μg/mL for selection of transformed S. lividans cells. For growth of S. lividans harbouring pMS115, thiostrepton was added at a concentration of 15 μg/mL in liquid medium or at 20 μg/mL in solid medium and cultures were grown on TSB or NMMP media [23] at 30°C for 48 h with shaking.

E. coli strains used were TOP10 for cloning of PCR products into pCR-Blunt vector (Invitrogen) and DH5α as host strain for plasmid propagation. Cultures were grown at 37°C in Luria-Bertani medium in the presence of ampicillin (50 μg/mL) when required.

2.2. Plasmids and cloning procedures

The 276 bp SacI-BamHI fragment from pIJ4070 harbouring the constitutive ermE-up promoter [22] was subcloned in the pMS39 plasmid [10] carrying the long synthetic signal peptide (LSSP) giving the pMS41 plasmid. Then the 410 bp KpnI-HincII fragment (PermE-up-LSSP) from pMS41 was cloned in the KpnI-HincII sites of pIJ4070 yielding the pMS51 plasmid (PermE-up-LSSP).

The sequence coding for the A-domain of the rat leukocyte β2 integrin CR3 αM subunit (CD11b) was amplified by PCR using the following two primers: S43 5‘-CTCGATAATCTGGTCTACGAGGAGACAC-3‘ harbouring an EcoRV site and S44 5‘-CTCTGCGCTAGT-CAGTCAGAGACAC-3‘ harbouring a PstI site and as template the pGEX-2T plasmid containing the CD11b A-domain fused to the glutathione S-transferase (GST) [20]. (PCR conditions were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 68°C, and 35 s at 72°C. Amplification was carried out using the Pfu DNA polymerase from Stratagene.) The 622 bp PCR fragment corresponding to the A-domain coding sequence was cloned into the pCR-Blunt vector to produce the pMS52 plasmid. Then the 610 bp EcoRV-PstI fragment from pMS52 was cloned in the EcoRV-PstI sites of pMS51 giving the pMS62 plasmid. Finally, the 1030 bp BglII fragment from pMS62 carrying the cassette “PermE-up-LSSP-A-domain” was ligated to the pIJ699 vector cut by BglII [24]. After transformation of the S. lividans 1326 protoplasts, the recombinant strain carrying the resulting pMS115 plasmid was called S. lividans 1326/pMS115.

2.3. DNA isolation and manipulation

Plasmid preparation and DNA manipulation from the S. lividans 1326/pMS115 strain were carried out according to Hopwood et al. [23] and from E. coli as described by Sambrook et al. [25].

2.4. RNA isolation and RT-PCR

The S. lividans 1326/pMS115 strain was precultured at 30°C on TSB medium [23] then inoculated at 1/10 in the same conditions. Cultures were stopped at the end of the exponential phase (36 h), early stationary phase (50 h), and late stationary phase (60 h). Then the cells were harvested by centrifugation (7500 xg, for 15 min at 4°C). RNAs were prepared from these cultures as described by Hopwood et al. [23] except that a DNase I (Promega) treatment was used in addition to salt precipitation. This RNA preparation was used in RT-PCR experiments. cDNA corresponding to the A-domain
was synthesized from the isolated RNAs (treated with DNase) using the AMV reverse transcriptase (AMV-RT) from Amersham as follows: 2 μL of RNA (2 μg), 2.5 μL of primer S259 at 10 μM, and 9 μL H2O RNase-free. Reactions were incubated in a thermocycler at 65°C for 5 min then placed on ice and the following components were added in each sample: 4 μL of AMV-RT 1X buffer, 1 μL of AMV-RT (1 U/μL), 1 μL of RNasin (Amersham), and 0.5 μL of dNTP 10 mM each. Reactions were incubated at 42°C for 90 min and then at 70°C for 15 min.

10% of the synthesized cDNA (2 μL) was amplified by PCR using the internal primers S258-forward, 5′-CAGGA-GAGCAACATTGCCTTC-3′, and S259-reverse, 5′-GTGAT-CACCAGCTGGCTTAGA-3′. As control, we have performed a PCR reaction using as matrix 1 μg of RNA treated with DNase. The cycling parameters were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 30 s. In all cases, 10 μL from 50 μL of the PCR reactions were analyzed on a 1.2% agarose gel.

2.5. Western blot analysis

*S. lividans* 1326/pMS115 strain was precultured on TSB medium then inoculated at 1/10 in NMMP medium [23] with of 1% (W/V) of glucose and grown for 48 h at 30°C. After culture centrifugation (7500 × g, for 15 min at 4°C), supernatant was lyophilized, resuspended in a minimum amount of phosphate buffered saline (PBS) at pH 7.4, and dia- lyzed for 24 h at 4°C against the same buffer.

Protein content was determined using Bradford’s method with serum albumin as the standard.

15 μg of secreted proteins were loaded and resolved on a 15% SDS denaturing polyacrylamide gel run in a Tris glycine electrophoresis buffer as described by Laemmli [26].

The GST/CD11b A-domain fusion protein and the A-domain released from the fusion protein by thrombin cut were used as controls [20]. The gel was transferred to an immunobilon-P membrane. Unspecific binding of the an- tibody anti-rat CD11b (clone OX42) purchased from Pharmingen and diluted to 1/1000 in TBS containing 1% dried skimmed milk. Immunoreactive bands were revealed using peroxidase conjugated anti-rat IgG antibodies.

3. RESULTS AND DISCUSSION

3.1. The ermE-up promoter drives an efficient expression of the rat α integrin CD11b A-domain in*S. lividans*

To investigate the potential of *S. lividans* 1326 to express α integrin A-domain as a secreted recombinant protein, the A-domain of the rat leukocyte β2 integrin αM subunit (CD11b), coding sequence was amplified by PCR and fused to the long synthetic signal peptide (LSSP) [10] of the pMS51 plasmid harbouring the *ermE-up* promoter as described in “Materials and Methods” (Figure 1). Finally the cassette “PermE-up-LSSP-A-domain” was cloned in the plJ699 vector yielding the pMS115 plasmid.

Figure 1: Construction of plasmid pMS115. The constitutive *ermE-up* promoter from plJ4070 was subcloned as a SacI-BamHI fragment in the pMS39 plasmid carrying the long synthetic signal peptide (LSSP) giving the pMS41 plasmid. Then the KpnI-HincII fragment carrying *PermE-up-LSSP* from pMS41 was cloned in plJ4070 yielding the pMS51 plasmid. PCR product of the rat CD11b A-domain was cloned into the pcR-Blunt vector to produce the pMS52 plasmid. The fragment EcoRV-PstI corresponding to the A-domain from pMS52 was cloned in pMS51 giving the pMS62 plasmid. Finally, the BglII fragment from pMS62 carrying the cassette “PermE-up-LSSP-A-domain” was cloned in the plJ699 vector yielding the pMS115 plasmid.
obtained from A-domain messengers since no amplification was observed when PCR reaction was performed directly with DNase-treated RNA. This result confirms the expression in \textit{S. lividans} 1326/pMS115 of the rat \(\alpha\)M subunit (CD11b) A-domain sequence under the control of the constitutive \(erm\)\textsuperscript{E-up} promoter from \textit{Streptomyces erythraeus} [22]. Indeed, this strong and constitutive promoter was widely used for gene expression in various \textit{Streptomyces} species [10, 27–31].

### 3.2. The LSSP signal peptide permits the production of the CD11 A-domain as an extra cellular protein in \textit{S. lividans}

The \textit{S. lividans} 1326/pMS115 was grown for 48 hours on NMMP medium with glucose as the carbon source. Then, the supernatant culture was lyophilized, dialyzed against PBS buffer and electrophorized on an SDS polyacrylamide gel. As shown in Figure 3, the size of the recombinant A-domain (21 kDa) expressed by \textit{S. lividans} was the same of its homologous expressed in \textit{E. coli} as a GST/CD11b A-domain fusion protein (48.5 kDa) and released by thrombin cut [20]. Since it is known that \textit{E. coli} is unable to glycosylate the polypeptide, and given the fact that the size of the recombinant protein expressed in each system is similar, we can conclude that \textit{S. lividans} did not glycosylate the A-domain, even though the sequence of the rat CD11b displays a putative N glycosylation site.

Furthermore, the yield of the recombinant rat CD11b A-domain secreted by \textit{S. lividans} was estimated at 8 mg per litre. This yield is based on the comparison of band intensity as shown in Figure 3 and takes into consideration the 50x concentration of the supernatant prior to loading on to electrophoresis gel. The estimated A-domain represents 8% of the total extracellular proteins (100 mg/L). This relatively low extracellular protein level could be explained by the use of a minimal medium (NMMP) useful for the further analysis. Western blot analysis of the supernatant of the \textit{S. lividans} culture showed (Figure 3) a protein band with a molecular mass of about 21 kDa corresponding to the secreted rat CD11b A-domain expressed in \textit{S. lividans}. Binding of the function blocking antibody implied that this secreted recombinant form is properly folded and suggests strongly that the protein would be functional as previously shown with its counterpart produced in \textit{E. coli}.

As described by Mhiri et al. [10], the long synthetic signal peptide (LSSP) contains two positive charges on the N-terminal region and carries two ribosome binding sites (RBS) as well as two translational initiation codons which are known to contribute to increasing the yield of the secreted protein [15, 32]. This signal peptide was successfully used for the secretion of the \textit{Streptomyces} sp. TO1 amyrase.
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