Expression and purification of recombinant methylated HBHA in Mycobacterium smegmatis

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Received 7 June 2004; received in revised form 22 July 2004; accepted 10 August 2004
First published online 26 August 2004
Edited by R.S. Buxton

Abstract

The Heparin-Binding Haemagglutinin (HBHA) is a mycobacterial adhesin involved in the dissemination of Mycobacterium tuberculosis from the site of primary infection and a potential candidate for the development of a new vaccine against tuberculosis. Methylation of HBHA is a novel post-translational event that imparts important immunological properties to the protein. Since recombinant HBHA expressed in Escherichia coli is not methylated, we investigated the possibility of producing recombinant methylated HBHA in fast growing mycobacteria for use in immunological and biochemical studies. The complete coding sequence of HBHA was cloned in the plasmid pMV206, under the control of a strong promoter (hsp60) or its own promoter. The constructs generated were electroporated into Mycobacterium smegmatis and the recombinant strains obtained were analyzed for the presence of the HBHA protein using the anti-HBHA monoclonal antibodies D2 and E4. Our results indicate that expression of high amounts of intact protein can be toxic for the mycobacteria, that methylated HBHA can be obtained in M. smegmatis only when using a promoter sequence weaker than hsp60 and that the expression of the complete structural gene is required in order to obtain methylated HBHA. We constructed a recombinant M. smegmatis strain (pMV3-38) that expresses a histidine-tagged methylated HBHA that can be easily purified. The use of fast-growing strains of M. smegmatis to obtain significant amounts of purified HBHA protein within a short timeframe, should be an effective strategy for the evaluation of a new HBHA-based vaccine candidate for tuberculosis.

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Keywords: Mycobacteria; HBHA; Tuberculosis; Mycobacterium; Vaccine; Protein expression; Methylation

1. Introduction

Tuberculosis (TB) remains one of the deadliest infectious diseases affecting mankind and it has been estimated that one-third of the world’s population is presently infected with Mycobacterium tuberculosis. Most of the TB cases occur in developing countries, where tuberculosis poses a major health, economic and...
The development of new and more effective vaccines would provide a powerful tool to fight this ancient disease [3,4]. In the last decade, genomics and proteomics have helped to identify new proteins and other molecular components involved in the pathogenesis of *M. tuberculosis* [5]. Among them is the heparin-binding haemagglutinin (HBHA), a 199-amino-acid protein that ranks as one of the few well characterized adhesion factors of *M. tuberculosis* [6,7].

HBHA is a mycobacterial cell surface protein that mediates the interaction of the tubercle bacilli with epithelial cells and components of the extracellular matrix [6,8,9]. In vivo experiments have shown that HBHA is directly involved in the dissemination of *M. tuberculosis* from the site of primary infection [7]. Since dissemination may represent a fundamental step in the pathogenesis of TB [10], the development of prophylactic tools that specifically target HBHA is an innovative approach to fighting TB infection [7,11,15].

Biochemical and molecular studies have demonstrated that the C-terminal domain of HBHA mediates bacterial interactions with host cells [8,12], and that HBHA undergoes a novel post-translational modification resulting in the methylation of several lysines present at the C-terminus of the protein [13]. Methylation has been shown to affect the immunological properties of the protein, as indicated by differences in the reactivity of the native vs. the recombinant protein obtained in *E. coli* using Mabs [13,14].

Purification of the native HBHA from *M. tuberculosis* H37Ra or from *M. bovis* BCG for biochemical and immunological studies, is a complex and time consuming procedure [6,8]. The development of a new and improved strategy for producing a methylated recombinant HBHA would accelerate efforts to test the effectiveness of HBHA as a vaccine candidate or as a component of a new diagnostic [15].

### 2. Materials and methods

#### 2.1. Cloning strategies

The *hbhA* gene, its selected fragments, the promoter sequences and the promoter plus signal sequence regions were amplified from the H37Rv genomic DNA [16] using the set of primers indicated in Table 1, the Vent Polymerase (New England Biolab, Beverly, MA) and procedures as previously indicated [8]. Each primer bears an adapter sequence for a restriction endonuclease. The amplified fragments were subcloned in pCR-Blunt using the Zero Blunt Cloning system (Invitrogen, San Diego, CA) then cut with the appropriate restriction endonucleases and inserted into the pMV206 construct [17]. The sequences coding for the histidine-tagged HBHA and its C-terminal domain were

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Adapter</th>
<th>Noted Construct</th>
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<tbody>
<tr>
<td>HAwh5Nh</td>
<td>5'-ACC GCT AGC ATG GCT GAA AAC TCG AAC ATT GAT GAC-3'</td>
<td>NheI</td>
<td>Starts at +1 bp pMV1-25, pMV3-26</td>
</tr>
<tr>
<td>HA + 295Nh</td>
<td>5'-ACC GCT AGC ATG GAG TTG ATC ACG AAC CTG CGT GAG-3'</td>
<td>NheI</td>
<td>Starts at +85 bp pMV3-21, pMV3-28, pMV3-17</td>
</tr>
<tr>
<td>HA + 875Nh</td>
<td>5'-ACC GCT AGC ATG CTC GAG GCC GCG ACT AGC CGG TAC-3'</td>
<td>NheI</td>
<td>Starts at +262 bp pMV3-22, pMV3-29</td>
</tr>
<tr>
<td>HBst3Bm</td>
<td>5'-ACC GGA TCC CTA CTT CTG GGT GAC CTT CTT GGC CGC-3'</td>
<td>BamHI</td>
<td>All</td>
</tr>
<tr>
<td>HApr5Hn</td>
<td>5'-ACC AAG CTT CCG TTG GCC GGC TGT TTT GCG CAC CGG-3'</td>
<td>HincII</td>
<td>Starts at /C0 357 bp pMV1-21, pMV3-21, pMV3-22</td>
</tr>
<tr>
<td>HApr3Nh</td>
<td>5'-ACC GCT AGC TCC TTT CAT TAA CTA TTT TCT GAT ATC-3'</td>
<td>NheI</td>
<td>Reverse, starts at /C0 7 bp pMV3-21, pMV3-22</td>
</tr>
<tr>
<td>64pr5Hn</td>
<td>5'-ACC AAG CTT CGT CGA GCA CCA CGC GAC ACC GGG CC-3'</td>
<td>HincII</td>
<td>Starts at /C0 175 bp pMV3-26, pMV3-17</td>
</tr>
<tr>
<td>64pr3Xb</td>
<td>5'-ACC AAG CTT CGT CGA GCA CCA CGC GAC ACC GGG CC-3'</td>
<td>XbaI</td>
<td>Reverse, starts at /C0 7 bp pMV3-26, pMV3-17</td>
</tr>
<tr>
<td>HSpr5Hn</td>
<td>5'-ACC AAG CTT CCG ATG CCG GTG TTG GCG CCG GTG-3'</td>
<td>HincII</td>
<td>pMV1-25, pMV3-28, pMV3-29</td>
</tr>
<tr>
<td>HSpr3Xb</td>
<td>5'-ACC AAG CTT CCG ATG CCG GTG TTG GCG CCG GTG-3'</td>
<td>XbaI</td>
<td>Reverse, starts at /C0 7 bp pMV1-25, pMV3-28, pMV3-29</td>
</tr>
</tbody>
</table>

- The first two letters indicate the gene: HA, *hbhA* (Rv0475); 64, mpt64 (Rv1980); HS, heat shock protein (Rv0350).
- The promoter sequences are indicated in Table 1.
- The restriction endonuclease that cuts at the adapter consensus sequence.
- The pMV-based plasmids obtained starting from the gene sequences amplified using that primer.

**Table 1** List of primers used in this study to amplify the gene and sequences of interest
obtained from the pET15b-based plasmids previously described [8], by cutting with the *Nco*I and *Bam*HI restriction endonucleases. These fragments were inserted into the plasmids pMV1-21 cut with the *Nco*I and *Bam*HI enzymes to obtain constructs pMV3-38 and pMV3-39.

2.2. Transformation and isolation of recombinant *M. smegmatis* strains

Preparation of *M. smegmatis* competent cells and electroporation procedures were performed following standard procedures [18]. Briefly, *M. smegmatis* strain mc^155 was grown in 7H9/ADC/Tw (7H9 containing 10% ADC (Microbiol, Cagliari, Italy) and 0.05% Tween 80 (Sigma–Aldrich, Saint Louis, MO)) until mid-log phase. Cells were then harvested and washed twice with cold 10% glycerol and resuspended in the same buffer. 0.2 ml of the competent cells was electroporated following standard setting: 1.5 kV, 1000 Ohm, 25 μF using the Gene Pulser apparatus (Biorad). Cells were harvested with 1 ml of warm 7H9/ADC/Tw, incubated for 4 h at 37 °C in gently shaking, and differential dilutions of the culture were plated in 7H11/OADC plates containing 50 μg/ml of hygromycin (Sigma–Aldrich). Plates were incubated at 37 °C for 3–4 days to obtain the recombinant strains.

2.3. Preparation of cell lysates

The selected *M. smegmatis* recombinant strains were grown in 7H9/ADC/Tw containing 50 μg/ml of hygromycin up to late log phase. An aliquot of 0.2 ml was transferred in 20 ml of Sauton media containing hygromycin at the final concentration of 50 μg/ml, and cultures were incubated at 37 °C with gently shaking. Cells were harvested three days later, washed once with cold PBS (phosphate buffered saline) and resuspended in the same buffer. 0.2 ml of the competent cells was electroporated following standard setting: 1.5 kV, 1000 Ohm, 25 μF using the Gene Pulser apparatus (Biorad). Cells were harvested with 1 ml of warm 7H9/ADC/Tw, incubated for 4 h at 37 °C in gently shaking, and differential dilutions of the culture were plated in 7H11/OADC plates containing 50 μg/ml of hygromycin (Sigma–Aldrich). Plates were incubated at 37 °C for 3–4 days to obtain the recombinant strains.

2.4. Purification of histidine-tagged HBHA from *M. smegmatis*

The *M. smegmatis* (pMV3-38) recombinant strain expressing the histidine-tagged HBHA was grown in 200 ml of Sauton media [19] containing 50 μg/ml of hygromycin for 4 days at 37 °C in gently shaking. Cells were harvested by centrifugation and the pellet was washed twice in cold PBS. Cells were resuspended in 20 ml of PBS containing 0.05% Tween 80 (PBST) containing the protease inhibitor P-8849 and 0.1 mg/ml of lysozime (Sigma–Aldrich) and incubated on ice for 15 min. Five cycles of sonication (3′ each) were performed on a Sonifier Cell Disruptor (Branson Sonic Power, Danbury Connecticut) (duty cycle 50%, microtip limit 55) to lysate cells. Cell lysate was centrifuged at 1500g and the supernatant transferred to a fresh tube and then mixed with 8 ml of Ni-NTA matrix (X-Press, Invitrogen) previously prepared as indicated by the manufactures. Incubation was carried out for 30′ at 4 °C and the matrix was washed three times with 30 ml of NBB (500 mM NaCl, NaHCO3, NaH2CO2, pH 7.8), five times with NBW (500 mM NaCl, NaHCO3, NaH2CO2, pH 6.0), and once with NBW containing 50 mM imidazole. The Ni-NTA matrix was then transferred to a column and elution was obtained with NBW containing increasing concentration of imidazole (200, 350 and 500 mM). The eluted fractions were analyzed in SDS–PAGE and Coomassie brilliant blue (CBB) staining and the fractions containing the purified protein were pooled and dialyzed using the centrifugal filter devices (Amicon, Redford, MA). Protein concentration of the final preparation was determined by the BCA method.

2.5. SDS–PAGE and immunoblots

The cell lysates of the *M. smegmatis* recombinant strains were analyzed in immunoblot following standard procedures [20]. About 200 μg of total protein was separated by 12% SDS–PAGE, transferred to a nitrocellulose membrane and analyzed in immunoblot using the Mab E4 and D2 [6,21].

3. Results and discussion

3.1. Construction of hbhA expression plasmids

To obtain a mycobacterial strain that expresses significant amounts of native HBHA, the *hbh*A gene was cloned in the pMV206 plasmid under the control of a number of mycobacterial promoter sequences (Fig. 1(a)). Since the C-terminal lysine-rich domain is involved in the interaction with the host components and antibodies that target this region inhibit the binding of the bacilli to epithelial cells [8,12,14], we constructed recombinant strains expressing truncated forms of the protein lacking the putative transmembrane region of the protein (29 aa), or lacking the transmembrane plus most of the coiled coil region (87 aa) [8]. The pMV206-based expression constructs [17] were obtained by fusing a promoter sequence with the *hbh*A-derived fragments containing the ATG start codon to make all the constructs shown in Fig. 1(b).
3.2. Lessons learned from the expression of the HBHA protein in M. smegmatis

The pMV206-based constructs were electroporated into the fast-growing, non-pathogenic M. smegmatis mc^2155 [22] strain which lacks the \textit{hbh}A gene, to obtain a mycobacteria recombinant strain that could be easily manipulated in most laboratories. Colonies of \textit{M. smegmatis} transformed with the plasmid pMV1-25, expressing the \textit{hbh}A gene under the control of the strong \textit{hsp}60 promoter of \textit{M. tuberculosis} [17,23], appeared very small compared to controls and could not be grown in minimal media. Conversely, colonies of \textit{M. smegmatis} strains expressing a fragment of the HBHA protein lacking the putative trans-membrane domain (pMV3-28), or expressing the C-terminal domain (pMV3-29), appeared normal in solid media and could be grown in minimal media. These data suggest that the expression of high levels of intact HBHA protein may be toxic for \textit{M. smegmatis}, while expression of significant levels of truncated forms of HBHA appear not to be toxic.

To obtain \textit{M. smegmatis} strains capable of expressing HBHA protein without affecting cell viability, a number of plasmids expressing whole or truncated forms of HBHA under the control of weaker promoter sequences (\textit{pr::mpt}64 and \textit{pr::hbh}A) were generated, as indicated in Fig. 1(b), and used to transform \textit{M. smegmatis}. The recombinant \textit{M. smegmatis} strains that grew normally in solid media were grown in Sauton media and the cell lysates obtained analyzed in immunoblot using the two anti-HBHA monoclonal antibodies D2 and E4 [6,21].

As indicated in Fig. 2(a) and (b), the two MAbs recognize a major band around 32 kDa in the control strain \textit{M. smegmatis} (pMV1-18) (lane 1) corresponding to the Laminin Binding Protein (LBP) which has been shown to have epitopes that cross-react with HBHA [13]. As shown in Fig. 2(a), the MAbE4 recognized a band of 20 kDa in the cell lysate of the \textit{M. smegmatis} (pMV3-
28) strain (lane 5) and a band of 14 kDa in strain *M. smegmatis* (pMV3-29) (lane 6) which were not recognized by MabD2 (Fig. 2(b)). Since the MabD2 recognizes only native methylated HBHA [6,14], these truncated forms of HBHA do not appear properly methylated.

Expression of the complete *hbhA* gene under the control of the *mpt*64 promoter sequence (plasmid pMV3-26), resulted in a recombinant strain that grew normally. A protein of the expected molecular weight was recognized in the corresponding *M. smegmatis* cell lysates by both monoclonal antibodies. A representative immunoblot of at least two different cell lysate preparations is shown.

Three *hbhA* gene fragments were inserted downstream of the sequence containing the *hbhA* promoter (pMV1-21, pMV3-21 and pMV3-22). The corresponding recombinant strains grew normally in liquid media and each of them expressed a protein that was recognized by MabE4 (Fig. 2(a), lanes 2–4). The protein expressed by the *M. smegmatis* (pMV1-21) strain migrated at the expected MW for full-length HBHA and was also recognized by MabD2 suggesting that it is methylated. Conversely, the two proteins expressed by the strains *M. smegmatis* (pMV3-21) and *M. smegmatis* (pMV3-22) were not recognized by MabD2. Similar to what was observed for the *M. smegmatis* (pMV3-28) and *M. smegmatis* (pMV3-29) strains, the presence of the HBHA putative transmembrane region appears to be required for methylation.

Taken together these results suggest that: (a) methylation of HBHA can occur in *M. smegmatis*, suggesting that quite different mycobacterial species share the same methylation process; (b) the full-length HBHA sequence is required in order for the methylation to occur. It has been previously shown that the targets of the methylation are the lysines present at the C-terminus end of the protein [13]. Since we demonstrated that the N-terminal domain of HBHA is required for proper methylation, it may be hypothesized that the N-terminal domain is required for the recognition by the methyltransferase(s) or to target the protein to the proper cellular compartment where methylation occurs. Interestingly, while expression of recombinant HBHA did not affect the expression rate of the LBP in strain *M. smegmatis* (pMV3-26), in strain *M. smegmatis* (pMV1-21), the expression of LBP was reduced. Since these two plasmids differ only in the promoter region upstream from the *hbhA* gene, and since HBHA and LBP share significant gene homology [13], it may be that expression of these two proteins is regulated by a common mechanism.

3.3. Successful expression of a histidine-tagged HBHA protein in *M. smegmatis*

In order to simplify the purification of recombinant HBHA from *M. smegmatis* cells, the full-length *hbhA* gene, or the *hbhA* gene fragment encoding the C-terminal domain, were fused to a sequence coding for repeated histidine residues as previously described [8]. Cloning was carried out so to obtain a fusion at N-terminal of the histidine-tag, to reduce any sterical interference with the functionally active C-terminal domain of the protein. The two fragments were fused to the sequence containing the *hbhA* promoter region to obtain plasmids pMV3-38 and pMV3-39, as shown in Fig. 1(b). Immunoblot analysis of the cell lysates of these two *M. smegmatis* strains (Fig. 3) indicated that the two proteins were expressed and migrated on SDS–PAGE at the expected MW. The *M. smegmatis* (pMV3-38) strain (lane 2) expressing the full-length HBHA protein fused to the histidine-tag was also recognized by MabD2, while the protein expressed by the *M. smegmatis* (pMV3-39) (lane 3) was not recognized by MabD2. Hence, fusion of a full-length HBHA at the N-terminus with the histidine-tag can result in the expression of a methylated protein. Conversely, as observed for the other truncated
forms of HBHA, fusion of the C-terminal domain with the histidine-tag did not result in a methylated protein.

3.4. Purification of the histidine-tagged HBHA from M. smegmatis

The M. smegmatis strain (pMV3-38) was grown in Sauton medium in large volumes and processed as indicated in Section 2 to purify the histidine-tagged protein. The entire protocol to obtain the purified protein required 5 days for the M. smegmatis strain to grow and only ~8 h to process the cell lysate and to purify the protein by nickel chromatography. By comparison, bacterial growth and purification of native HBHA from BCG or M. tuberculosis H37Ra takes several weeks.

The SDS–PAGE shown in Fig. 4 indicated that the protocol for the purification of the histidine-tagged HBHA protein from M. smegmatis (pMV3-38) was very effective and produced a very pure protein preparation (lane 2), that was similar to the protein preparation obtained from E. coli expressing the recombinant histidine-tagged HBHA (lane 3) [8]. When starting from a 200-ml culture of strain M. smegmatis (pMV3-38) yields obtained were in the range 0.7–0.8 mg of pure protein. Analysis of these HBHA preparations by immunoblots suggests that the recombinant HBHA protein obtained from M. smegmatis (pMV3-38) (lane 2) has immunologically properties similar to the native HBHA obtained from M. tuberculosis H37Ra (lane 1), since it is recognized by both MabE4 and MabD2, but different from the HBHA protein obtained in E. coli (lane 3), that is not recognized by MabD2 (Fig. 4(b)).

In summary, we have demonstrated that expression of full-length HBHA using a strong promoter can be toxic for M. smegmatis cells harboring this plasmid, but expression of methylated HBHA can be achieved in M. smegmatis by placing the hbbA gene under the control of weaker promoter sequences. Methylation of only full-length HBHA occurs in M. smegmatis and is dependent upon the presence of the N-terminal domain of the protein. In this study, a M. smegmatis strain expressing a methylated histidine-tagged HBHA was developed and significant amounts of pure protein were purified using a relatively simple procedure. The results described here for the recombinant M. smegmatis strains should provide useful information for the successful expression of recombinant proteins in mycobacterial strains that contain important post-translational modifications. This may lead toward a better understanding of the structure, function and immunogenicity of mycobacterial antigens [24].

Acknowledgements

This work has been sponsored by the AERAS Global TB Vaccine Foundation (formerly the Sequella Global TB Foundation) through the Vaccine Innovation Program (VIP 2001-004) to G.D. and by a grant from the National Vaccine Program Office to M.J.B.

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