Expression of SA5K, a secretion antigen of *Mycobacterium tuberculosis*, inside human macrophages and in sputum from tuberculosis patients

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Abstract

An 8.3 kDa protein (SA5K), secreted by *Mycobacterium tuberculosis*/Mycobacterium bovis bacillus Calmette–Guérin (BCG) in culture filtrate, has been previously described in our laboratory. In the present study, analysis of the distribution of SA5K gene (Rv1174c) among *M. tuberculosis* strains, isolated from a wide variety of clinical specimens, revealed that the gene is present in all clinical isolates analyzed (29/29). SA5K expression inside human macrophages infected with BCG was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) on RNA extracted from bacterial cells following 24 and 48 h of infection. In addition, in order to evaluate whether SA5K gene was also expressed at the site of infection in the lung, an nested RT-PCR assay was developed to detect specific mRNA in sputum samples collected from smear positive tuberculosis patients. SA5K mRNA was detected in all the samples containing high numbers of tubercle bacilli demonstrating that the corresponding gene is expressed during the course of clinical infection.

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1. Introduction

Among diseases with bacterial etiology tuberculosis (TB) is still the one causing most deaths worldwide [1]. Currently several measures are under investigation for improving the control of the disease including a rapid diagnosis and identification of cases [2], the development of new antituberculous drugs, the establishment of a new TB vaccine. All the above-mentioned strategies for the control of TB require an in-depth knowledge of the pathogenesis of TB infection and the identification of the proteins that are specifically expressed by *Mycobacterium tuberculosis* in the intracellular environment or in the sites of infection which, possibly, represent virulence factors involved in the survival of the microorganism in the host.

Over the last few years several efforts have been made to identify mycobacterial genes specifically expressed or induced inside human macrophages cultured in vitro and a number of proteins involved in intracellular survival have been described [3–9]. In contrast, very little is yet known about the genes and the corresponding protein products required by *M. tuberculosis* for growth in the human host [10]. Detection of gene transcripts directly in sputum samples, has successfully been used for the analysis of virulence genes expressed by *Pseudomonas aeruginosa* or by *Staphylococcus aureus* in the course of pulmonary infection of patients with cystic fibrosis [11–13]. Recently, analysis of genes expressed in sputum samples from patients with TB has also been performed and it has been suggested as a suitable marker to detect viable *M. tuberculosis* for rapid diagnosis of pulmonary TB [14,15].

An 8.3 kDa protein (SA5K), secreted by *M. tuberculosis* and *Mycobacterium bovis* in the extracellular environment, was previously identified and purified in our laboratory [16,17]. The protein is expressed during intracellular growth of recombinant *Mycobacterium smegmatis* and seems to be involved in intracellular survival mechanisms
Moreover, a recombinant form of SA5K was found to induce proliferation of CD4+ T lymphocytes from healthy donors sensitized to mycobacterial antigens [18].

Aims of the present study were: (i) analyze the distribution of the SA5K gene (Rv1174c) [19] in clinical isolates of M. tuberculosis; (ii) evaluate SA5K expression during growth of M. bovis Calmette–Guérin (BCG) into the human monocytic cell line THP-1; (iii) develop a nested reverse transcription-polymerase chain reaction (RT-PCR) assay for detection of SA5K mRNA in sputum samples to establish whether SA5K gene was expressed in the lung of patients with pulmonary TB. The same sputum specimens were also evaluated for the presence of mRNA for Ag85B, one of the major extracellular M. tuberculosis proteins [20,21] which has been found to be expressed in the lung of TB patients [22,23]. Finally, ribosomal 16S RNA was selected as a target for control of RNA extraction and amplification procedures. The results obtained demonstrated that SA5K gene is widely distributed in clinical isolates of M. tuberculosis and is expressed during intracellular growth and in the lung of the majority of TB patients.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Twenty-nine M. tuberculosis strains were isolated and identified at the Mycobacteriology Section of the Microbiology Laboratory of the University Hospital of Pisa (Italy). Most of the strains were recovered from respiratory specimens: sputum (15), bronchoalveolar lavage (two), caseous liquid (one). The remaining strains were isolated from other specimens: blood (one), urine (two), synovial fluid (one), lymph-node aspirate (four), cutaneous swabs (three). IS6110-based DNA restriction fragment length polymorphism assay [24], performed on all M. tuberculosis strains, revealed a marked heterogeneity in their banding patterns (data not shown) suggesting that the isolates were actually distinct strains and not the result of a nosocomial transmission of few strains. Mycobacterium avium, strain 35712, was obtained from ATCC. BCG, strain Pasteur, was supplied by Pasteur Merieux (Lyon, France).

All mycobacterial strains were cultured on Lowenstein–Jensen slants (Becton Dickinson) at 37°C for 14–21 days before being subjected to DNA extraction. For infection of THP-1 macrophage cell line BCG was grown in Sauton’s modified medium [25] in rolling bottles for 8 days. Colony forming units (CFU) of BCG grown in broth were determined by plating 10-fold dilution, in duplicate, on Middlebrook 7H11 agar supplemented with oleic acid albumin dextrose complex (OADC) (Becton Dickinson). Colonies were counted after incubation at 37°C for 15 days.

2.2. Infection of the THP-1 human macrophage cell line and evaluation of intracellular SA5K expression

Culture and activation of THP-1 cells were carried out as previously described [6]. BCG was added to the cells at a multiplicity of infection of 1:10 (bacteria:cells). After 3 h incubation at 37°C, infected monolayers were repetitively washed and incubated in RPMI medium supplemented with 5% (v/v) fetal calf serum (FCS), 2 mM L-glutamine for 2 days. Macrophages were lysed by osmotic shock [6] and total RNA was obtained from macrophage-grown BCG using Trizol reagent (Gibco) following the manufacturer’s instructions. RT-PCR for the detection of SA5K mRNA was carried out using the access RT-PCR system (Promega) as previously described [6].

2.3. DNA isolation from mycobacteria

Genomic DNA from M. tuberculosis clinical isolates, BCG and M. avium was prepared following standard procedures for mycobacteria [24].

2.4. Analysis of the distribution of SA5K gene in M. tuberculosis clinical isolates by PCR amplification and Southern blot hybridization

SA5K gene from genomic DNAs was amplified by PCR. Amplification reactions were performed as follows: denaturation for 1 min at 94°C; primer annealing for 1 min at 60°C (Table 1); primer extension for 1 min at 72°C. After 30 cycles, a final extension was carried out at 72°C for 10 min. Primer sequences and the corresponding annealing temperatures are indicated in Table 1. The 330 bp PCR products were electrophoresed in a 0.8% agarose gel, de-natured and transferred by capillarity onto a nylon membrane (Hybond-N filter, Amersham Pharmacia) following standard procedures [26]. Southern blotting analysis was performed using enhanced chemiluminescence (ECL) system (Amersham Pharmacia) according to the manufacturer’s instructions. A 258 bp SA5K fragment obtained by PCR on BCG genomic DNA was used as a specific probe (Table 1).

2.5. Sputum collection and processing

Expression of M. tuberculosis selected target genes was evaluated in 12 sputum samples obtained from patients with symptomatic pulmonary TB. All sputum specimens were collected before the initiation of anti-TB treatment. Each sputum sample was divided into two aliquots: one aliquot was further divided in 500 µl aliquots which were immediately frozen in liquid nitrogen until RNA extraction; a second aliquot was subjected to standard procedures for homogenization and decontamination and further processed for microscopic examination and for culture of M. tuberculosis by inoculation in BACTEC
12B vials [27]. After microscopic examination of Ziehl–Neelsen-stained smear, each sputum sample was assigned a positivity index (from 0 to 4+) according to the guidelines of the American Lung Association for reporting numbers of acid fast bacilli in stained smears [28]. Species identification of the BACTEC 12B positive cultures was performed using DNA probes following standard procedures [27]. To determine the limit of detection of M. tuberculosis specific mRNAs by nested RT-PCR assay, pooled sputa from M. tuberculosis negative subjects were divided in several aliquots and each of them was spiked with serial 10-fold dilutions of BCG grown in broth (from $10^7$ to $1 \text{ CFU ml}^{-1}$). The different aliquots were processed as test specimens.

2.6. RNA extraction from sputum

RNA was isolated from microscopy positive sputum specimens as follows: briefly, two aliquots of frozen sputum samples, 500 µl each, were thawed and 1 ml of Trizol LS reagent (Gibco) and 500 µl of glass beads (0.1 mm) were added to each of them. Mycobacteria were lysed in a spin/rotation instrument (Bead Beater) with a speed setting of 2500 rpm and a time setting of $6 \times 20$ s. After lysis, 800 µl of chloroform was added to each aliquot and the aqueous phase was collected after centrifugation for 15 min at 4°C at 12,000×g. After addition of 4 µg of glycogen, 0.1 volume of 5 M ammonium acetate and an equal volume of isopropanol, RNA was precipitated for 1 h at −70°C. The resulting RNA pellets were washed with 75% ethanol and resuspended in 165 µl of deethyl pyrocarbonate (DEPC)-treated H2O. RNA preparations obtained from two different aliquots were pooled. To exclude the possibility of DNA contamination, all the RNA samples were subjected to nested PCR, with target specific primers, without prior RT.

2.7. Nested RT-PCR

RNA (5 µl of the total volume) was reverse transcribed using random primer N6. The cDNA synthesis was performed using the RT-PCR system (Promega) as follows: denaturation of RNA at 70°C for 10 min, primer annealing at 25°C for 10 min, RT at 42°C for 45 min.

5 µl of cDNA were used as target for the first amplification reaction and 4 µl of the amplification product were used as target for the second amplification stage. Specific outer and inner primers for SAK, Ag85B genes, and 16S rRNA are indicated in Table 1 and were used at a final concentration of 0.037 and 0.75 mM, respectively. PCR products were electrophoresed in 1.5% agarose gel stained with 0.5 µg ml$^{-1}$ of ethidium bromide and visualized by ultraviolet (UV) transillumination.

3. Results and discussion

3.1. Analysis of the distribution of SAK gene in M. tuberculosis clinical isolates by PCR/Southern blotting

As frequent variations have recently been described at the genetic level among different clinical isolates of M. tuberculosis [29,30] the presence of SAK gene in distinct M. tuberculosis strains was evaluated by PCR/Southern blot. As shown in Fig. 1 a single hybridization band was detected in all clinical isolates tested (29/29) demonstrating that such a gene is widely distributed among M. tuberculosis strains. Amplification and Southern blot

<table>
<thead>
<tr>
<th>Table 1</th>
<th>PCR primers and probe used in this study</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Employment</td>
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<td>---------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>SAK</td>
<td>outer primers for nested PCR and</td>
</tr>
<tr>
<td></td>
<td>genomic DNA amplification</td>
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<td></td>
<td>inner primers for nested PCR and</td>
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<tr>
<td></td>
<td>M. tuberculosis probe</td>
</tr>
<tr>
<td>Ag85B</td>
<td>outer primers for nested PCR</td>
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<tr>
<td></td>
<td>inner primers for nested PCR</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>outer primers for nested PCR</td>
</tr>
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<td></td>
<td>inner primers for nested PCR</td>
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hybridization on genomic BCG DNA (Fig. 1, line 12) gave rise to a band of similar molecular mass of that obtained from each of the *M. tuberculosis* genomic DNAs tested in agreement with the fact that *SA5K* gene sequence is identical in the two species [18]. No hybridization signal was observed after amplification of *M. avium* DNA (Fig. 1, line 13) confirming previous observations that such mycobacterial species does not produce SA5K protein [16].

3.2. Expression of *SA5K* gene in human macrophage THP-1 cell line infected with *M. bovis* BCG

*SA5K* gene has been previously cloned in *M. smegmatis*, a rapid growing non-pathogenic mycobacterium which does not contain the gene for the protein, and its expression was found to correlate with an accelerated intracellular multiplication of such heterologous host [6]. To evaluate whether the *SA5K* gene was expressed also by the natural host, BCG, during growth inside human macrophages, total RNA was extracted from intracellularly growing BCG at 24 and 48 h after infection, and was subjected to RT-PCR with *SA5K* specific primers. A single 258 bp fragment was detected after RT-PCR of RNA extracted from macrophage-grown BCG both at 24 and 48 h after infection (Fig. 2). The length of the amplified fragment was in agreement with that deduced by the *SA5K* nucleotide sequence. No amplification signal was detected after PCR, excluding the possibility of DNA contamination. Such results suggest that the SA5K protein is expressed during growth in the intracellular milieu.

3.3. Limits of detection of *M. tuberculosis* mRNAs in sputum samples by nested RT-PCR

To evaluate the sensitivity limit of the nested RT-PCR assays used, sputum samples from *M. tuberculosis* non-infected subjects were pooled and spiked with known CFU numbers of BCG grown in broth to reach a final concentration from 10⁷ to 1 CFU ml⁻¹ of sputum. The different aliquots were then processed as test specimens. Total RNA was extracted and subjected to nested RT-PCR to detect the selected RNA targets (*SA5K* mRNA, *Ag85B* mRNA and 16S rRNA). For each target the limit of detection was calculated by determining the greatest dilution at which a band, corresponding to the specific amplicon, was observed on agarose gel. The value obtained was corrected for dilution factors such as volume of sputum used for RNA extraction, volume of RNA used for retrotranscription, and volume of cDNA used in the amplification reaction. Fig. 3A and B show the limits of detection obtained for *SA5K* mRNA and *Ag85B* mRNA, respectively. For *SA5K*, a single 258 bp fragment was detected after nested RT-PCR on RNAs extracted from samples containing as few as 10⁴ CFU ml⁻¹. The sensitivity limit for *SA5K* mRNA, calculated after dilution correction, was 1.25 × 10⁵ CFU ml⁻¹ sputum. The sensitivity of the assay for *Ag85B* mRNA was 10 times lower than that obtained for *SA5K* mRNA, indicating that the efficiency of RT and/or amplification reaction may vary according to the target chosen. Influence of the nucleotidic sequence of amplified genes on the efficiency of PCR reaction has previously been reported [31]. In accordance
with the higher stability and abundance of rRNA as compared to specific mRNAs [14], the sensitivity of the assay for 16S rRNA was much higher than that observed for SA5K and Ag85B mRNAs, the specific amplification product being detectable in sputum sample spiked with 1 × 10^4 CFU ml⁻¹ already after the first PCR reaction (data not shown).

3.4. SA5K and Ag85B expression in sputum samples of TB patients

SA5K and Ag85B gene expressions were analyzed by nested RT-PCR in 12 sputum samples smear and culture positive for M. tuberculosis. A representative agarose gel of nested RT-PCR for the detection of SA5K specific mRNA is depicted in Fig. 4. As shown in Table 2, all the samples resulted positive for the presence of rRNA 16S indicating the effectiveness of the RNA extraction procedure and the amplifiability of the samples.

Ten out of 12 and four out of 12 sputum samples resulted positive for the presence of SA5K and Ag85B mRNAs, respectively (Table 2). All the samples negative for SA5K and/or Ag85B mRNAs were characterized by a low positivity index (+1), determined by direct microscopic examination of Ziehl–Neelsen-stained smears. As the two samples negative for SA5K mRNA were also negative for Ag85B mRNA, no definitive conclusion can be drawn about SA5K gene expression in these two specimens. In fact, the negative results might be due to the actual lack of SA5K expression or, alternatively, to an amount of mycobacteria in the sample below the detection limit of the nested RT-PCR assay. Although six out of eight of the samples negative for Ag85B mRNA were positive for SA5K mRNA, the higher sensibility of detection of the latter transcript, again, does not allow to definitively discriminate between low number of bacteria or lack of expression of the Ag85B gene in these samples. As it has been estimated that smear positive sputum specimens contain at least 10^4 acid fast bacilli per ml [27], the sensitivity limit of the nested RT-PCR assays developed in the present study should have been sufficient to detect SA5K and Ag85B mRNAs in all microscopy positive sputum samples. However, a difference between the sensitivity of the RT-PCR assay evaluated using sputum spiked with broth-grown microorganisms and that observed directly in patients’ sputa has been previously described for Ag85B mRNA [15]. It has been suggested that such a

Table 2
SA5K, Ag85B mRNAs and 16S rRNA detection in sputum specimens by nested RT-PCR assays

<table>
<thead>
<tr>
<th>Patient</th>
<th>Microscopic examination</th>
<th>SA5K</th>
<th>Ag85B</th>
<th>16S rRNA</th>
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<tr>
<td>1</td>
<td>+4</td>
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<td>2</td>
<td>+4</td>
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<td>9</td>
<td>+1</td>
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<td>+1</td>
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<td>11</td>
<td>+1</td>
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<tr>
<td>12</td>
<td>+1</td>
<td>-</td>
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<td>+</td>
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difference might be due to incomplete recovery or partial degradation of RNA between the time of sputum collection and processing. Furthermore, as sputum aliquots used for RNA extraction were not fluidized and homogenized, the actual amount of tubercle bacilli may not exactly correspond to that estimated by microscopic examination. It is also likely that many (or all) mRNAs are expressed at different levels during in vitro or in vivo growth, under the stimuli of the natural and specific defense mechanisms of the host. Finally it is possible that viable bacteria represent only a small proportion of the total number of bacilli in the sample estimated by microscopic observation. Thus, despite the recent development of sensitive techniques for detecting mycobacterial mRNAs, analysis of M. tuberculosis gene expression in sputum samples seems, at the moment, feasible only for specimens containing a high number of bacilli.

In conclusion, the results obtained in the present study indicate that SA-5K gene is constantly present in M. tuberculosis clinical isolates and is expressed during intracellular growth and in the lung of TB patients, suggesting a role of the protein during clinical infection.

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