Brazilian Journal of Medical and Biological Research (2000) 33: 1275-1282
ISSN 0100-879X

Evidence for the expression of native *Mycobacterium tuberculosis* phospholipase C: recognition by immune sera and detection of promoter activity

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Research supported by FAPESP (No. 97/06506-6). T. Matsui was the recipient of a CAPES fellowship.

Part of a Master's thesis presented by T. Matsui to the Departamento de Microbiologia, Imunologia e Parasitologia, UNIFESP, São Paulo, SP, Brazil.

Received February 8, 2000
Accepted July 27, 2000

Abstract

The genome of *Mycobacterium tuberculosis* H37Rv contains three contiguous genes (*plc-a*, *plc-b* and *plc-c*) which are similar to the *Pseudomonas aeruginosa* phospholipase C (PLC) genes. Expression of mycobacterial PLC-a and PLC-b in *E. coli* and *M. smegmatis* has been reported, whereas expression of the native proteins in *M. tuberculosis* H37Rv has not been demonstrated. The objective of the present study was to demonstrate that native PLC-a is expressed in *M. tuberculosis* H37Rv. Sera from mice immunized with recombinant PLC-a expressed in *E. coli* were used in immunoblots to evaluate PLC-a expression. The immune serum recognized a 49-kDa protein in immunoblots against *M. tuberculosis* extracts. No bands were visible in *M. tuberculosis* culture supernatants or extracts from *M. avium*, *M. bovis* and *M. smegmatis.* A 550-bp DNA fragment upstream of *plc-a* was cloned in the pJEM12 vector and the existence of a functional promoter was evaluated by detection of ß-galactosidase activity. ß-Galactosidase activity was detected in *M. smegmatis* transformed with recombinant pJEM12 grown *in vitro* and inside macrophages. The putative promoter was active both *in vitro* and *in vivo*, suggesting that expression is constitutive. In conclusion, expression of non-secreted native PLC-a was demonstrated in *M. tuberculosis*.

Key words
- *Mycobacterium tuberculosis*
- Phospholipase C
- Gene expression
- Promoters

Introduction

Tuberculosis is an ancient disease, but its immunopathogenic mechanisms are still not well understood. Fundamental questions still exist about which antigens are involved in the generation of a protective immune response in humans and their participation in pathogenicity. Several mycobacterial antigens have been recently characterized, some of them expressed exclusively in bacteria of the *Mycobacterium tuberculosis* complex, which consists of four pathogenic members (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*) and one non-pathogenic member (*M. bovis* BCG) (1). Differences in biological characteristics essential for pathogenesis, potentially determined by expression of species-specific proteins, are probably responsible for the unique pathogenic properties and world distribution of *M. tuberculosis*. Identification and characterization of *M. tuberculosis* species-specific proteins is an essential step in research directed at the elucidation of the specific immunopathogenic mechanisms of tuberculosis.

A 3.0-kb *Bam*HI DNA fragment was identified in an *M. tuberculosis* genomic library and sequenced (2,3). The analysis of its nucleotide sequence revealed the existence of two open reading frames (ORF) coding for highly similar proteins. A comparative search also showed great similarity between these ORFs and two phospholipase C (PLC) genes of *Pseudomonas aeruginosa* (3-5). The *M. tuberculosis* H37Rv genome project revealed the existence of three similar consecutive genes - *plc-a*, *plc-b* and *plc-c* (6). The host range of the *plc* site is still a matter of debate, but probes derived from the 3.0-kb fragment did not hybridize with genomes of other mycobacteria, including members of the *M. tuberculosis* complex (2).

PLC-a was expressed in *Escherichia coli* and recombinant clones were found to present hemolytic activity (3). PLC-a and PLC-b were expressed in *M. smegmatis* and the recombinant clones, as well as *M. tuberculosis* H37Rv, were found to have PLC activity (7).

PLC has been described as a virulence factor of *Pseudomonas aeruginosa* (8), *Bacillus cereus* (9), *Listeria monocytogenes* (10) and *Clostridium perfringens* (11,12). An enzyme with this activity may have an active role in tuberculosis, participating in different pathogenic processes attributed to *M. tuberculosis* infection, such as contact-dependent cytolytic activity (13) and lysis of the phagolysosome membrane (14). It may participate in the acquisition of phosphate, as was demonstrated for *P. aeruginosa* PLC (15). It could also modify protein kinase C cell signaling by generation of diacylglycerol, interfere with the activation of phagocytic cells and amplify the inflammatory response by arachidonic acid production. Apoptosis by induction of cytotoxic activity has been observed with *P. aeruginosa* (16) and *M. tuberculosis* (17).

For this reason, it is of fundamental importance to determine if the proteins coded by the *plc-a*, *plc-b* and *plc-c* genes are expressed by *M. tuberculosis* and under which conditions. Here, the expression of native PLC-a was demonstrated in *M. tuberculosis* by immunoblot. Promoter activity of a 550-bp fragment from the upstream region of *plc-a* was detected in recombinant mycobacteria grown *in vitro* and *in vivo*. These results confirmed that the first PLC gene, *plc-a*, is expressed, suggesting that this protein could be biologically relevant during the course of the disease.

Material and Methods

Bacterial strains, plasmids, and macrophage cell line

Escherichia coli JM101 and *E. coli* BL21(DE3) (Novagen, Madison, WI, USA) were grown in Luria Bertani (LB) medium (18) at 37ºC in a shaking incubator. *M. smegmatis* mc^{2}155 was grown in 7H9 liquid medium (Difco, Detroit, MI, USA) or 7H10 agar (Difco) plates supplemented with oleic acid, albumin, dextrose, and catalase (OADC; Difco). Ampicillin (100 µg/ml; Bayer, São Paulo, SP, Brazil) and kanamycin (15 µg/ml; Sigma Chemical Co., St. Louis, MO, USA) were the antibiotics used for selection purposes. X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) (Gibco-BRL, Rockville, MD, USA) was used as a substrate for ß-galactosidase. Bacterial strains were stored at -20ºC in liquid medium plus 15% glycerol.

M. tuberculosis H37Rv, *M. bovis* AN5, and *M. avium* D4 were a gift from Dr. Eliana Roxo, Instituto Biológico, São

Paulo, SP, Brazil.

The plasmid used for expression was pET23a (Novagen). pJEM12, an *E. coli*-mycobacterial shuttle plasmid containing a promotorless ß-galactosidase gene, was a gift from Dr. Brigitte Gicquel, Pasteur Institute, Paris, France (19).

The J774 mouse macrophage cell line was maintained in RPMI medium 1640 (Gibco-BRL) supplemented with 10% fetal calf serum in an incubator with 5% CO_2, at 37ºC.

Cloning and expression strategies

A 1533-bp DNA fragment containing the *plc-a* ORF region was amplified by PCR with primers PET1: 5'-GCAAGgatcCGC AAGCCCAC-3' [positions 434 to 543 of the sequence reported in (3)] and PET2: 5'-GGTGTGGaTcCCTGAGTTGG-3' [1966 to 1947] (positions modified to create *Bam*HI restriction sites are in lower case letters and *Bam*HI sites are underlined). The amplicon was cloned into the *Bam*HI-restricted pET23a vector. Recombinant clones were introduced into *E. coli* BL21(DE3) by electroporation. Isolated ampicillin-resistant colonies were grown in LB-ampicillin liquid medium and PLC-a expression was induced with 0.1 mM isopropylthio-ß-galactoside (IPTG) for 4 h at 37ºC.

A 550-bp fragment containing the initial 125 nucleotides of the *plc-a* coding region was amplified by PCR using primers JEM1: 5'-TCAGCCGgatCCACCAGAGTC-3' [positions 14 to 34] and JEM2: 5'-CCCGGatCCGTAGGCCTTTTC-3' [positions 543 to 563]. The fragment was cloned into *Bam*HI-digested pJEM12. Competent *E. coli* JM101 were transformed with the ligation product by electroporation and recombinant clones were selected on LB-kanamycin agar plates. Recombinant plasmids were purified by minipreparation and introduced into competent *M. smegmatis* by electroporation (20).

The orientation of the cloned fragments was confirmed by restriction analysis and DNA sequencing using an Automated Laser Fluorescence Sequencer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). using the Sequenase kit and primers labeled with FluorePrime fluorescein Amidite (Amersham Pharmacia Biotech).

Localization and purification of recombinant PLC-a

After induction with IPTG, recombinant *E. coli* BL21(DE3) was collected by centrifugation at 3000 *g*, washed twice with phosphate-buffered saline (PBS) and resuspended in 1/50 volumes PBS. Bacteria were sonicated with a Branson Sonifier 450 (Branson Sonic Power Co., Danbury, CT, USA) with pulses of 15-s duration and centrifuged at 12000 *g* for 5 min. Whole extracts, pellet and supernatant were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels. Periplasmic proteins were extracted from 2 ml induced *E. coli* cultures by adding 15 µl of chloroform to the bacterial pellet and incubating at room temperature for 15 min. Seventy-five microliters of 10 mM Tris-HCl, pH 8.0, was added, the solution was centrifuged and the supernatant analyzed by SDS-PAGE.

The recombinant protein was cut from preparative SDS-PAGE gels, eluted with 25 mM Tris-HCl, pH 8.3, 250 mM glycine, 0.1% (w/v) SDS for 24 h, dialyzed against PBS and concentrated with a Speed-vac apparatus (Savant Instruments Inc., Farmingdale, NY, USA).

Animal immunization

Six female BALB/c mice were immunized with the eluted recombinant protein by subcutaneal injections at four sites. Fifty micrograms of protein in PBS, emulsified with incomplete Freund's adjuvant (v/v), was injected per site on days 0, 30, 45 and every two months thereafter.

Immunoblots

Recombinant *E. coli* induced with IPTG was centrifuged, resuspended in loading buffer, and submitted to SDS-PAGE. *M. tuberculosis* H37Rv, *M. bovis* AN5, *M. avium* D4, and *M. smegmatis* mc^2155 were grown in Sauton medium (21) at 37ºC until the surface of the medium was covered. Bacilli were inactivated at 80ºC for 30 min, centrifuged at 3000 *g* for 15 min and resuspended in PBS at a concentration of 1 g/ml. One hundred milligrams of whole cell extract was submitted to electrophoresis on 10% SDS-polyacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose filters (Hybond-C, Amersham), which were first incubated with immune serum (1:100 in PBS plus 0.05% Tween 20) for 2 h and then with goat anti-mouse peroxidase-conjugated antibodies (Promega Corp., Madison, WI, USA) (1:7500 in PBS-Tween 20). The reaction was developed with ECL reagents (Amersham) or with 4-chloro-1-naphthol (Sigma).

Detection of promoter activity

M. smegmatis transformed with recombinant and non-recombinant pJEM12 was grown in 7H9-OADC-kanamycin liquid medium for 2 days at 37ºC in a shaker. Bacteria were distributed on 7H10-OADC-kanamycin-X-gal agar plates and incubated for 2 to 3 days at 37ºC.

Liquid cultures ($A_{600\ nm}$ 0.6 to 1) were centrifuged at 3000 *g* for 15 min and resuspended in 150 µl of lysis buffer from the ß-galactosidase enzyme assay system (Promega). Five microliters of chloroform and SDS at a final concentration of 0.1% were added and the tubes were incubated at 37ºC for 5 min. Assay buffer (150 µl) from the kit was added and the reaction was incubated at 37ºC until the development of yellow color. A standard curve was obtained using ß-galactosidase (Promega). The reaction was stopped with 0.5 ml 1 M Na_2CO_3, samples were centrifuged at 12000 *g* for 5 min and the absorbance at 420 nm was read in an Ultrapec-Plus spectrophotometer (Pharmacia). ß-Galactosidase units (U) were calculated using the formula:

$$\frac{A_{420\ nm} \times 1000}{T \times V \times A_{600\ nm}} = \text{ß-galactosidase (U)}$$

where T = incubation (h) and V = culture volume (ml).

Macrophages cultured on 30-mm culture Petri dishes (Falcon, Becton Dickinson and Co., Lincoln Park, NJ, USA) were infected with recombinant and non-recombinant *M. smegmatis* from liquid cultures at a 10:1 infection rate for 4 h at 37ºC. Extracellular bacteria were killed with gentamicin, 50 µg/ml, for 2 h and 5 µg/ml for an additional 24 h. Cells were washed several times in sterile PBS and fixed with 0.5% glutaraldehyde (Sigma) in PBS for 16 h at 4ºC. Two milliliters of 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 1 mg/ml X-gal were added and the plates were incubated overnight at 37ºC. Macrophages were observed under an Axovert S100 inverted microscope (Zeiss, Jena, Germany) and ß-galactosidase activity was detected as a blue precipitate inside the cells.

Results

To demonstrate expression of native PLC-a in *M. tuberculosis* H37Rv, a specific anti-PLC-a immune serum was produced by immunization of mice with recombinant PLC-a expressed in *E. coli*. To obtain the recombinant protein, the *plc-a* insert was amplified by PCR and cloned into the pET23a expression vector. A 59-kDa insoluble recombinant protein with a poly-histidine C-terminal tail, not present in the periplasmic fraction, was expressed after induction with IPTG (Figure 1). The recombinant protein was insoluble, which made its purification by affinity to nickel ions difficult. Recombinant PLC-a could be solubilized with ionic detergent (sarcosyl) or in 20 mM Tris-HCl, pH 11, 0.5 M NaCl, plus 6 M urea, but subsequent purification on an Ni-nitrilo-triacetic acid resin column (Ni-NTA, Qiagen Inc., Valencia, CA, USA) resulted in very low binding and elution yields (data not shown). Finally, the protein was eluted from acrylamide gels and used for immunization.

Immune sera obtained from 6 animals were tested for anti-PLC-a activity in ELISA against eluted PLC-a. The most reactive serum (limit dilution, 1:50,000) was used in the experiments described below.

The specificity of the anti-PLC-a mouse serum was evaluated using immunoblots against different recombinant and non-recombinant *E. coli*. The immune serum recognized recombinant PLC-a expressed from pET23a and also from previous pGEX-5T constructions (3). No cross-reactivity with *E. coli* proteins was observed (Figure 2).

When this serum was used in immunoblots against extracts of different mycobacteria, a single 49-kDa band was detected exclusively in *M. tuberculosis* H37Rv extracts (Figure 3). No bands were visible in extracts from *M. avium*, *M. bovis* or *M. smegmatis* or in a 4-week *M. tuberculosis* culture supernatant, suggesting that PLC-a is an *M. tuberculosis*-specific non-secreted protein.

To confirm expression of native PLC-a, an upstream *plc-a* ORF fragment was cloned in pJEM12 and a functional promoter present in this insert was identified by detection of ß-galactosidase activity in *M. smegmatis* transformed with recombinant pJEM12. Blue colonies were produced on 7H10-OADC-kanamycin-X-gal agar plates (Figure 4A) and ß-galactosidase production was confirmed by tests performed with the ß-galactosidase enzyme assay system (Table 1). Colonies of *M. smegmatis* harboring a plasmid with the insert in the inverted orientation showed no blue precipitate on X-gal agar plates and showed ß-galactosidase activity similar to that of non-recombinant *M.*

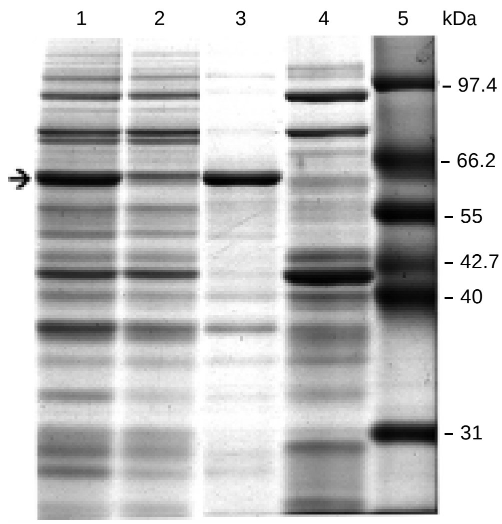

Figure 1 - Polyacrylamide gel (10% acrylamide) stained with Coomassie blue showing the localization of recombinant PLC-a expressed from pET23a. *Lane 1*, *E. coli* BL21(DE3) transformed with pET23a harboring a PLC-a ORF - whole cell extract after sonication. *Lane 2*, Sonicated extract supernatant. *Lane 3*, Sonicated extract pellet. *Lane 4*, Periplasmic fraction proteins. *Lane 5*, Mid-range protein molecular weight marker (Promega). The arrow shows the recombinant protein.

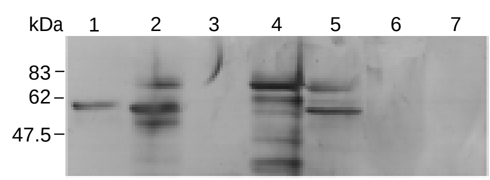

Figure 2 - Immunoblots with anti-PLC-a mouse serum. *Lane 1*, Recombinant protein eluted from the gel. *Lane 2*, Extract of *E. coli* BL21(DE3) transformed with pET23a harboring the complete *plc-a* fragment. *Lane 3*, *E. coli* BL21(DE3) transformed with non-recombinant pET23a. *Lane 4*, *E. coli* JM101 transformed with pGEX-5T harboring the complete *plc-a* ORF fragment. *Lane 5*, *E. coli* JM101 transformed with pGEX-5T harboring the *plc-a* ORF fragment without the 5' region coding for the putative signal peptide. *Lane 6*, *E. coli* JM101 transformed with non-recombinant pGEX-5T. *Lane 7*, Non-transformed *E. coli* JM101. Numbers at left indicate molecular mass in kDa.

Table 1 - ß-Galactosidase expression.

A: *M. smegmatis* mc²155, B: *M. smegmatis* transformed with non-recombinant pJEM12, C: *M. smegmatis* transformed with recombinant pJEM12 containing the insert in correct orientation, D: *M. smegmatis* transformed with recombinant pJEM12 containing the insert in inverted orientation.

Bacteria	$A_{420\ nm}$	ß-Galactosidase (U)
A	0.128	0.95
B	0.056	0.41
C	0.321	2.38
D	0.170	1.26

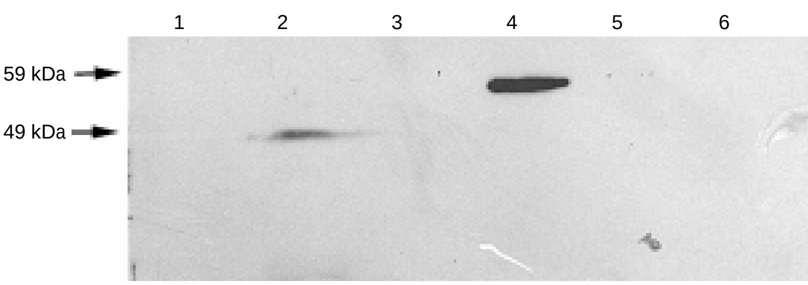

Figure 3 - Immunoblotting with anti-PLC-a serum. *Lane 1*, Four-week *M. tuberculosis* culture supernatant; *lane 2*, *M. tuberculosis* whole cell extract; *lane 3*, *M. bovis* whole cell extract; *lane 4*, recombinant PLC-a; *lane 5*, *M. avium* whole cell extract, and *lane 6*, *M. smegmatis* whole cell extract.

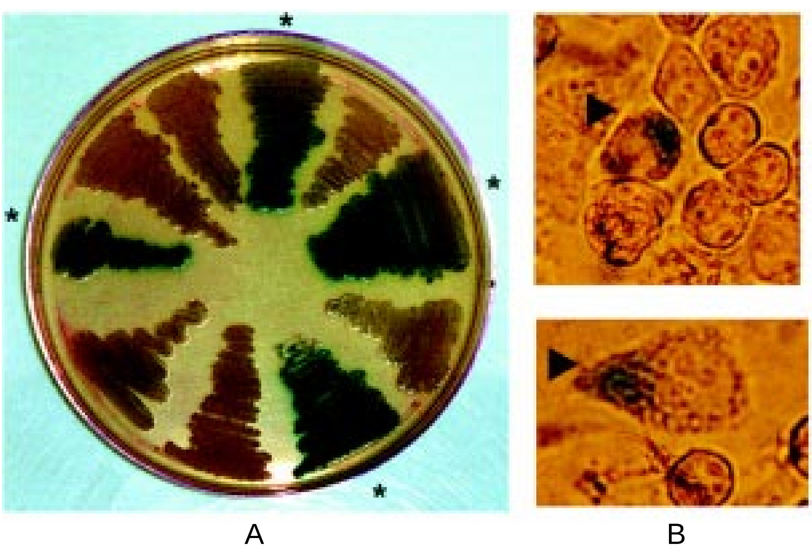

Figure 4 - Expression of ß-galactosidase in recombinant *M. smegmatis*. *A*, 7H10-OADC-kanamycin-X-gal agar plates. *M. smegmatis* mc²155 transformed with pJEM12 containing the insert in correct orientation is indicated by asterisks. *B*, J774 macrophages infected with *M. smegmatis* transformed with pJEM harboring the insert in correct orientation. Arrowsheads show cells with a blue precipitate indicative of ß-galactosidase activity.

smegmatis in the liquid assay. These results substantiated the existence of a promoter in a *plc-a* upstream region, which is active in bacteria grown *in vitro*, indicating that the *plc-a* gene is transcribed in this condition.

Promoter activity in bacteria growing inside cells was evaluated in J774 macrophages infected with recombinant *M. smegmatis*. Bacteria turned blue after substrate addition (Figure 4B) confirming activity of the *plc-a* promoter, and consequently gene transcription, both *in vitro* and *in vivo*. Non-recombinant *M. smegmatis* and recombinant *M. smegmatis* harboring the plasmid with the insert in inverted orientation showed no blue precipitate (data not shown).

The 550-bp fragment contains the first 125 bp from the *plc-a*-coding region. A sequence that matches 9 of 18 residues with an *E. coli* Pho box was previously identified between positions 369 and 386 (3). Four hexamers similar to the *E. coli* TATAAT-10-consensus sequence were identified at positions 89 (TAGTTT), 205 (TAATTT), 212 (TACTGT), and 388 (TAACGT).

These results provide evidence that PLC-a is a non-secreted, constitutively expressed *M. tuberculosis*-specific protein.

Discussion

Multigene families have been described in mycobacteria, i.e., 85 complex, PE-PPE, and PLC genes, and could be a source of antigenic variation representing important elements for bacterial survival. The absence of *plc* genes in other mycobacteria led to the hypothesis of their participation in the pathogenic mechanisms of *M. tuberculosis*. The demonstration of expression of PLC-a shown here is evidence that PLC participates in *M. tuberculosis* metabolism or virulence. The fact that *M. bovis*, lacking the three *plc* genes, can also be pathogenic for men and animals could be explained by the existence of a fourth *plc* gene, *plc-d*, that was deleted from the genome of *M. tuberculosis* H37Rv, but is present in the genomes of other members of the *M. tuberculosis* complex (6).

In order to demonstrate that PLC-a was expressed in *M. tuberculosis*, the protein had to be purified. Purification of native mycobacterial proteins is a technically difficult process because large amounts of bacilli are necessary and the complex wall requires complex and laborious lysis and purification steps. Recent advances in mycobacterial genetics and availability of antigens in recombinant form have provided the opportunity to evaluate, for example, specific immune responses directed against them (22). Expression of mycobacterial antigens in nonpathogenic organisms like *E. coli* and *Saccharomyces cerevisiae* can be enhanced by placing genes under the control of strong promoters, and has the additional advantage of permitting safer working conditions.

We have previously shown that *E. coli* transformed with recombinant pGEX-5T expressing PLC-a had hemolytic activity, suggesting that the protein was active when expressed in this system (3). The GST-fusion PLC-a was insoluble, which impaired purification by affinity chromatography with glutathione or nickel. The recombinant protein could be solubilized with ionic detergents such as sarcosyl, suggesting that interaction with membranes could be an important contribution to the insolubility of this protein. A different PLC-a fragment lacking the first 78 nucleotides, coding for an N-terminal hydrophobic region, was also expressed in pGEX-5T and presented the same solubility impairment, suggesting that the N-terminal region was not responsible for the insolubility (data not shown). Expression in *S. cerevisiae* resulted also in an insoluble protein (data not shown), indicating that this was not a specific phenomenon when *E. coli* was used as a host. Non-fused PLC-a, expressed in pET23a, shown here, was also insoluble. Bacterial phospholipases must gain access to membranes for phospholipid hydrolysis. The precise mechanism by which these enzymes bind to membranes remains unclear, but presumably they possess hydrophobic regions that can be exposed after conformational changes take place upon interaction with the membrane surface (23).

Considering the difficulty in obtaining pure soluble recombinant PLC-a, mice were immunized with the protein eluted from the gels. Recognition of PLC-a by these immune sera was highly specific as shown by immunoblotting against proteins expressed from different vectors in which different *plc-a* fragments were cloned. Figure 2 shows that immune serum recognized more than one band in recombinant clones (lanes 2, 4, and 5). As no cross-reactivity with *E. coli* proteins was observed (lanes 3, 6, and 7), the most plausible explanation for the recognition of more than one band is protein degradation, a frequent observation with recombinant proteins (24).

Absorbance values obtained by ELISA with the immune serum against *M. tuberculosis* whole cells were higher than against *M. smegmati*s whole cells, but the difference was not statistically significant (data not shown). The most reactive immune serum did not detect the protein in the culture supernatant by immunoblot. These results suggest that the native protein can be expressed on the bacterial surface and that it is not secreted. No bands were visible in *M. avium*, *M. bovis* or *M. smegmatis* extracts, confirming the *M. tuberculosis* specificity previously reported (2).

The calculated mass of the protein recognized in *M. tuberculosis* extracts was 49 kDa, as shown in Figure 3. Since GTG at position 438 is the initiation codon, the calculated mass of PLC-a was 56 kDa. Differences in molecular mass (mobility) of the native protein could be partly explained by hydrolysis of the putative signal sequence (3), which would result in a 52-kDa protein.

Detection of promoter activity in the *plc-a* upstream region confirmed gene transcription and represented secondary evidence for expression of native PLC-a in *M. tuberculosis*. Demonstration of promoter activity in bacteria grown *in vitro* and also after infection of macrophages suggests that this is a constitutively expressed protein that could be important for *M. tuberculosis* housekeeping.

Acknowledgments

We acknowledge Rui Pereira Serafim for DNA sequencing.

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