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Further biochemical characterization of *Mycobacterium leprae* laminin-binding proteins

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Abstract

It has been demonstrated that the α2 chain of laminin-2 present on the surface of Schwann cells is involved in the process of attachment of *Mycobacterium leprae* to these cells. Searching for *M. leprae* laminin-binding molecules, in a previous study we isolated and characterized the cationic proteins histone-like protein (Hlp) and ribosomal proteins S4 and S5 as potential adhesins involved in *M. leprae*-Schwann cell interaction. Hlp was shown to bind α2-laminins and to greatly enhance the attachment of mycobacteria to ST88-14 Schwann cells. In the present study, we investigated the laminin-binding capacity of the ribosomal proteins S4 and S5. The genes coding for these proteins were PCR amplified and their recombinant products were shown to bind α2-laminins in overlay assays. However, when tested in ELISA-based assays and in adhesion assays with ST88-14 cells, in contrast to Hlp, S4 and S5 failed to bind laminin and act as adhesins. The laminin-binding property and adhesin capacity of two basic host-derived proteins were also tested, and only histones, but not cytochrome *c*, were able to increase bacterial attachment to ST88-14 cells. Our data suggest that the alanine/lysine-rich sequences shared by Hlp and eukaryotic H1 histones might be involved in the binding of these cationic proteins to laminin.

Key words
- *Mycobacterium leprae*
- Laminin
- Adhesion
- Schwann cell
- Ribosomal protein
- Histone

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Introduction

Mycobacterium leprae peripheral nerve tropism leads to disabilities and deformities observed in over 25% of individuals affected with leprosy (1). In the nerves, the leprosy bacillus is essentially found inside Schwann cells that seem to constitute perfect niches in which this pathogen can survive and replicate (2). The molecular basis of nerve pathogenesis in leprosy continues to be poorly understood. Therefore, there is an urgent need in leprosy research to investigate the molecular mechanisms involved in *M. leprae*-Schwann cell interaction, which would necessarily include the effects of *M. leprae* invasion on the physiology and metabolism of Schwann cells and the ways in which these effects might be related to the progressive and irreversible degenerative process within peripheral nerves. A better understanding of this interaction would inevitable lead to the development and testing of new strategies for the therapy and prevention of nerve damage.

A critical event in the process of Schwann cell invasion by *M. leprae* is the attachment of the bacteria to the cell surface. A recent study (3) showed that this step is essentially dependent on laminin-2 molecules present in the basal lamina that covers Schwann cells (4). Moreover, it was demonstrated that bacterial adherence was mediated by the globular domain of the α2 chain of the laminin-2 molecule (3) and that the membrane laminin receptor α-dystroglycan serves as a Schwann cell receptor for *M. leprae* (5). The binding of laminin-2 to mycobacteria was then analyzed in more detail and the identification, cloning and expression of a laminin-binding protein from *M. leprae* was described (6). This protein, designated ML-LBP21, was demonstrated to avidly bind the recombinant globular domain of the α2 chain-containing laminins and to be abundantly present on the bacterial surface. Moreover, recombinant ML-LBP21 was able to promote the binding and internalization of polystyrene beads into primary Schwann cells, suggesting a role for this protein as adhesin in the process of *M. leprae* nerve colonization *in vivo*.

In a recent report, we isolated, cloned and characterized a laminin-binding protein from the *M. leprae* cell wall (7). This protein was identified as a 21-kDa histone-like protein (Hlp), a highly conserved cationic protein present in other species of mycobacteria. Sequence alignments revealed that Hlp is identical to the ML-LBP21 laminin-binding protein previously described (6). However, we showed that mutation in the *hlp* gene was unable to affect the capacity of mycobacteria to bind to ST88-14 Schwann cells, suggesting that alternative adhesins and/or pathways might be used by mycobacteria during the process of adherence to these cells.

In our previous study, two other cationic proteins, the S4 and S5 ribosomal proteins, were isolated from the *M. leprae* cell wall and identified as potential laminin-binding adhesins (7). In the present study, these proteins were cloned and expressed in *Escherichia coli* and, together with the host basic proteins cytochrome *c* and histones, were evaluated for their capacity to bind laminin and to act as adhesins. The observation that only histone-related proteins were able to enhance *M. leprae* attachment to Schwann cells suggests that common sequences and/or structural features shared by these proteins might be directly involved in their binding to laminin.

Material and Methods

Reagents

Human merosin (a mixture of the α2-containing laminins [isoforms -2 and 4]) was purchased from Gibco BRL (Rockville, MD, USA). Histones from calf thymus and cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, MO, USA). α2-Laminins were labeled with biotin using the FluoReporter Mini-biotin XX protein-labeling kit (Molecular Probes, Eugene, OR, USA) according to manufacturer instructions.

M. leprae and Schwann cells

M. leprae was purified from livers and spleens of infected armadillos as previously described (8). The ST88-14 schwannoma cell line was isolated from a patient with neurofibromatosis type I (9) and kindly donated by Prof. Jonathan Fletcher, Harvard University, Boston. The cells have been maintained in RPMI medium (Gibco BRL) supplemented with 15% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in an incubator at 37°C with 5% CO_2.

PCR and cloning

The primers for the *s5* gene were constructed to correspond to the 5' and 3' ends of

the open reading frame previously identified as *M. leprae s5*. The primers to amplify *s4* were designed based on the reported nucleotide sequence of the *M. tuberculosis s4* open reading frame, since the sequence of the corresponding *M. leprae* gene was not available at that time. The sequence of 5' primers for *s4* and *s5* were 5'-GAGACCATGGCTC GTTACACCGGAC-3' and 5'-GCTACCA TGGCGGCGCAGTCAGC-3', respectively. The 3' primers were 5'-GGGCTTGAGT AGTACTCGACGATCAG-3' and 5'-CCCT GCCTGTTCCCTCACGCGCC-3', respectively. The 5' primers of *s4* and *s5* incorporate *Nco*I sites for cloning into the *E. coli* expression vector pTYB4 (New England Biolabs Inc., Beverly, MA, USA).

The genes were PCR amplified from *M. leprae* genomic DNA with Vent DNA polymerase. The touchdown PCR method was used in a Perkin Elmer Geneamp PCR System 2400 at an initial annealing temperature of 64°C with a 1°C decrease for the first 10 cycles followed by 25 more cycles at 54°C annealing temperature. PCR products were gel purified and digested with the respective restriction enzymes and followed by ligation with *Nco*I- and *Sma*I-digested pTYB4. Ligation products were transformed into electrocompetent *E. coli* DH5α cells. The sequences of the cloned *s4* and *s5* genes were confirmed by automated nucleotide sequencing (ABI; model 377).

Overexpression and purification of S4 and S5 proteins

Overexpression and purification of the *M. leprae* S4 and S5 proteins were performed according to the manufacturer's instruction manual for the IMPACT T7 one-step protein purification system. Briefly, *E. coli* ER2566 (New England Biolabs) harboring the plasmid constructs was grown up to an OD_{600} 0.5 in ampicillin-containing Luria-Bertani medium. IPTG was added to a final concentration of 500 µM and grown at room temperature for another 3 h. Cells were harvested and stored at -80°C. For purification, cells were resuspended in 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 0.5 mM EDTA and 0.5% CHAPS (buffer A) and disrupted by sonication on ice. Debris were removed by centrifugation at 15,000 *g* for 20 min and the supernatant was loaded on a chitin bead column pre-equilibrated with buffer A. Unbound proteins were removed by washing the column with 20 column volumes of buffer A. Finally, 4 column volumes of 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 30 mM DTT were passed through the column and left at 4°C for 72 h for cleavage. Proteins were eluted in 50 mM Tris-HCl, pH 8.0, and dialyzed against PBS (10 mM phosphate buffer, pH 7.2, 0.15 M NaCl) with two changes at 4°C to remove DTT. Purity of the fractions was checked by SDS-PAGE and protein concentration was measured with the Coomassie plus protein assay reagent (Pierce Chemical Company, Rockford, IL, USA) using BSA as standard.

SDS-PAGE and laminin overlay assay

Recombinant Hlp was obtained as previously described (7). Purified rHlp, rS4 and rS5 proteins (0.5 µg/ml) were loaded on each lane and SDS-PAGE was performed under reducing conditions in a 15% gel (10). Proteins were silver stained (11) or transferred onto nitrocellulose membranes and blots were blocked overnight with Tris-buffered saline containing 0.05% Tween 20 (TBS/T) and 2% BSA. Membranes were incubated with biotin-labeled α2-laminins at 2.5 µg/ml in TBS/T-1% BSA for 3 h at 37°C. Blots were washed with TBS/T and incubated with streptavidin peroxidase (0.04 µg/ml) in TBS/T-1% BSA for 30 min at room temperature. The reacting bands were developed with the chemiluminescent Supersignal Substrate (Pierce). The specificity of the reaction was assessed by omitting laminin in control membranes.

Laminin-binding ELISA-based assay

To monitor the binding of soluble laminin to rHlp, rS4 and rS5, 2.5 µg/ml of each protein in 0.1 M carbonate buffer, pH 9.6 (50 µl), was used to coat the wells of polystyrene microplates (Corning, New York, NY, USA). Plates were incubated overnight at 4°C. The wells were then washed with PBS and blocked for 2 h with 100 µl PBS-2% BSA at room temperature. Upon washing with PBS/0.05% Tween 20 (PBS/T), 50 µl of different concentrations of biotinylated α2-laminins were added to the wells and incubation was performed at room temperature for 3 h. The wells were rinsed with PBS/T and incubated with streptavidin-peroxidase (Pierce) at 0.5 µg/ml. Peroxidase activity was revealed with hydrogen peroxide and *O*-phenylenediamine. The reaction was stopped with HCl and read at 490 nm in a TitertekPlus microplate reader (ICN Biomedicals, Inc., Costa Mesa, CA, USA). In control wells, laminin was omitted and specific laminin binding was determined by subtracting the absorbance resulting from nonspecific binding detected in the control wells. Additionally, control wells coated with BSA were included in all binding assays. In parallel, wells coated with the proteins and blocked with 2% BSA were incubated with mouse polyclonal antibodies (1:500 in PBS/T) for 2 h at room temperature. The wells were rinsed with PBS/T and incubated with goat anti-mouse IgG-peroxidase (Sigma) for 1 h at room temperature. Peroxidase activity was developed and read as mentioned above.

***In vitro* adherence assays**

ST88-14 cells (7 x 10^4 cells/ml) were plated onto 24-well plates containing glass coverslips. After 24 h of incubation, the culture was washed three times with a solution of 25 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 0.15 M NaCl (buffer B) and blocked with 1% BSA in buffer B for 1 h at 37°C. FITC-labeled mycobacteria were prepared as described (12) and pretreated with B/BSA buffer containing or not each of the recombinant Hlp, S4, S5 proteins, cytochrome *c* or histones for 1 h at 37°C. The bacteria were added at a multiplicity of infection of 50:1. After incubation for 1 h at 37°C, cells were washed at least six times with buffer B, stained with 50 µg/ml ethidium bromide for 10 min, and fixed briefly with cold methanol. The number of bacteria attached to 100 cells was counted by fluorescence microscopy (Optiphot-2 Nikon, Tokyo, Japan).

Antibodies to *M. leprae* recombinant proteins

To prepare specific antibodies to Hlp and S5, 6-12-week old female BALB/c mice were injected subcutaneously at two sites in the abdomen with 20-40 µg of the proteins in an emulsion with incomplete Freund's adjuvant (0.2 ml per mouse) and boosted 15 days later. Five days later, serum was examined for antibodies to Hlp and S5 by immunoblotting. The mice were boosted again and bled after 10 days.

Results and Discussion

In a previous study, laminin overlay assays with *M. leprae* cell wall allowed the identification of a single 25-kDa band (7). This band was excised from the gel and subjected to in-gel proteolytic digestion with trypsin. The peptides were then analyzed by mass spectrometry (LC-MS-MS) and were found to map with 8 of the gene products recently defined by the *M. leprae* genome project. Since the laminin-binding proteins were never detected by conventional 2-D gel electrophoresis, Hlp and two other proteins, the S4 and S5 ribosomal proteins, were selected as potential laminin-binding proteins based on their extreme pI. Hlp was shown to bind α2-laminins and to greatly enhance the attachment of mycobacteria to ST88-14 Schwann cells (7). In the present study we

investigated the laminin-binding capacity of the other two proteins, the ribosomal proteins S4 and S5.

To analyze the laminin-binding capacity of S4 and S5, the coding regions of their genes were successfully amplified by PCR from *M. leprae* genomic DNA according to the primers and conditions described in the Material and Methods section. The amplified fragments were purified from a 1.0% agarose gel, appropriately digested with restriction enzymes, and cloned into the pTYB4 vector of the IMPACT T7 expression system. The recombinant plasmids were used to transform *E. coli* ER2566 cells for expression. The recombinant S4 and S5 proteins were purified from crude extracts of induced cells by binding the fusion protein to a chitin affinity column followed by the release of the target protein from the column by intein-mediated self-cleavage. The purified proteins were analyzed by silver-stained SDS-15% PAGE showing single bands of 25 and 26 kDa corresponding to S4 and S5, respectively (Figure 1A). The discrepancy between the calculated molecular mass of S4 and S5 (23.5 and 22.6 kDa, respectively) and that estimated by SDS-PAGE is most probably due to the highly basic nature of these proteins, which results in aberrant migration during electrophoresis (13). To analyze the laminin-binding capacity of the recombinant products, proteins were transferred onto a nitrocellulose membrane and probed with biotinylated α2 chain-containing laminins. Figure 1B shows that, similarly to rHlp, rS4 and rS5 bind laminins. Since Hlp, S4 and S5 share the property of being cationic proteins, these results suggest that they were binding to laminin by nonspecific electrostatic interactions.

The recombinant S4 and S5 were tested in two additional assays to evaluate their capacity to bind α2-laminins and act as adhesins. Initially, using an ELISA-based assay, the recombinant proteins were immobilized in wells of microtiter plates and the binding of soluble biotinylated α2-laminins was assessed with the streptavidin peroxidase complex. Soluble biotinylated α2-laminins bound to Hlp in a dose-dependent manner, reaching a plateau at a concentration of 2.5 µg/ml (Figure 2A). In contrast, S4 and S5 were unable to bind α2-laminins even at laminin concentrations of 5 µg/ml. To rule out the possibility that the results obtained could be the consequence of a lower efficiency of the S4 and S5 proteins to bind to microtiter wells, some wells coated with Hlp and S5 were developed with specific polyclonal antibodies produced against these proteins. As shown in the inset of Figure 2A, equivalent amounts of these proteins seem

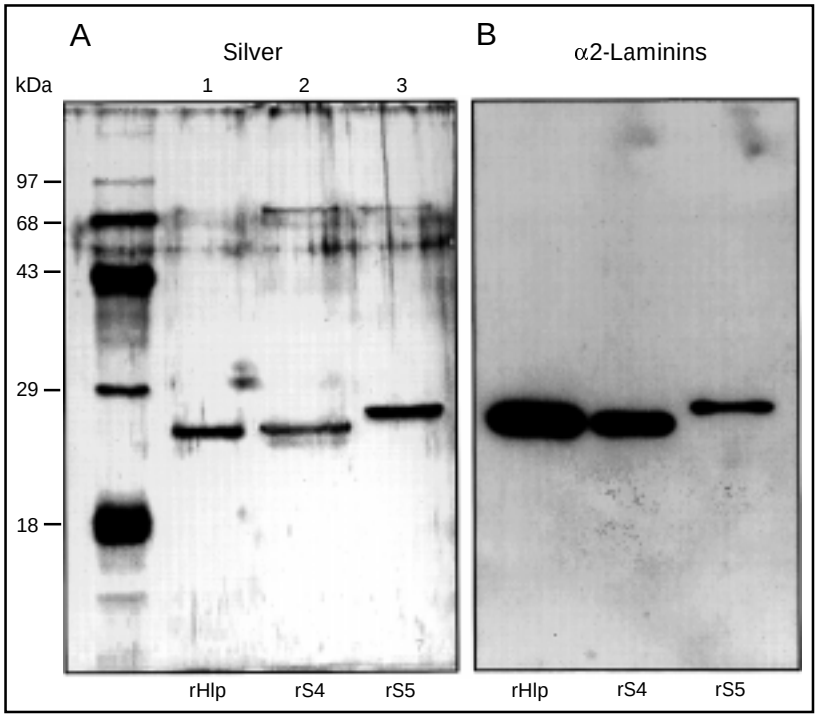

Figure 1 - Expression and laminin-binding capacity of *M. leprae* S4 and S5. The coding regions of the *s4 and s5* genes were amplified by PCR and cloned into pTYB4 and the recombinant plasmids were used to transform *E. coli* ER2566 cells. The recombinant proteins were purified from crude extracts of induced cells by binding the fusion protein to a chitin affinity column followed by the release of the target protein from the column by intein-mediated self-cleavage. *A*, The purified rHlp (lane 1), rS4 (lane 2) and rS5 (lane 3) were analyzed by SDS-15% PAGE with silver staining. *B*, Binding of α2-laminins to rHlp, rS4 and S5. Proteins were separated by SDS-15% PAGE and transferred to a nitrocellulose membrane. Nitrocellulose transfers were overlayed with 2.5 µg/ml biotinylated laminin and developed with the streptavidin peroxidase complex using an enhanced chemiluminescence substrate. Molecular mass markers are indicated on the left.

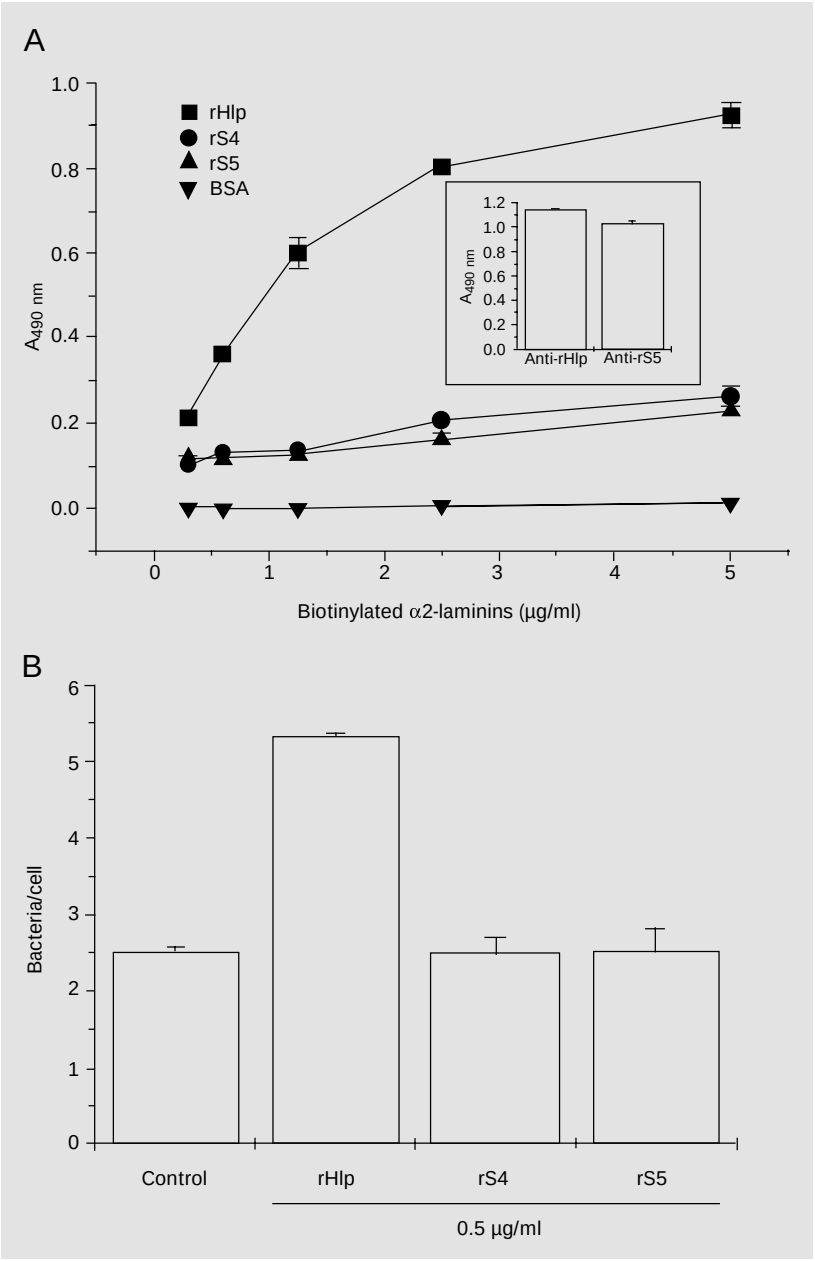

Figure 2 - *A*, Laminin-binding capacity of *M. leprae* S4 and S5 analyzed by ELISA. Microtiter wells coated with *M. leprae* rHlp, rS4, rS5 and BSA were incubated with different concentrations of biotinylated α2-laminins. In the *inset*, microtiter wells coated with *M. leprae* rHlp and rS5 were incubated with mouse polyclonal antibodies (1:500) anti-rHlp and anti-rS5, respectively. Binding is expressed as absorbance units at 490 nm. Data represent the mean ± SEM of five experiments carried out in triplicate. *B*, Recombinant Hlp but not S4 and S5 enhanced *M. leprae* adherence to Schwann cells. Recombinant Hlp, S4 and S5 were purified from a bacterial extract of the recombinant clone using the IMPACT system. ST88-14 cells were cultivated on 24-well tissue culture plates and incubated with FITC-labeled *M. leprae* pretreated with 0.5 µg/ml of rHlp, rS4 and rS5. After 1 h of incubation, unbound bacteria were removed by washing and cells were stained with ethidium bromide and analyzed under a fluorescence microscope. Results are reported as the average number of bacteria per cell. Data represent the mean ± SD of a typical experiment carried out in duplicate. Five experiments were performed with similar results.

to be present in the wells.

To investigate whether S4 and S5 can mediate *M. leprae* adherence to Schwann cells, they were compared with Hlp for their ability to affect bacterial attachment to the ST88-14 human schwannoma cell line. Bacteria were labeled with FITC and pre-incubated with the recombinant proteins for 1 h prior to adding the mixture to confluent cultures of ST88-14. In agreement with the ELISA-based assay, Figure 2B shows that, in contrast to Hlp, S4 and S5 failed to enhance *M. leprae* binding to ST88-14 cells. These results can be at least partially explained by the differences in the conformational states of these proteins in these assays. While in the ELISA-based assay and in the cell adhesion assay the native conformation of these proteins was preserved, overlay assays were always run under denatured conditions. Thus, it seems that S4 and S5 bind laminin only in their denatured forms, but not in their native conformation. These results contradicted our initial hypothesis that the binding of these cationic proteins to α2-laminins would be mediated by nonspecific electrostatic interactions.

To better understand the laminin-binding property of Hlp, testing was done to ascertain whether two basic proteins also bind laminins and can act as adhesins. One of them, a mixture of calf thymus histones containing histone H1, shares sequence homology with Hlp at the C-terminal region of the protein. The other one, cytochrome *c*, was a protein not related to Hlp. Figure 3A shows that both histones as well as cytochrome *c* were able to bind laminin in overlay assays, similarly to Hlp, S4 and S5. However, interestingly, only histones, but not cytochrome *c*, were able to increase bacterial attachment to ST88-14 cells to the levels obtained when the same assay was performed with purified rHlp (Figure 3B).

Mycobacterial Hlp correspond to typical DNA binding proteins and have recently been found to bind DNA *in vitro* (14). How-

ever, they have a unique structure sharing sequence and structural similarities with both prokaryotic Hlp and eukaryotic class H1 histones. Mycobacterial Hlp are about twice the size of other bacterial Hlp. The N-terminal half of *M. leprae* Hlp shares 49.4 and 55.1% identity with HU from *E. coli* and Hb from *Bacillus subtilis*, respectively. The second half of mycobacterial Hlp has an unusual amino acid composition owing to a high alanine and lysine content (22 and 18.5% for *M. leprae* Hlp, respectively), resembling in this way the C-terminal region of eukaryotic class H1 histones. This abundance in alanine and lysine favors the formation of expanded α-helix chains in *M. leprae* Hlp as predicted by the Plotstructure software of the Genetics Computer Group software package (data not shown) that are also present in H1 histones.

In conclusion, we have shown that the *M. leprae* ribosomal proteins S4 and S5 were unable to act as adhesins in the context of *M. leprae*-ST88-14 Schwann cell interaction. On the other hand, the observation that only histone-related proteins were able to enhance *M. leprae* attachment to Schwann cells suggests that common sequences and/or structural features shared by these proteins might be directly involved in their binding to laminin. Therefore, we hypothesize that the alanine/lysine-rich sequences shared by Hlp and H1 histones might be involved in the binding of these proteins to laminin and in their capacity to act as adhesins. Currently, truncated Hlp molecules corresponding to the C-terminal and N-terminal regions of the protein are being produced to test this hypothesis.

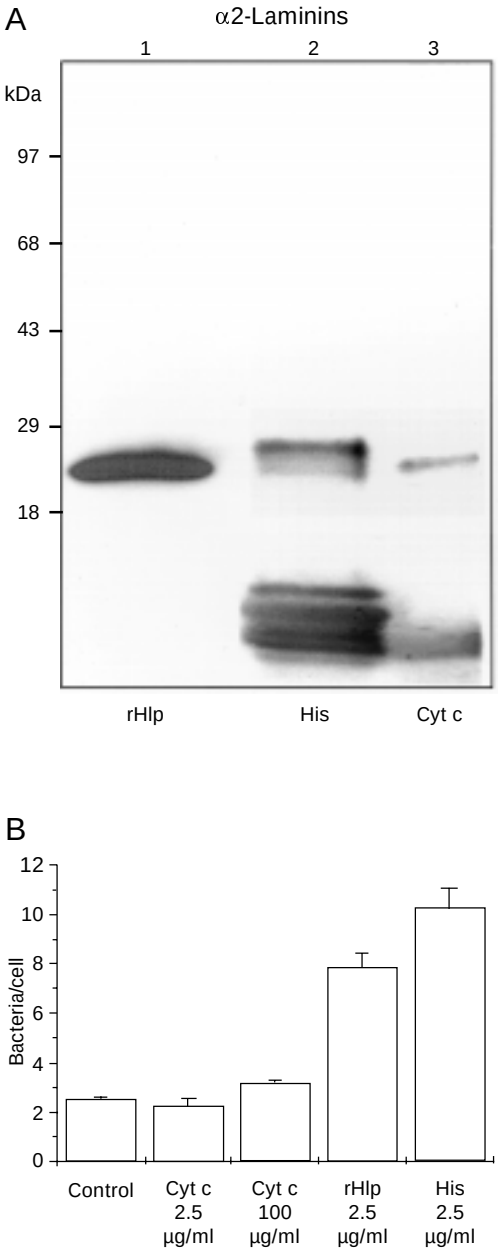

Figure 3 - Histone-related proteins enhanced *M. leprae* adherence to Schwann cells. *A*, rHlp (0.5 µg; lane 1), histones (His, 1 µg; lane 2) and cytochrome *c* (Cyt c, 1 µg; lane 3) were fractionated by SDS-PAGE and proteins were transferred to nitrocellulose membranes. Blots were incubated with 2.5 µg/ml of biotinylated α2-laminins and developed with the streptavidin peroxidase complex using an enhanced chemiluminescence substrate. In control blots laminin was omitted. Molecular mass markers are indicated in kDa on the left. *B*, FICT-labeled *M. leprae* was pre-incubated with 2.5 µg/ml and 100 µg/ml Cyt c, 2.5 µg/ml *M. leprae* rHlp and 2.5 µg/ml His. The number of bacteria bound to the cells was counted by fluorescence microscopy. Data represent the mean ± SD of a typical experiment carried out in duplicate. Two experiments were performed with similar results.

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