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Cloning, expression and characterization of *Mycobacterium tuberculosis* lipoprotein LprF

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ABSTRACT

Lipoproteins are well known virulence factors of bacterial pathogens in general and of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis, in particular. Lipoprotein lipidation between Gram-positive and Gram-negative bacteria differs significantly as these are di- and triacylated, respectively. Little is known about the lipid anchor of mycobacterial lipoproteins. We reported recently that mycobacterial LppX, a lipoprotein involved in synthesis of cell wall components is triacylated, although mycobacteria are classified as GC-rich Gram-positive bacteria. We here exploited the model organism *Mycobacterium smegmatis* for the expression of *Mtb* LprF and characterized N-terminal modifications at the molecular level. LprF is a putative lipoprotein of *Mtb* involved in signaling of potassium-dependent osmotic stress. LprF is extensively modified in a mycobacterium-specific manner by a thioether-linked diacylglyceryl residue with one ester-bound tuberculostearic- and one C16:0 fatty acid and additionally by a third *N*-linked C16:0 fatty acid, and a hexose.

Introduction

Tuberculosis is a major cause of death around the world, with 9.3 million new cases and 1.8 million deaths occurring in 2007, which is the highest rate claimed by a single bacterial pathogen [1]. The causative agent of the disease is *Mycobacterium tuberculosis (Mtb)*, an acid-fast bacillus that is primarily transmitted via the respiratory route. The reasons for the pathogens extraordinary success are diverse: it is slow-growing which makes antibiotic treatment complicated and lengthy, it has a thick waxy cell wall and therefore is resistant to different kinds of mechanical and chemical stress and it evades the immune system by parasitizing the macrophages of its host.

The high immunogenic potential of *Mtb* is based on its unusual cell envelope which is exceptionally rich in lipids, glycolipids and polysaccharides [2]. Among others, uncommon cell wall components like mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan (LAM) and arabinogalactan trigger inflammatory host reactions [2]. On the other hand, *Mtb* is able to subvert the immune response of the host by inhibiting its innate defense by several mechanisms. It prevents inflammasome activation [3], delays phagosome maturation in macrophages [4] and suppresses MHC class II antigen presentation [5]. Suppression of MHC II antigen expression is conferred by several lipoproteins, e.g., the 19 kDa lipoprotein (LpqH) [6].

Lipoproteins are a subclass of proteins found in the cell envelope of all bacteria. Lipoproteins are either di- (in case of Gram-positive bacteria) or triacylated (in case of Gram-negative bacteria) on a highly conserved cysteine located at the N-terminus, which is part of the lipobox [LVI][ASTVI][GAS][C] [7]. By the consecutive action of the three enzymes pre-prolipoprotein diacyl glyceryl transferase (Lgt), prolipoprotein signal peptidase (LspA) and apolipoprotein *N*-acyltransferase (Lnt), lipoproteins are post-translationally modified after translocation over the cytoplasmic membrane. Lgt attaches a diacylglyceryl residue to the universally conserved cysteine in the lipobox by thioether-linkage. Then LspA removes the lipoprotein signal peptide and Lnt attaches a third acyl chain to the amino group of the modified cysteine. Despite the fact that *N*-acylation in *Bacillus subtilis* and *Staphylococcus aureus* was reported [8–10], Lnt was found exclusively in Gram-negative bacteria. Even though *Mtb* is deemed to be a Gram-positive bacterium because of its staining properties, it has been shown recently that mycobacteria have a periplasmic-like structure [11]. Furthermore, it has been shown that mycobacteria express a functional Lnt as at least one lipoprotein, LppX was found to be triacylated [12].

The functions of lipoproteins are manifold; they may be involved in protein export and folding, in antibiotic resistance, in ABC transporter systems, act as substrate-binding proteins and

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are involved in cell signaling. Examples of mycobacterial lipoproteins which have been explored are Mpt83, LppX, LpqW and the 19 kDa lipoprotein. Mpt83 is assumed to be an adhesin and it has been shown that RNA encoding Mpt83 induces protective immune responses against *Mtb* infection [13]. LpqW and LppX have been shown to be key players in synthesis and transport of unique components of the mycobacterial cell envelope. While LppX is involved in translocation of phthiocerol dimycocerosates (DIM) to the outer membrane [14], LpqW has been shown to be essential in the synthesis of the cell wall components phosphatidyl-*myo*-inositol mannoside (PIM) and LAM [15,16]. The 19 kDa lipoprotein has been described as an adhesin [17]. It induces IL-1, IL-2 and TNF-α through TLR2-signaling in macrophages. Overall, lipoproteins are important in host–pathogen interactions and they have a high pathogenic potential which has been proven by disruption of *lspA*, the lipoprotein signal peptidase. An *lspA*-deficient strain of *Mtb* exhibited reduced multiplication in mouse macrophages and reduced number of colony forming units in a mouse model by 3–4 logs [18].

The putative lipoproteins LprF and LprJ of *Mtb* recently have been described to interact with the histidine kinase KdpD in a yeast two-hybrid screen [19]. Both lipoproteins have been suggested to form ternary complexes with the histidine kinase domain of KdpD which in turn seems to be activated after potassium-dependent sensing of environmental osmotic stress and activates a signal transduction pathway.

Even though advances in the past few years contributed to the knowledge of the function of lipoproteins, there is scarcely known anything about the chemical composition of the lipid modifications. Modern tools like mass spectrometry instead of incorporation of radioactive precursors provide support to understand post-translational modifications by lipidation and glycosylation of lipoproteins. Mycobacterial lipoprotein LppX is the first and only lipoprotein characterized at the molecular level [12]. Investigations in other bacteria indicate that lipids of lipoproteins may differ significantly within one species [9,10]. Therefore, we extended our studies on mycobacterial lipoproteins.

In this study, we cloned, expressed and purified LprF of *Mtb* in *Mycobacterium smegmatis*, a non-pathogenic but phylogenetically close relative of *Mtb*. We show for the first time that a putatively membrane-localized lipoprotein of *Mtb* is *N*-acylated and determine the predominant fatty acids by matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS).

Materials and methods

Bacterial strains and growth conditions. Mycobacterium smegmatis strains were cultivated in LB medium or on LB agar. If necessary, gentamicin was added at a final concentration of 5 μg/ml. Strains used in this study were *M. smegmatis* SmR5 [20], a strain carrying a non-restrictive *rpsL* mutation conferring streptomycin resistance, and *M. smegmatis lnt::aph* mutant [12].

Expression of recombinant lipoprotein LprF. LprF of *Mtb* was expressed under control of the 19 kDa antigen (*lpqH*) promoter. The recombinant protein was generated by fusion PCR; fragment 1 contains the sequence of the 19 kDa promoter, fragment 2 contains the functional domain of the LprF lipoprotein including the leader peptide and the sequence of a C-terminal thrombin cleavage site and a HA-epitope tag (hemagglutinin protein), followed by a 6× His-epitope tag. Fragment 1 was amplified with primers 1a (5′-GG**GTTAACGAATTC**TACATTG-3′, bold letters indicate sites of restriction enzymes *Hpa*I and *Eco*RI) and 1b (5′-*CTTGTGAGAT CAAGCCATTCAT*CCTGTGCTCCT-3′, italic letters indicate the linker for *lprF*) from plasmid pMV261-Gm-FusLppX [12]. Fragment 2 was amplified with primers 2a (5′-AGGAGCACAGG*ATGA ATGGCTT-GATCTCACAAG*-3′, italic letters indicate the linker for *lprF*) and 2b (5′-CC**GTTAACGAATTC**TAGTGGTGGTGGTGGTGAGTGGCGTAGT CGGGGACGTCGTAGGGGTAACTACCACGTGGAACTAGTCCCGCCGGG TTCGG-3′, bold letters indicate sites of restriction enzymes *Hpa*I and *Eco*RI, underlined letters indicate thrombin cleavage site, HA- and His-tag) from *Mtb* genomic DNA. Fusion PCR was performed with primers 1a and 2b. The recombinant *lprF* gene was cloned into pMV261-Gm (a shuttle vector replicating in *Escherichia coli* as well as in mycobacteria, derived from pMV261) using the *Eco*RI-restriction sites. The resulting plasmid pMV261-Gm-*lprF* was transformed into *M. smegmatis* SmR5 and *M. smegmatis lnt::aph*.

Preparation of cell extracts and Western blot analysis. Bacteria transformed with pMV261-Gm-*lprF* were cultured in 2 L LB medium for 3 days at 37 °C. The cultures were harvested (4400 rpm, 1 h) and resuspended in PBS containing Complete EDTA-free tablets (Roche). Cells were lysed by three French press cycles (American Instrument Company) at 1.1×10^6 Pa. Extracts were treated with 2% sodium *N*-lauroylsarcosine (SLS) for 1 h at room temperature, and for 16 h at 4 °C thereafter. Soluble and insoluble fractions were separated by centrifugation at 30,000*g* for 1 h at 4 °C. Extracts corresponding to 1–5 μg of total protein were separated by a 12.5% SDS–PAGE and subsequently analyzed by Western blot using anti-HA-antibodies (1:300, Roche).

Protein fingerprinting. Proteins were digested with trypsin and dissolved in 25 μl 0.1% formic acid. Samples were desalted by using a Ziptip C18 column, mixed 1:1 with matrix solution (5 mg/ml α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) in 0.1% trifluoroacetic acid, 50% acetonitrile) and spotted onto the target.

FPLC protein purification. The soluble fraction of cell extracts expressing epitope-tagged proteins was mixed with dilution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4) and loaded on a HisTrap™ HP column (GE Healthcare) previously equilibrated with equilibration buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 0.2% SLS, 20 mM imidazole, pH 7.4). Proteins were eluted applying an imidazole gradient (0.125–0.5 M).

As a further purification step, the column flow through from His-tag purification was dialyzed against equilibration buffer (20 mM Tris-hydroxymethyl-aminomethane, 0.1 M NaCl, 0.1 mM EDTA, pH 7.5), loaded onto an anti-HA-affinity matrix (Roche), washed with a buffer containing 20 mM Tris-hydroxymethyl-aminomethane, 0.1 M NaCl, 0.1 mM EDTA, 0.05% (v/v) Tween 20, pH 7.5 and eluted with column regeneration buffer (0.1 M glycine, pH 2.0).

MALDI-TOF/TOF. 100–200 pmol of purified lipoprotein were prepared and analyzed according to Ujihara et al. [21]. Lipoproteins were digested with AspN. Extracted peptides were dried and dissolved in 5 μl 0.1% trifluoroacetic acid, 50% acetonitrile. Samples were mixed 1:1 with matrix solution (5 mg/ml α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) in 0.1% trifluoroacetic acid, 50% acetonitrile) and spotted onto the target. The MALDI-TOF/TOF mass spectra were recorded on an Ultraflex II MALDI-TOF/TOF instrument with smartbeam laser upgrade (Bruker Daltonics). The laser was set to a repetition rate of 100 Hz and the ion acceleration voltage was 29.5 kV. The mass measurements were performed in the positive ion reflector mode.

Edman degradation. For N-terminal sequencing, proteins were separated by 12.5% SDS–PAGE, blotted onto a PVDF membrane and stained with Coomassie Brilliant Blue (0.03% (w/v) Coomassie Brilliant Blue R-250, 25% (v/v) isopropanol, 10% (v/v) acetic acid) for 5 min. The membrane was destained (10% acetic acid, 35% methanol) and washed once with Aqua B. Braun (Ecotainer®). Visible bands were cut out and analyzed by Edman degradation (Procise 492 cLC protein sequencer, Applied Biosystems), according to the manufacturer's instructions.

Results and discussion

To analyze the putative lipoprotein LprF from *Mtb* concerning the lipoprotein specific modifications with fatty acids we generated the expression vector pMV261-Gm-*lprF*. Plasmid pMV261-Gm-*lprF* was transformed into *M. smegmatis* SmR5 wildtype and an isogenic *lnt*::*aph* mutant strain, lacking a functional apolipoprotein-*N*-acyl-transferase (Lnt). Recombinant LprF from whole cell extracts was purified using the His-epitope and subsequently analyzed by Western blot using anti-HA-antibodies. Depending on the kinetics of the enzymes of the lipoprotein biosynthesis pathway [7], the cell extract may contain different forms of the lipoprotein, the pre-prolipoprotein, prolipoprotein, apolipoprotein and the mature lipoprotein. The theoretically calculated molecular masses for LprF are in the range of 26–30 kDa. However, the apparent molecular masses estimated from an SDS–PAGE may differ significantly. Analysis of the elution fractions from wildtype and *lnt::aph* mutant by Western blot and Coomassie stained SDS–PAGE showed bands with an apparent size of 30 and 35 kDa in both strains (data not shown).

Protein analysis

Fingerprint analysis of the 30 and 35 kDa proteins from wildtype and *lnt::aph* mutant confirmed these proteins as LprF from *Mtb*. For characterization and identification of the N-terminal modifications of LprF only the mature lipoprotein is needed. To characterize the 30 and 35 kDa forms of LprF which have been detected after His-tag purification we applied several methods. Edman degradation provides a suitable method to identify the pre-prolipoprotein, prolipoprotein and apolipoprotein. *N*-acylation blocks Edman degradation. Therefore, MALDI-TOF/TOF MS was used to confirm *N*-acylated LprF forms and to identify modifications at the molecular level.

Edman degradation

The proteins with an apparent size of 35 kDa (Fig. 1A) revealed the sequence MNGLI, which is an LprF sequence starting with the initial methionine of the signal peptide (Fig. 1C) thereby confirming these forms as pre-pro-LprF or pro-LprF in both strains. The proteins with a size of 30 kDa (Fig. 1A) revealed the sequence KKPTT in wildtype and TVVAG in the *lnt::aph* mutant. These determined sequences start at position +3 and at position −5, respectively, in relation to the cysteine (+1) of the lipobox (Fig. 1C). These results indicate that the 30 kDa forms of LprF are not the desired LspA-cleaved LprF but LprF forms cleaved by other proteases. The 30 kDa protein form isolated from the *lnt::aph* mutant and starting with the sequence TVVAG contains potentially diacylglycerol modified cysteine and therefore was subjected to MALDI-TOF/TOF MS analysis. The 30 kDa band isolated from the wildtype strain may be a mixture of triacylated LprF and truncated LprF with the N-terminal residues KKPTT. As triacylated lipoprotein is not accessible to Edman degradation due to the blocked N-terminus, the 30 kDa band from wildtype was also subjected to MALDI-TOF/TOF MS.

MALDI-TOF/TOF MS analysis

The copper chloride stained SDS–PAGE of His-tag-purified LprF showed the same bands detected before on Western blot and Coomassie stained SDS–PAGE (Fig. 1A). AspN-digested peptides from the 30 kDa recombinant LprF of both strains were analyzed with MALDI-TOF/TOF MS to confirm the N-terminal sequence of mature LprF and to characterize the predominant modifications occurring

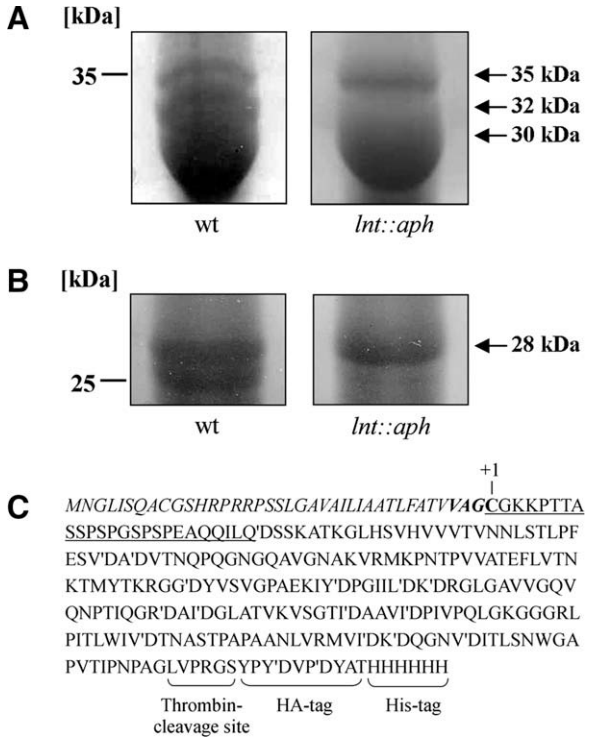

Fig. 1. Copper chloride stained SDS–PAGE and amino acid sequence of purified LprF. (A) HisTrap™-column purified LprF from *M. smegmatis* wildtype and *lnt::aph* mutant. (B) HA-affinity matrix purified LprF from *M. smegmatis* wildtype and *lnt::aph* mutant. Proteins at 25 kDa were identified as histone-like protein HupB from *M. smegmatis*. (C) Amino acid sequence of recombinant LprF. Italic letters indicate the signal peptide cleaved by LspA. Bold letters indicate the lipobox including the conserved cysteine at position +1 modified by Lgt and Lnt. Inverted commas indicate AspN cleavage sites. The modified N-terminal peptide after AspN digestion found in *M. smegmatis* wt and *lnt::aph* mutant is underlined.

in *M. smegmatis* lipoprotein LprF at the molecular level. The calculated monoisotopic *m*/*z* value for the AspN digested unmodified N-terminal peptide of the LspA-cleaved LprF is *m*/*z* = 2496.2 (Fig. 1C). The fatty acids found in mycobacterial phospholipids are palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) and tuberculostearic acid (10-methyloctadecanoic acid) (C19:0) [22]. Since fatty acids of membrane phospholipids are used for *N*-acylation of lipoproteins in *E. coli* [23,24], we calculated the theoretical mass of the N-terminal peptide of LprF with all possible combinations of the above mentioned four fatty acids. Lipoproteins sometimes are glycosylated [25] and putative sites for *O*-glycosylation are also present in the N-terminal AspN-fragment of LprF. Therefore, we also calculated the mass with several hexose modifications. However, no signals corresponding to the free or acylated N-terminal peptides (with or without glycosylations) were found in the mass spectrum (data not shown). So, the analyzed 30 kDa proteins of LprF are most likely the non-modified but truncated proteins, as indicated by the results of the Edman degradation.

Analysis of HA-tag purified LprF

Since the 35 kDa LprF clearly was identified as the (pre-) pro-LprF and the 30 kDa forms were not the LspA-processed LprF we assumed that the expected mature forms were not yet isolated. Concentrating again on the Western blots and Coomassie stained SDS–PAGE from His-tag purification, we found another distinct band on the Western blots from the column flow throughs. This band had an apparent size of 28 kDa possibly corresponding to the LspA-cleaved LprF (data not shown).

Due to the fact that this protein form did not bind to the HisTrap™ HP column, we used the HA-epitope in the recombinant LprF to purify this protein from the flow through fractions. After purification with the HA-affinity matrix, the proteins with the size of 28 kDa in wildtype and *lnt::aph* mutant were observed on Western blot, Coomassie and copper chloride stained SDS–PAGE as well (Fig. 1B).

Edman degradation of HA-tag purified LprF

To determine whether these 28 kDa forms from wildtype and *lnt::aph* mutant are the LspA-cleaved LprF or LprF at all, Edman degradation was performed although for the mature lipoprotein from wildtype N-terminal sequencing is expected to be blocked by the modified N-terminus. The analyses revealed the unique sequence xxKKP in LprF indicating that these proteins start with the cysteine at position +1 (Fig. 1C) thereby confirming these proteins as the LspA-cleaved LprF in the wildtype and the mutant. As the resolution of the SDS–PAGE is not sufficient to separate triacylated and diacylated lipoproteins, the 28 kDa band from wildtype may be a mixture of both forms.

MALDI-TOF/TOF MS analysis of HA-tag purified LprF

For the identification of the lipoprotein specific modifications at the conserved cysteine the AspN digested peptides from the 28 kDa LprF from wildtype and *lnt::aph* mutant were investigated by MALDI-TOF MS. Instead of the $[M+H]^+$ signal at $m/z = 2496.2$ as calculated for the AspN digested, unmodified N-terminal peptide we found a signal at $m/z = 3488.9$ for the modified N-terminal peptide of LprF from wildtype (Fig. 2A). In the *lnt::aph* mutant we found a signal at $m/z = 3250.5$ (Fig. 2B), indicating a smaller size of the N-terminal peptide and thus suggesting an Lnt dependent modification in the wildtype. The difference in molecular mass between the unmodified N-terminal peptide ($m/z = 2496.2$) and the peptide found in the *lnt::aph* mutant ($m/z = 3250.5$) is 754.3 Da indicating a diacylglyceryl modification with ester-linked tuberculostearic acid and C16:0 fatty acid (592.5 Da) and a glycosylation with one hexose (162.2 Da, $\Sigma = 754.7$). The difference in molecular mass of 238.4 Da between wildtype ($m/z = 3488.9$) and mutant indicates an additional modification of the N-terminal peptide with a C16:0 fatty acid in the wildtype.

In order to confirm the modifications of the AspN digested N-terminal peptide of LprF from wildtype ($m/z = 3488.9$) and *lnt::aph* mutant ($m/z = 3250.5$), the structure of the N-terminal peptide was analyzed with MALDI-TOF/TOF MS. In wildtype (Fig. 3A) the ion at $m/z = 3326.7$ corresponds to the cleavage of a hexose ($\Delta = 161.2$ Da). The ion at $m/z = 2698.7$ is the most intense ion and corresponds to the release of the diacylthioglyceryl carrying both, an *O*-linked tuberculostearic and a C16:0 fatty acid ($\Delta = 628.0$ Da). The release of 257.1 Da from the ion at $m/z = 3326.7$ corresponds to the elimination of a C16:0 fatty acid and the release of 370.9 Da from the ion at $m/z = 3069.6$ corresponds to the elimination of a tuberculostearic acid α-thioglyceryl ester. The difference in molecular mass between the MS signal for the N-terminal peptide from wildtype and *lnt::aph* mutant is 238.4 Da which indicates a third acylation with a C16:0 fatty acid in the wildtype peptide in an Lnt dependent manner.

In the *lnt::aph* mutant (Fig. 3B) the ions at $m/z = 3088.8$ and 2461.9 correspond to the release of a hexose ($\Delta = 161.8$) and a diacylthioglyceryl carrying both *O*-linked tuberculostearic and C16:0 fatty acid ($\Delta = 626.9$), respectively. Of note – a loss of the His-epitope in this 28 kDa LprF forms was assumed to be responsible for the failure to extract this proteins by the His-tag purification.

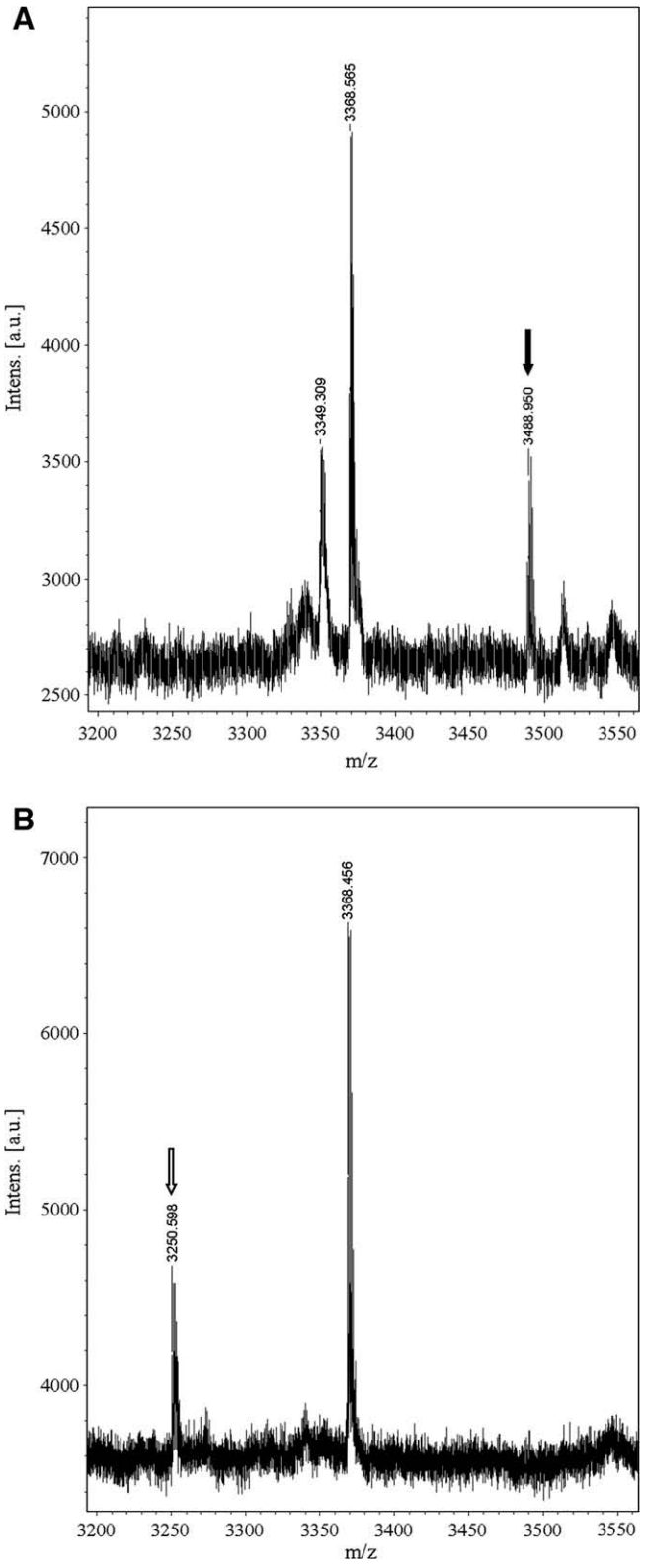

Fig. 2. MALDI-TOF MS analysis of AspN digested purified LprF (28 kDa). MS data of AspN digested LprF peptides from (A) *M. smegmatis* wildtype and (B) *M. smegmatis lnt::aph* mutant. Black arrow indicates triacylated monoglycosylated N-terminal peptide. Open arrow indicates diacylated monoglycosylated N-terminal peptide. The mass at $m/z = 3368.5$ corresponds to an internal AspN peptide of LprF (residue 201–232 of the mature lipoprotein).

But, the presence of the tag was confirmed by MALDI-TOF/TOF MS analysis (data not shown).

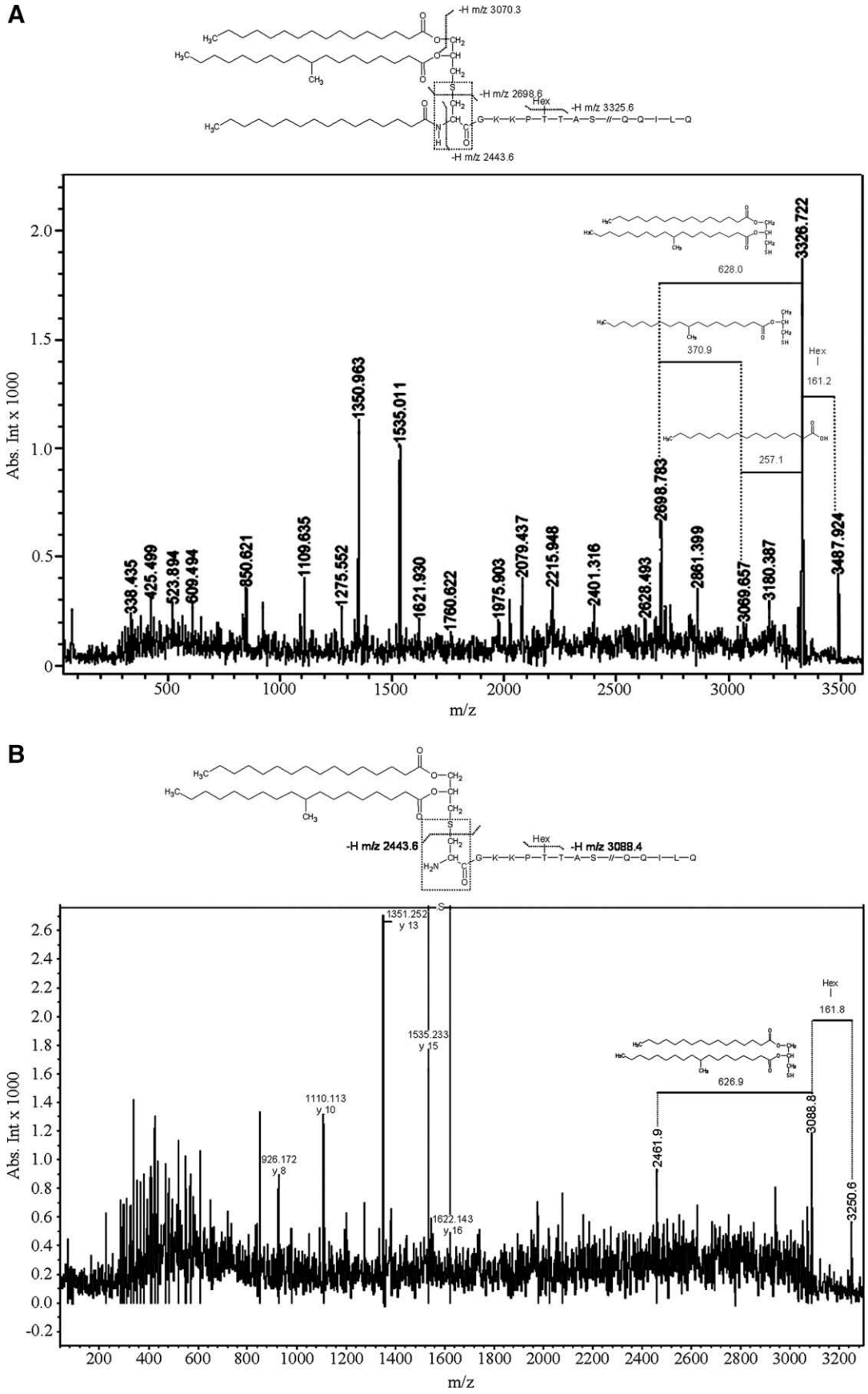

Fig. 3. MALDI-TOF/TOF MS analysis of N-terminal peptides of LprF. MS/MS data of the N-terminal peptides of LprF from (A) *M. smegmatis* wildtype and (B) *M. smegmatis lnt::aph* mutant. Schematic drawings of the structure of the modified N-terminal peptide of LprF are shown in the upper part of each MS/MS spectrum. Dotted frames indicate the modified conserved cysteine at molecular level. Cleavage sites of each identified *m/z* signal are indicated. Eliminated fragments of LprF modifications are depicted in the spectra.

Conclusions

This study shows for the first time the modifications of a putative membrane located lipoprotein of *Mtb* on the molecular level. The LprF of *Mtb* wildtype is a triacylated and glycosylated lipoprotein carrying a thioether-linked diacylglyceryl residue with an ester-bound tuberculostearic- and C16:0 fatty acid and a third C16:0 fatty acid most likely at the amino terminal cysteine residue. These results together with the recent analysis of LppX [16] indicate that *N*-acylation seems to be a common motif in mycobacterial lipoproteins. As expected for the *lnt::aph* mutant the cysteine in LprF is modified with the diacylglyceryl residue, but is missing the third acylation (only glycosylated and modified with the diacylglyceryl residue), thereby confirming lipoprotein acylation by MSMEG_3860 in mycobacteria. Due to the close phylogenetic relation between *M. smegmatis* and *Mtb* the same acylation pattern of LprF in *Mtb* is assumed. Although glycosylation of the N-terminal AspN peptide of LprF was identified, the exact glycosylation site of seven possible sites within the peptide could not be determined.

Fingerprinting and Edman degradation identified several proteins isolated either by His- or HA-affinity purification as LprF. However, the N-terminal lipidated LprF could not be purified by His-tag purification suggesting that the lipidation interferes with His-purification despite the presence of the His-tag which was proved by analysis of the C-terminus (data not shown) or somehow reduces the binding affinity of the protein. Beside the pre-pro-lipoprotein, apolipoprotein and the mature LprF a protein with an apparent size of 32 kDa found only in the wildtype was confirmed by fingerprint as LprF as well, but was not subjected to further analysis in this study. This LprF may result from cleavage by proteases other than LspA.

Acknowledgments

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