Demonstration by Mass Spectrometry that Purified Native Treponema pallidum Rare Outer Membrane Protein 1 (Tromp1) Has a Cleaved Signal Peptide

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Purified native Tromp1 was subjected to mass spectrometric analysis in order to determine conclusively whether this protein possesses a cleaved or uncleaved signal peptide. The molecular masses of Tromp1, three Treponema pallidum lipoproteins, and a bovine serum albumin (BSA) control were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The molecular masses of all of the T. pallidum lipoproteins and BSA were within 0.7% of their respective calculated masses. The molecular mass of Tromp1 was 31,510 Da, which is consistent with a signal-less form of Tromp1, given a calculated mass of unprocessed Tromp1 of 33,571 Da, a difference of 2,061 Da (a 6.5% difference). Purified native Tromp1 was also subjected to MALDI-TOF analysis in comparison to recombinant Tromp1 following cyanogen bromide cleavage, which further confirmed the identity of Tromp1 and showed that native Tromp1 was not degraded at the carboxyl terminus. These studies confirm that Tromp1 is processed and does not contain an uncleaved signal peptide as previously reported.

Treponema pallidum subsp. pallidum, the etiologic agent of venereal syphilis, has for over a decade been known to possess a unique outer membrane containing an extremely low density of membrane-spanning surface-exposed protein (17, 20). It is believed that these T. pallidum rare outer membrane proteins, termed TROMPs (6), are the only surface-exposed antigens on this organism and therefore represent the key surface targets for protective host immune mechanisms which develop during syphilitic infection.

In our previous attempts to identify potential TROMP candidates, two proteins of 31 and 28 kDa were found to be markedly enriched in outer membranes isolated from T. pallidum (5). The 31-kDa protein, termed Tromp1, was found to have properties consistent with those of an outer membrane porin protein, including amphiphilicity following phase separation in the detergent Triton X-114 and electrical conductivity when analyzed in planar lipid bilayers (3). It was also determined that recombinant Tromp1, when expressed, exported, and targeted to Escherichia coli outer membranes, also exhibited porin activity similar to that measured for native Tromp1 (4). Tromp1 has also been found to have 26 to 28% sequence identity to adhesin proteins found in the streptococcal family (4). Tromp1 has also been found to have 26 to 28% sequence identity to adhesin proteins found in the streptococcal family (4). Tromp1 has also been found to have 26 to 28% sequence identity to adhesin proteins found in the streptococcal family (4).

While our studies have shown that Tromp1 is a porin protein, recent studies by Hardham et al. (10) have found that Tromp1 is also part of an operon which possesses similarities to ABC transporter systems and that Tromp1, also called TroA, might be a periplasmic binding protein. This is supported by the fact that the sequence identity to periplasmic binding proteins of these ABC transporter operons is 28% sequence identity between the demonstrated outer membrane location and porin activity of Tromp1 and the suggestion that TroA is a periplasmic binding protein from homology comparisons is an area of research which is currently being investigated. It has also been recently reported by Akins et al. (2) that Tromp1 possesses an uncleaved signal peptide, which these investigators conclude anchors Tromp1 to the inner membrane and accounts for its demonstrated hydrophobicity. Because Tromp1 possesses an N terminus blocked to Edman sequencing, the conclusion that Tromp1 is uncleaved was based upon a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)-based size comparison of native Tromp1 and that generated from an in vitro translation product. Thus, the findings of these studies have resulted in one current view that Tromp1 is not an outer membrane protein, but rather a periplasmic binding protein anchored to the inner membrane by an uncleaved signal peptide.

In order to begin to address the controversy surrounding Tromp1, we have isolated and purified the hydrophobic form of native Tromp1 for mass spectrometric analysis to determine conclusively whether this protein possesses a cleaved or uncleaved signal peptide. The findings reported here demonstrate conclusively that Tromp1 has a cleaved signal peptide and is therefore not anchored to the inner membrane by an uncleaved signal peptide as previously reported (2). Also important in these findings is that Tromp1 is not due to an uncleaved signal peptide, as reported previously, but is rather an inherent property of this protein, which we believe is consistent with its outer membrane location.

Isolation and purity of native Tromp1 and three other T. pallidum hydrophobic proteins. Native hydrophobic forms of Tromp1, the 47-kDa lipoprotein, the MglB homolog lipoprotein (41 kDa), and the TmpC lipoprotein (35 kDa) were isolated from approximately 2 × 1011 T. pallidum cells as follows. T. pallidum subsp. pallidum (Nichols strain) was extracted in phosphate-buffered saline (PBS; pH 7.2) from 20 intrathecally infected rabbits as previously described (13). Approximately 800 ml of treponemal extract was centrifuged twice at 400 × g for 10 min each...
time in order to pellet gross tissue debris and then at 20,000 \times g for 30 min in order to pellet the treponemes. The treponemal pellet was washed in 200 ml of PBS and then recentrifuged at 20,000 \times g for 30 min. The final treponemal pellet was resuspended in 26 ml of ice-cold PBS to which was added 4 ml of ice-cold 10% hydrogenated Triton X-114 (Calbiochem, San Diego, Calif.) to yield a final detergent concentration of 2%. The suspension was next incubated on a rocker at 4°C for 2 h in order to solubilize the outer membrane. After this incubation, the suspension was centrifuged at 20,000 \times g in order to remove *T. pallidum* protoplasmic cylinders and the supernatant was removed and warmed to 37°C for 5 min, which resulted in cloud formation of the detergent. The suspension was then centrifuged at 13,000 \times g in order to yield separated hydrophobic (bottom) and aqueous (top) phases. The hydrophobic phase was recovered (approximately 2 ml), extracted twice with 40 ml of warm PBS, and then centrifuged as described above. The final extracted hydrophobic phase (approximately 2 ml) was then combined with 30 ml of ice-cold acetone, incubated for 2 h at 4°C, and then centrifuged at 10,000 \times g for 30 min to recover precipitated protein. The protein pellet was then subjected to two-dimensional SDS-PAGE as previously described (5). After electrophoresis, proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, Mass.) as previously described (18), stained with 0.2% amido black in water for 20 min, and destained by using distilled water. Protein spots corresponding to Tromp1, the 47-kDa lipoprotein, the MglB lipoprotein, and the TmpC lipoprotein were identified based upon their respective molecular weights and isoelectric points as previously described (15).

In order to elute the proteins from the membrane, the section of the PVDF membrane corresponding to each of these proteins was cut out and placed in 250 \mu l of 2% SDS–1% hydrogenated Triton X-100–50 mM Tris, pH 9.0 (1). The suspensions were incubated on a shaker for 16 h at room temperature and then centrifuged at 13,000 \times g for 10 min. Samples from each of the supernatants were analyzed by SDS-PAGE (12% polyacrylamide) and identified by silver staining (Bio-Rad). The total amount of protein recovered from each of the eluted samples was estimated to be 3 to 5 \mu g.

As shown in Fig. 1, 1/25 (approximately 200 ng) of each sample analyzed showed a single band corresponding to a molecular mass consistent with that of Tromp1 (31 kDa), the 47-kDa lipoprotein, the 41-kDa MglB homolog lipoprotein, and the 35-kDa TmpC lipoprotein. In addition, Tromp1 was further analyzed by two-dimensional immunoblotting using specific anti-Tromp1 serum and enhanced chemiluminescence as previously described (4). As shown in Fig. 2, the amido black-stained immunoblot showed a doublet at 31 kDa (Fig. 2A) corresponding to the molecular mass and pI (6.6) reported previously for Tromp1 (3). The stained 31-kDa doublet reacted specifically with anti-Tromp1 serum (Fig. 2B), confirming that the isolated protein was Tromp1.

**MALDI mass spectrometry of purified native Tromp1.** To obtain an accurate measurement of the molecular mass of Tromp1, its mass spectrum was recorded by using matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry. MALDI-TOF mass spectrometry was performed by using a Voyager RP machine (Perceptive Biosystems, Framingham, Mass.) operating in linear mode. The three other *T. pallidum* membrane lipoproteins and bovine serum albumin (BSA) were also analyzed as controls. For sample preparation, 0.3 \mu l of chloroform–methanol-precipitated protein (21) (1 to 10 pmol) dissolved in 60% HCOOH (2 to 4 \mu l) was mixed with 0.5 \mu l of a 20-mg/ml matrix solution (2,5-dihydroxybenzoic acid; Al-
For CNBr treatment of native and recombinant Tromp1, chloroform-methanol-precipitated protein pellets were dissolved in 35 μl of a 1-g/ml saturated solution of CNBr (Sigma, St. Louis, Mo.) in 90% formic acid (Fisher, Fair Lawn, N.J.) to which was added 15 μl of water. A final CNBr/Met molar ratio of 500 was exceeded in all digestions. CNBr digestion was carried out for 4 h in the dark at room temperature. The reaction mixture was then dried (SpeedVac), redissolved in 250 μl of 0.1% trifluoroacetic acid (Pierce, Rockford, Ill.), and dried again. For MALDI-TOF mass spectrometry, 0.3 μl of the CNBr-digested samples (1 to 2 pmol) dissolved in 60% HCOOH (2 to 4 μl) was mixed with 0.5 μl of a 10-mg/ml matrix solution (α-hydroxycinnamic acid; Aldrich) in 0.1% trifluoroacetic acid–70% CH₃CN and dried immediately on the MALDI plate. Bovine insulin was used as a standard.

Table 2 summarizes the results of MALDI-TOF mass spectrometric analysis of CNBr-treated recombinant and native Tromp1. Central to the interpretation of the mapping experiments is the appearance of peptides derived from the N and C termini of the protein. The C-terminal peptide (peptide 8) was observed in both cases, providing conclusive evidence that any processing was not at the C terminus. Internal peptides (peptides 3, 4, and 6) were well represented in both proteins, proving that the protein analyzed was Tromp1. In addition, the set of the native Tromp1 peptides used to search the National Center for Biotechnology Information protein databases was found to match only Tromp1 and TroA, confirming that the peptides analyzed were from Tromp1. In native Tromp1, N-terminal data set peptides with masses agreeing well with those of peptide 2 and hybrid peptide 2/3 were observed, whereas only peptide 2 was observed in the recombinant protein. In the case of native Tromp1, the masses of the peptides that were observed support a model with cleavage between residues 19 and 20 generating a novel set of CNBr fragments that were not observed in the map of the uncleaved protein.

The results presented here, in contrast to the previous report by Akins et al. (2), conclusively demonstrate that Tromp1 has a processed and cleaved signal peptide. Mass spectrometry analysis of native Tromp1 resulted in a molecular mass of 31,510 Da, consistent with a processed form of this protein given a calculated mass of unprocessed Tromp1 of 33,571 Da, a difference of 2,061 Da, which is the average size of a 19-residue signal peptide (19). This result was also reproducible whether an internal BSA protein control was included in or excluded from the sample containing Tromp1. Further, mass spectrometry peptide analysis of CNBr-treated Tromp1 con-
firmed that the sample tested was Tromp1 and that it was not degraded from the carboxy terminus. In addition, three *T. pallidum* lipoproteins, isolated the same way as Tromp1 from the hydrophobic phase of a Triton X-114 detergent extract, were also analyzed in order to confirm the validity of the molecular mass results obtained for *T. pallidum* proteins isolated by this method. The molecular masses obtained for the three lipoproteins were all within 0.7% of their calculated values given the addition for each of these lipoproteins of a covalent association of glycerol and three molecules of the fatty acid palmitate. Therefore, mass spectrometry analysis, which provides the most reliable assessment of molecular mass, has proven that native Tromp1 possesses a cleaved signal peptide.

Of the three potential leader peptidase I cleavage sites present at the carboxy terminus of the Tromp1 signal peptide, which include (from the N to the C terminus) threonine-histidine-alanine (THA), alanine-phenylalanine-glycine (AFG), and alanine-alanine-alanine (AAA), theoretical cleavage following THA results in a calculated molecular mass of the mature protein having the closest agreement with the mass spectrometry result of native Tromp1 (1% error). THA is also the cleavage site predicted for the Tromp1 signal peptide by the SignalP analysis program (14). However, given that a mass accuracy of 0.1% is expected for the MALDI-TOF technique, prediction of the cleavage site based upon this level of accuracy would place the cleavage site N terminal to the threonine of a Gly residue.
the THA motif. It is unlikely that this is the actual N terminus of the mature protein because this would require the upstream sequence of threonine-glycine-phenylalanine to be recognized as the cleavage motif, which is not a classic leader peptidase I cleavage recognition site (19).

Purified native Tromp1 and a signal-less form of recombinant Tromp1 were also analyzed in this study by mass spectrometry following CNBr cleavage. The intact-protein mass data combined with the CNBr mapping data provide overwhelming evidence that the observed protein is indeed Tromp1 and that again it is cleaved to a significantly shorter length than that predicted from the uncleaved protein. CNBr mapping data can be accommodated with cleavage between amino acids 19 and 20 or thereabout, bearing in mind the N-terminal modification implied by blockage to Edman sequencing. Definitive structural assignment of the N terminus of Tromp1 requires further biochemical analyses. Central to these analyses will be electrospray-ionization mass spectrometry, which has recently been applied to membrane proteins (23) and has, indeed, been used to measure the molecular weight of recombinant Tromp1 (22). Resolution by electrospray-ionization mass spectrometry should solve the heterogeneity of Tromp1 apparent in gels.

In the concluding study by Akins et al. (2), their conclusion that Tromp1 possesses an uncleaved signal peptide was used to explain the hydrophobicity of Tromp1 and suggest its anchoring to the inner membrane. In contrast, our latest studies have indicated that the hydrophobicity of Tromp1 is due to reasons other than an uncleaved signal peptide. Further, our previous studies have shown that treatment of T. pallidum with low concentrations of the nonionic detergent Triton X-114, which completely solubilizes the T. pallidum outer membrane without solubilizing the inner membrane (7, 16), resulted in the complete release of Tromp1 with no residual detection of Tromp1 in the inner membrane proteolipid cylinder complex (3). Such findings are consistent with the idea that this hydrophobic protein has an outer membrane origin rather than being anchored to the inner membrane.

It was also reported by Akins et al. (2), who used a purified recombinant, that Tromp1 did not show any porin activity by the liposome swelling assay (8). Similarly, we have also found that with the exception of recombinant Tromp1 targeted to E. coli outer membranes (4), no soluble recombinant form of Tromp1 tested has shown porin activity when planar lipid bilayers were used. It should be emphasized, however, that this is in direct contrast to purified native Tromp1, which we have found to have consistent and reproducible porin activity following isolation by isoelectric focusing (3) and more recently by fast-performance liquid chromatography (9). We believe that the difference in this demonstrable porin activity between the native and recombinant proteins may be due to conformation.

In summary, the findings presented in this study conclusively show that native Tromp1 does, indeed, possess a processed signal peptide and is therefore an exported protein. These findings, therefore, indicate that Tromp1 is not anchored to the inner membrane and support the possibility that Tromp1 is a bona fide outer membrane protein of T. pallidum.

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REFERENCES


