Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain

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Abstract

A novel antigenic protein of pathogenic Leptospira, Loa22, was identified by using the PhoA fusion method followed by immunoblotting with convalescent mouse sera. Loa22 was shown to be a lipoprotein having a C-terminal OmpA consensus domain. Loa22 was detected among pathogenic leptospires but not among non-pathogenic leptospires, suggesting the possible involvement of this protein in virulence. The results of three different experiments suggested that Loa22 is located in the outer membrane and a small portion is exposed on the cell surface. Thus, Loa22 may be a candidate for a novel vaccine against infection with pathogenic leptospires.

Keywords: Leptospira; Antigen; Lipoprotein; OmpA

1. Introduction

Leptospira is the causative agent of leptospirosis, an important zoonotic disease throughout the world. There are more than 230 serovars among the pathogenic leptospires [1]. The variation in the carbohydrate moiety of lipopolysaccharide (LPS) accounts for the antigenic diversity of pathogenic leptospires. It has been well demonstrated that leptospiral LPS is a serovar-specific protective immunogen [1]. In contrast to LPS, proteins extracted from a pathogenic Leptospira can confer protective immunity against the challenge with a heterologous serovar strain of Leptospira in an experimental animal model [2]. This result suggests that leptospiral protein(s) can be candidates for a new vaccine that prevents infection with a broad spectrum of Leptospira. Some outer membrane proteins of Leptospira, lipoproteins LipL32 (also known as Hap1), LipL41 and the porin OmpL1, have been found to be protective immunogens that are conserved among various pathogenic leptospires [3–7].

In this study, we identified a novel lipoprotein with a C-terminal OmpA domain, Loa22, which reacts with convalescent mouse sera and is highly conserved among pathogenic leptospires.

2. Materials and methods

2.1. Leptospira strains and culture conditions

Leptospira interrogans serovar manilae strain UP-MMC and Leptospira reference strains were cultivated in liquid modified Korthof medium with 10% rabbit serum at 30°C [1,8].

2.2. Construction of PhoA fusion library

Genomic DNA was prepared from L. interrogans UP-MMC using a cetyltrimethylammonium bromide precipitation method [9]. The DNA was partially digested with Sau3AI, purified through sucrose gradient centrifugation, and fragments ranging from 0.5 to 4 kb were isolated and ligated to BamHI-digested pJDT1, pJDT2 and pJDT3. These vectors contain a phoA gene lacking a signal peptide in a different reading frame with respect to the BamHI site [10]. Escherichia coli JM109 was used as the host strain to make a PhoA fusion library. The transformants were
plated on LB agar containing 100 µg ml⁻¹ of ampicillin and 40 µg ml⁻¹ of 5-bromo-4-chloro-3-indolyl phosphate (XP: Sigma). DNA sequencing was performed by the di-deoxyribonucleotide chain termination method using the Big-Dye Terminator Cycle Sequencing Ready Reaction kit ver. 2.0 (Applied Biosystems).

2.3. Convalescent mouse sera and immunoblotting experiments

Nine-week-old C3H/HeJ mice were inoculated intraperitoneally with 1 × 10⁷ UP-MMC. The mice were killed on the 28th day after challenge, and their sera were harvested for immunoblotting experiments [8]. All animal experiments were approved by the animal research committee of the National Institute of Infectious Diseases.

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [11] and Western blotting [12]. The blotted membrane was incubated in 900-fold diluted convalescent mouse sera or in 1000-fold diluted rabbit anti-E. coli PhoA serum followed by incubation in horseradish peroxidase-conjugated secondary antibody. Positive signals were detected using the ECL Western blotting detection system (Amersham Biosciences). According to the PhoA activity and the reactivity with both anti-PhoA serum and convalescent mouse sera, one clone (PA-2) was selected for further characterization.

2.4. IPCR

The 3'-terminal end of the loa22 gene was amplified by the method of IPCR [13]. PCR was performed with Taq DNA polymerase (Ex-Taq, TaKaRa) using ClaI-digested/self-ligated genomic DNA of L. interrogans UP-MMC as the template, and the sense primer (5'-GGTTGTGCGGAAGTCG-3') and the antisense primer (5'-GGGGTTTGGTATCAGAGTGT-3').

2.5. Expression and purification of recombinant GST/Loa22 fusion proteins

The loa22 gene was amplified with the GeneAmp High Fidelity PCR System (Applied Biosystems) using the upstream primer Loa22F/GST (5'-GGGTTTGGTATCAGAGTGT-3') and the downstream primer Loa22R/GST (5'-GGGTCAAGAAGATCCTGAATCTG-3'). These primers contain a BamHI site and a XhoI site, respectively, near their 5' ends (underlined). L. interrogans UP-MMC genomic DNA was used as the template. The PCR products were purified with a High Pure PCR product purification kit (Roche), digested with BamHI and XhoI, and cloned into the GST vector pGEX-6P-1 (Amersham Biosciences). To express the GST fusion protein, E. coli JM109 was transformed with the plasmid harboring a gene for GST/Loa22 fusion protein. The GST fusion protein expressed in E. coli was purified using glutathione Sepharose 4B (Amersham Biosciences) according to the supplier's instruction.

2.6. Production of anti-Loa22 serum

The Loa22 protein was cleaved from the GST/Loa22 fusion protein with PreScission protease (Amersham Biosciences), and purified according to the supplier's instruction. Antiserum against Loa22 was raised in mice by injecting 10 µg of purified Loa22 with complete Freund's adjuvant and booster injections of the same amount of protein without the adjuvant 2 and 4 weeks later.

2.7. Labeling of Loa22 protein with [3H]palmitate

[3H]Palmitate radiolabeling was performed as described by Akins et al. [14], except that 1% casamino acids were included in the medium during the cultivation.

2.8. Triton X-114 extraction of L. interrogans

L. interrogans UP-MMC cells were extracted with Triton X-114 (TX114) as described by Zuerner et al. [15], except that 0.5% protease inhibitor cocktail (Sigma) was added during the extraction procedure.

2.9. Surface immunoprecipitation

About 2 × 10⁹ L. interrogans UP-MMC cells were pelleted at 4000×g for 15 min and resuspended in 1 ml of Tris-buffered saline containing 2% bovine serum albumin. The suspension was mixed with 5 µl of heat-inactivated (30 min at 56°C) anti-Loa22 serum, and then gently shaken for 1 h at room temperature. The leptospires were pelleted at 4000×g for 15 min, resuspended in 1 ml of phosphate-buffered saline (PBS) containing 5 mM MgCl₂, pelleted again at 4000×g for 15 min, and then resuspended in 450 µl of Tris–HCl (pH 8.0)–2 mM EDTA–0.5% protease inhibitor cocktail (Sigma). To this suspension, 50 µl of 10% Triton X-100 (TX100) was added, and then the mixture was agitated for 30 min at 4°C. The insoluble material was removed by centrifugation at 16000×g for 20 min. To the supernatant, 50 µl of 2% deoxycholate, 2.5 µl of 20% SDS, and 40 µl of a slurry of Dynabeads protein A (Dynal) were added. This mixture was gently agitated for 1 h at 4°C. The Dynabeads protein A–antibody–antigen complexes were washed three times in 10 mM Tris–HCl (pH 8.0) containing 0.01% TX100, and resuspended in SDS–PAGE sample buffer. Samples were subjected to SDS–PAGE and immunoblotting with anti-Loa22 serum. A control experiment was performed in parallel under the same conditions as described above except that the anti-Loa22 serum was added after solubilization of leptospiral cells with TX100.
2.10. Proteinase K digestion

Proteinase K (PK) digestion was performed as described previously [16,17]. About $5 \times 10^7$ L. interrogans UP-MMc cells were pelleted at 4000 x g for 15 min, resuspended in 1 ml of PBS containing 5 mM MgCl$_2$, pelleted again at 4000 x g for 15 min, and then resuspended in 50 µl of PBS containing 1 mM CaCl$_2$ and 5 mM MgCl$_2$. The suspensions were treated with PK (0.4 mg ml$^{-1}$), 0.01% TX100, PK plus 0.01% TX100, or PBS (as a control), and incubated for 30 min at 37°C followed by the addition of phenylmethylsulfonyl fluoride to 1 mg ml$^{-1}$. Lysates of untreated, PK-treated, TX100-treated, and PK/TX100-treated leptospires were analyzed by immunoblotting as described above.

3. Results and discussion

3.1. Identification of Loa22

In recent years, the identification of immunogenic outer membrane proteins of pathogenic Leptospira has become a major focus of Leptospira research. The expression of proteins as PhoA fusions is a commonly employed genetic strategy for dissecting membrane topology in various prokaryotes including the spirochetes Borrelia and Treponema [14,18]. In this study, we employed this method together with immunoblotting to identify leptospiral outer membrane proteins that react with convalescent mouse sera.

A genomic library of L. interrogans was constructed in pJD1, 2 and 3 plasmid vectors. Among 51 PhoA+ transfectants on XP medium, one clone (PA-2) reacted strongly with convalescent mouse sera on Western blotting (data not shown). Sequence analysis of the DNA insert in clone PA-2 (a derivative of pJD1; pLOAN) revealed the presence of putative -35 and -10 promoter regions and a ribosomal binding site immediately upstream of a part (123 amino acids) of an open reading frame (ORF) fused in-frame to PhoA (data not shown). The entire ORF was designated Loa22 (22-kDa Leptospira lipoprotein with OmpA domain). The hydrophobicity plot and the amino acid sequence (F-T-L-C) in the N-terminus of the predicted Loa22 protein suggested that this protein might be a lipoprotein. In order to confirm that Loa22 is a lipoprotein, E. coli harboring the plasmid pLOAN encoding Loa22/PhoA fusion was radiolabeled in vivo with $^3$H]palmitate. As shown in Fig. 1, the E. coli strain expressed a lipid-modified polypeptide with a molecular mass of 60 kDa on SDS-PAGE (lane 2) which co-migrated with the polypeptide recognized by both anti-PhoA serum (lane 3) and convalescent mouse sera (data not shown).

3.2. Sequence comparison of Loa22 with other proteins

Next, we performed IPCR to obtain the full-length loa22 gene. The amplified DNA fragment contained DNA sequences identical to the insert of the plasmid pLOAN. The entire ORF of the loa22 gene encoded a polypeptide of 195 amino acids with a calculated molecular mass of 20912 Da (accession number AB096686). Analysis of the Loa22 protein sequence using the NCBI database and BLAST revealed that Loa22 contains an OmpA consensus domain conserved in the C-terminus (pfam00691; identity, 36.5% (35/96); similarity, 57.3% (55/96); Fig. 2), while the N-terminus of Loa22 does not show significant similarity with any other proteins. This is the first description of a protein with an OmpA domain in Leptospira. Recently, the whole genome sequence of L. interrogans serovar lai was reported (http://www.chgc.sh.cn/gn/), which shows that there are five kinds of proteins with an OmpA domain in its genome. The carboxy-terminal OmpA consensus domain contains a peptidoglycan-associating motif [19], which is highly conserved in Loa22 (underline in Fig. 2). One of the functions of OmpA is to provide physical linkage between the outer membrane and the underlying peptidoglycan layer, which plays a critical role in the structural integrity of the bac-

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Fig. 1. Lipid modification of the Loa22/PhoA fusion expressed in E. coli and Western blotting with anti-PhoA serum. E. coli JM109 harboring plasmid pLOAN or pJD1 was metabolically radiolabeled with $^3$H]palmitate, and then the bacterial lysates were subjected to SDS-PAGE and Western blotting (lanes 1 and 2). The same protein samples prepared from E. coli JM109 harboring plasmid pLOAN or pJD1 were subjected to SDS-PAGE and Western blotting with anti-PhoA serum (lane 3). Lanes: 1, E. coli harboring pJD1 (vector only); 2 and 3, E. coli harboring the plasmid pLOAN.

Fig. 2. Alignment of the predicted amino acid sequence of Loa22 (accession number AB096686) and the consensus sequence of the OmpA domain (pfam00691). The numbering is based on the sequence of each protein. Identical amino acids are shown in bold letters. The underline indicates the position of the predicted peptidoglycan-associating motif (NX$_2$LSX$_2$RAX$_2$VX$_3$L).
The presence of Loa22 protein among a total of 17 pathogenic and non-pathogenic *Leptospira* strains was examined by immunoblot analysis with anti-Loa22 serum. As shown in Fig. 3, a positive signal reacting with anti-Loa22 serum could be detected at a position similar to that of Loa22 in all pathogenic strains, while no detectable level of the protein was observed in the non-pathogenic strains such as *L. biflexa* and *L. meyeri*. Thus, a strong correlation was observed between pathogenicity and the presence of Loa22, suggesting the involvement of this protein in *Leptospira* virulence.

3.3. Expression of Loa22 in *Leptospira* species

The presence of Loa22 protein among a total of 17 pathogenic and non-pathogenic *Leptospira* strains was examined by immunoblot analysis with anti-Loa22 serum. As shown in Fig. 3, a positive signal reacting with anti-Loa22 serum could be detected at a position similar to that of Loa22 in all pathogenic strains, while no detectable level of the protein was observed in the non-pathogenic strains such as *L. biflexa* and *L. meyeri*. Thus, a strong correlation was observed between pathogenicity and the presence of Loa22, suggesting the involvement of this protein in *Leptospira* virulence.

3.4. Cellular localization of Loa22

Based on the finding that lipoproteins in the outer membrane are partitioned selectively into the TX114 hydrophobic detergent phase [20], the localization of Loa22 was determined by the TX114 extraction method. Leptospiral cells were solubilized with 2% TX114 and the TX114-soluble fraction was further partitioned into two fractions, the aqueous phase and the detergent phase. Each fraction was analyzed by immunoblotting with anti-Loa22 serum. As shown in Fig. 4A, Loa22 existed in the detergent phase fraction but not the aqueous phase fraction (lanes A and D), suggesting the localization of Loa22 in the leptospiral outer membrane. Loa22 in the TX114-soluble fraction before partitioning (Fig. 4A, lane S) was almost intact, but most of the Loa22 in the detergent phase fraction after partitioning was degraded (Fig. 4A, lane D). We tried to prevent degradation by adding protease inhibitors (0.5% protease inhibitor cocktail; Sigma) but failed. This might be due to the existence of leptospiral protease(s) in the detergent phase.

We examined whether Loa22 was located on the cell surface by surface immunoprecipitation and PK digestion. Leptospiral cells labeled with anti-Loa22 serum were solubilized with TX100 and precipitated with protein A-conjugated Dynabeads. The amount of surface-exposed Loa22 was compared with the total amount of Loa22 immunoprecipitated (Fig. 4B, lanes 2 and 3). Only a small amount of the total Loa22 was found in the surface-labeled fraction (Fig. 4B, lane 3), suggesting that a limited amount of Loa22 is exposed on the cell surface. However, the possibility still remained that the antibodies in our antiserum were not directed against surface-exposed epitopes of Loa22. Then, we employed PK digestion to further examine the surface localization of Loa22. PK treatment of the intact cells did not affect Loa22 quantity (Fig. 4C, lanes 1 and 2), while PK treatment after solubilization of cells completely eliminated Loa22 (Fig. 4C, lane 4). This result suggested that the majority of Loa22 is located in the detergent fraction.
under the cell surface. As only a small portion of Loa22 is surface-exposed, the change in the amount of Loa22 after PK digestion might not be detected by Western blotting. Taken together, it was suggested that Loa22 is located in the outer membrane and only a small portion is exposed on the cell surface.

In *Borrelia burgdorferi*, outer membrane lipoproteins, OspE/F/Elp paralogs, OspA and OspC, are located both on the outer membrane surface and in the periplasmic space [21,22]. It has also been shown that these proteins are surface-exposed only in a subpopulation of *B. burgdorferi* [22]. Loa22 may behave like these proteins in *Leptospira*, but little is known about the mechanism of cellular localization of lipoproteins in spirochetes. Although OspA and OspC are partially exposed on the cell surface, these proteins are known to induce a protective effect against *Borrelia* infection [23,24]. Thus, the Loa22 protein may also be a novel vaccine candidate for protection against *Leptospira* infection. Further studies will be required to confirm the significance of Loa22 for the virulence and protective activity.

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References


