Cloning and Expression of an Immunogenic Membrane-Associated Protein of Helicobacter hepaticus for Use in an Enzyme-Linked Immunosorbent Assay

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Received 5 April 1999/Returned for modification 25 May 1999/Accepted 25 June 1999

Helicobacter hepaticus is a bacterial pathogen that causes chronic active hepatitis and inflammatory bowel disease in mice. The purpose of this study was to develop a recombinant antigen-based enzyme-linked immunosorbent assay (ELISA) to detect H. hepaticus-infected mice. A genomic library of H. hepaticus was constructed and was screened with sera from H. hepaticus-infected mice. A 459-bp open reading frame that coded for an 18-kDa immunoreactive protein, MAP18, was identified. The gene had high identity with genes coding for outer membrane proteins of other bacteria, and the predicted amino acid sequence of MAP18 had a putative membrane-trafficking signal sequence and a putative signal peptidase II cleavage site. The recombinant protein was expressed in Escherichia coli as a glutathione S-transferase (GST) fusion protein, GST-MAP18, and purified by affinity chromatography. The 44-kDa fusion protein was detected on Western blots probed with sera from H. hepaticus-infected mice but was not detected on blots probed with sera from mice infected with Helicobacter muridarum or Helicobacter bilis or with sera from mice free of Helicobacter infection. The GST-MAP18 fusion protein was used as an antigen in an ELISA to detect anti-H. hepaticus antibodies in sera from infected mice. This ELISA was compared to an H. hepaticus-specific ELISA that uses a detergent extract of H. hepaticus as the antigen. Sera from mice naturally and experimentally infected with H. hepaticus, H. bilis, or H. muridarum and sera from mice free of Helicobacter infection were evaluated. Both ELISAs performed with a high specificity (98%); however, the detergent extract-based ELISA performed with a higher sensitivity (89%) than the recombinant protein-based ELISA (sensitivity, 66%). These data indicate that H. hepaticus carries a gene that encodes an immunogenic 18-kDa membrane-associated protein; however, antibodies to this protein are not detected in all infected mice.

Helicobacter hepaticus is a helical, gram-negative, motile, microaerobic, bacterial pathogen of mice. It naturally colonizes the lower intestinal tract and can cause chronic active hepatitis or inflammatory bowel disease in a variety of immunocompetent mice (9–11, 13, 23, 24, 31, 39, 41) and immunodeficient mice (6, 8, 23, 40, 42). This bacterium is of concern to the scientific community because of its potential to invalidate studies that use infected mice (13). The hepatic and intestinal pathology induced by H. hepaticus can directly affect research involving these organ systems. Infected mice have abnormal liver function tests, as demonstrated by altered serum bile acid concentrations (39) and elevated levels of serum alanine aminotransferase (10, 11). Rectal prolapse, as a sequela to inflammatory bowel disease, may cause mice to be prematurely removed from an experiment. There is also evidence that H. hepaticus infections can disrupt host immune homeostasis. For example, infected mice mount a prompt and vigorous type 1 immune response against the bacterium (24, 42).

The potential for H. hepaticus to adversely affect scientific studies of mice is considerable, because infections in mouse colonies in the United States and Japan are widespread (30, 35). Since infected mice rarely display clinical signs of disease, accurate and inexpensive diagnostic tests to detect H. hepaticus infections are needed to effectively design and manage plans to eradicate this bacterium from mouse colonies. Diagnostic methods currently used to detect H. hepaticus-infected mice include histopathologic examination of silver-stained liver sections (39), culture of feces or livers (10), PCR analysis of liver, feces, or cecal tissue (3, 4, 27, 32), and enzyme-linked immunosorbent assay (ELISA) of serum (25) or feces (29) to detect anti-H. hepaticus specific antibodies.

Diagnostic ELISAs that use recombinant proteins as antigens to detect antibodies to bacterial pathogens have been described. Several of these recombinant protein-based ELISAs perform with high levels of sensitivity and specificity or function better than immunoassays that use alternative antigen sources (12, 14, 16, 17, 26). Recombinant protein antigens can be advantageous in bacterial ELISAs because they contain defined antigens that potentially have fewer cross-reactive epitopes than other antigen preparations. Also, large amounts of affinity-tagged recombinant proteins produced in prokaryotic or eukaryotic expression systems can be harvested and purified by affinity chromatography. In addition, the availability of recombinant antigens produced from fastidious organisms, such as H. hepaticus, eliminates the need to cultivate the bacteria.

In this report, we describe the identification and cloning of an immunogenic membrane-associated protein of H. hepaticus. This protein was expressed in and purified from Escherichia coli as a glutathione S-transferase (GST) fusion protein and used as an antigen to create an H. hepaticus-specific ELISA. The performance of this ELISA was compared to that of an H. hepaticus-specific ELISA that uses a detergent extract of H.

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**Helicobacter** as the antigen. The recombinant protein-based ELISA was highly specific for detecting *Helicobacter*-infected mice; however, this assay was less sensitive than the detergent extract-based *Helicobacter* ELISA.

**MATERIALS AND METHODS**

Bacteria, plasmids, and sera. An isolate of *Helicobacter* was obtained from an adult male C57BL/6 mouse from an endemically infected colony (39, 41) as previously described (25). The isolate was cultured under microaerobic conditions and was identified as *Helicobacter* based on its ultrastructural morphology and antigenic activity. The results of DNA sequence analysis of the 16S rRNA gene (32). 

**E. coli DH5α MCR (New England Biolabs, Beverly, Mass.) was used for subcloning procedures.** The plasmid pGEX-5X-3 (Pharmacia Biotech), which codes for an N-terminal GST moiety, was used to express fusion proteins in *E. coli*.

The *Helicobacter* genomic library was screened with serum that was produced in adult C57BL/6 mice that were inoculated intraperitoneally with $10^8$ viable *Helicobacter* expressing fusion proteins in Beverly, Mass.) was used for subcloning procedures. The plasmid pGEX-5X-3 was used to construct the genomic library, and the plasmid pUC18 (New England Biolabs, Beverly, Mass.) was used for subcloning procedures. The plasmid pGEX-5X-3 (Pharmacia Biotech), which codes for an N-terminal GST moiety, was used to express fusion proteins in *E. coli*.

**Construction of genomic library.** *Helicobacter* genomic DNA was isolated from cultured bacteria by using Genomic-tip (Qiagen, Inc., Valencia, Calif.) according to the manufacturer's protocol.

**Genomic DNA was partially digested with Sau3AI (New England Biolabs), and DNA was separated on NuSieve GTG low-melting-temperature agarose (FMC, Rockland, Maine). DNA fragments in the 4- to 10-kb size range were recovered with the Qiaex II gel extraction kit (Qiagen). Partially digested genomic DNA was ligated into *Bam*HI-digested Ready-To-Go pUC18 (Pharmacia Biotech), and *E. coli* DH5α MCR was transformed, plated on Luria-Bertani agar plates (33) containing 50 μg of ampicillin per ml and 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and incubated for 14 h at 36°C.

**Screening of genomic library.** Transformed bacteria were selected on nitrocellulose membranes (Nitrotype; Micron Separations Inc., Westborough, Mass.), and the signal was detected as described above.

**Western blot analysis.** Purified GST-MAP18 and GST or sonicated preparations of immunocompetent mice were blotted onto nitrocellulose membranes (Micron Separations Inc., Westborough, Mass.) and exposed to a 1:2,000 dilution of rabbit anti-*Helicobacter* serum (25). The blot was probed with a 1:200 dilution of alkaline phosphatase-labeled secondary antibody (Perry Laboratories, Gaithersburg, Md.). Immunoreactive clones were identified as described above.

**Nucleotide sequence accession number.** The GenBank accession number for the sequence for the 3.2-kb HindIII fragment and the MAP18 gene is AF134212.
RESULTS

Identification of immunoreactive protein. Immunoscreening of 8,000 white clones from the H. hepaticus genomic library with sera from H. hepaticus-infected mice revealed the three immunoreactive clones designated 1A, 1B, and B2, which contained the plasmids p1A, p1B, and pB2, respectively. Western blot analysis of all three positive clones demonstrated an 18-kDa immunoreactive protein when blots were probed with sera from H. hepaticus-infected mice (Fig. 1). The 18-kDa band was not visualized when clones were probed with sera from mice free of H. hepaticus infection (Fig. 1). Thus, subsequent experiments were focused toward identifying an ORF in the H. hepaticus genome contained in the plasmid p1A, p1B, or pB2 that coded for an H. hepaticus-specific 18-kDa immunoreactive protein. Restriction endonuclease digestion of the plasmids p1A, p1B, and pB2 revealed DNA inserts of approximately 8.5, 9.0, and 6.5 kb, respectively. Subcloning and immunoscreening of these plasmids revealed that a gene coding for an 18-kDa immunoreactive protein was located on a 3.2-kb piece of DNA generated by HindIII digestion of p1A. This subcloned plasmid, p1A2, was chosen for further study.

Identification of an ORF encoding an immunogenic protein. The DNA sequence of p1A2 was determined, and four ORFs that had putative ribosome binding sites and coded for predicted proteins of >7,000 Da were identified. The ORF 1 gene, which coded for a predicted 18,836-Da membrane-associated protein (MAP18), and the ORF 2 gene, which coded for a predicted 7,959-Da protein (P8), were generated by PCR, and the amplicons were directionally cloned in frame into the expression vector pGEX-5X-3 (Pharmacia Biotech) to create the plasmids pGEX-5X-3-MAP18 and pGEX-5X-3-P8, respectively. Colony lifts of E. coli harboring the plasmid pGEX-5X-3-MAP18 were immunoreactive when probed with sera from H. hepaticus-infected mice. Colony lifts of E. coli harboring pGEX-5X-3-P8 demonstrated no immunoreactivity when probed with sera from mice infected with H. hepaticus. Because ORF 1 was identified as the gene that coded for the 18-kDa immunoreactive H. hepaticus protein that was being sought, ORF 3 and ORF 4 were not evaluated for the production of immunoreactive proteins.

Gene sequence and predicted translated protein of ORF 1. The 459-nucleotide sequence of the ORF that coded for the 18-kDa immunogenic protein of H. hepaticus, ORF 1, is presented in Fig. 2. The gene had a putative ribosome binding site at positions −6 through −11, a TAA stop codon, and a G+C content of 40.3%. The ORF had a 59.8% identity in a 316-bp overlap with an ORF of the H. pylori genome that codes for a putative 18-kDa peptidoglycan-associated lipoprotein (GenBank nucleotide sequence accession no. AE000619) (37). The gene also had a 61.8% identity in a 338-bp overlap with a gene coding for a peptidoglycan-associated outer membrane lipoprotein of Campylobacter jejuni (GenBank nucleotide sequence accession no. U47617) (20) and had a high sequence similarity with genes coding for outer membrane proteins of other gram-negative bacteria. The predicted translated protein of ORF 1, MAP18, contained 153 amino acids and had a high identity with predicted outer membrane lipoproteins of other gram-negative bacteria. For example, MAP18 had a 48% identity with a lipoprotein of E. coli (GenBank accession no. U81017).

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identity over 104 amino acids with a predicted 18-kDa peptidoglycan-associated lipoprotein of H. pylori (GenBank accession no. AE000619) (37) and had a 49% identity over 107 amino acids with a predicted 18-kDa peptidoglycan-associated lipoprotein of C. jejuni (GenBank accession no. X83374). The N-terminal 19-amino-acid peptide fragment of the predicted translated protein of MAP18 (Fig. 2) was a predicted prokaryotic membrane lipoprotein signal sequence (19, 28, 38) that had a 52% identity with the leader sequence of a 20-kDa membrane-associated lipoprotein of H. pylori (GenBank accession no. AE000645) (7, 21). In addition, the Ser-Val-Gly-Cys sequence at amino acid positions 15 through 18 is a putative cleavage site for a specific lipoprotein signal peptidase, signal peptidase II (15), with the serine at position 19 serving as a probable outer membrane trafficking signal (44) and the cysteine at position 18 serving as a possible site for the protein to be attached to a membrane lipid (15). Thus, it is likely that MAP18 is a membrane-associated protein of H. hepaticus.

Western blotting of recombinant GST-MAP18. Western blots of affinity-purified GST-MAP18 demonstrated a 44-kDa fusion protein that reacted with sera from H. hepaticus-infected mice but did not react with sera from mice infected with H. bilis or H. muridarum or with sera from uninfected mice (Fig. 3). This 44-kDa recombinant protein, GST-MAP18, was composed of the 18-kDa immunoreactive protein of H. hepaticus (MAP18) coded for by ORF 1 fused with the 26-kDa GST protein affinity tag coded for by the expression vector.

Comparison of recombinant-antigen ELISA to detergent extract-based ELISA. The GST-MAP18 recombinant protein was expressed in and purified from E. coli and used as the antigen in an ELISA to detect anti-H. hepaticus specific antibodies in the sera of infected mice. This recombinant-antigen-based ELISA was directly compared to an established H. hepaticus-specific ELISA that uses a detergent extract of H. hepaticus as the antigen (25) (Table 1). The sensitivity and specificity of each ELISA were determined by evaluating sera from multiple strains of immunocompetent mice that were documented by fecal PCR assay to be infected with H. hepaticus or free of Helicobacter infections. Both ELISAs were highly specific (98%); however, the recombinant-protein-based ELISA was 66% sensitive in identifying mice naturally infected with H. hepaticus while the detergent extract-based ELISA was 89% sensitive in identifying these mice. To further compare the sensitivities and specificities of the two H. hepaticus-specific ELISAs, sera from mice that were orally gavaged with H. hepaticus, H. bilis, or H. muridarum were tested. When these sera were evaluated, the detergent extract-based ELISA and the recombinant protein-based ELISA were both 100% specific and 100% sensitive in identifying the H. hepaticus-infected mice. Thus, the recombinant-antigen-based ELISA was less sensitive than the detergent extract-based ELISA in detecting mice naturally infected with H. hepaticus while both assays were equally highly specific.

**DISCUSSION**

In this report we describe the identification and cloning of a gene that codes for an 18-kDa immunogenic membrane-associated protein (MAP18) of H. hepaticus. The protein was expressed in and purified from E. coli as a GST fusion protein, GST-MAP18, and was used as the antigen in an ELISA to detect anti-H. hepaticus specific antibodies in the sera of infected mice. This ELISA was directly compared to an H. hepaticus-specific ELISA that uses a detergent extract of H. hepaticus as the antigen. Both ELISAs performed with high specificity; however, the recombinant protein-based ELISA performed with a lower sensitivity than did the detergent extract-based ELISA in identifying mice naturally infected with H. hepaticus. The decreased sensitivity of the recombinant protein-based ELISA may have resulted from the presence of only a limited number of antigenic epitopes on the single protein antigen and the failure of all infected mice to generate a vigorous antibody response against this protein. Several immunoreactive bands were detected when Western blots of the H. hepaticus detergent extract antigen were probed with sera from H. hepaticus-infected mice (data not shown), suggesting that this antigen contains more immunogenic epitopes than the recombinant-protein antigen. Thus, the increased sensitivity of the detergent extract-based ELISA may result from its ability to identify several different H. hepaticus-specific antibodies.

**TABLE 1. Results for the performance of the two H. hepaticus-specific ELISAs**

| ELISAa | ELISA result | Helicobacter free (n = 41) | H. hepaticusb (n = 44) | H. hepaticus (n = 7) | H. bilisc (n = 7) | H. muridarum (n = 7) |
|--------|--------------|---------------------------|-----------------------|---------------------|-----------------|---------------------|
| Recombinant | Positive | 1 (2) | 29 (66) | 7 (100) | 0 (0) | 0 (0) |
| | Negative | 40 (98) | 15 (34) | 0 (0) | 7 (100) | 7 (100) |
| Detergent extract | Positive | 1 (2) | 39 (89) | 7 (100) | 0 (0) | 0 (0) |
| | Negative | 40 (98) | 5 (11) | 0 (0) | 7 (100) | 7 (100) |

a The recombinant-antigen-based ELISA used the fusion protein, GST-MAP18, as the antigen, and the detergent extract-based ELISA used a detergent extract of H. hepaticus as the antigen. Sera from mice free of Helicobacter infection, naturally infected with H. hepaticus, or experimentally inoculated with H. hepaticus, H. bilis, or H. muridarum were tested.

b Mice naturally infected with H. hepaticus.

c Mice experimentally inoculated with H. hepaticus, H. bilis, or H. muridarum.
that are produced in response to infection. The decreased sensitivity of the recombinant-protein-based ELISA may also have resulted from antigenic variation of MAP18 so that antibodies directed against the MAP18 proteins of other H. hepaticus isolates may not recognize the antigenic epitopes of the recombinant MAP18 protein used in our ELISA. Therefore, potential ways to increase the sensitivity of the ELISA are toclone other antigenic variants of MAP18 or to clone other immunoreactive proteins of H. hepaticus and to combine these proteins with GST-MAP18 to create a multivalent antigen preparation for use in an H. hepaticus-specific ELISA.

Our results suggest that MAP18 is a membrane-associated lipoprotein of H. hepaticus. The gene coding for MAP18, ORF 1, had a high sequence identity with genes coding for outer membrane lipoproteins of other gram-negative bacteria, including a putative 18-kDa outer membrane lipoprotein of H. pylori (37) and an 18-kDa outer membrane lipoprotein of C. jejuni (5), a bacterium from a closely related genus. Also, the predicted amino acid sequence of MAP18 had high identities with the amino acid sequences of these same proteins. In addition, the N-terminal 19-amino-acid peptide fragment of MAP18 contained a prokaryotic signal peptide that predicted that the protein would be trafficked to the bacterial membrane (19, 28, 38). The C terminus of the signal peptide had a putative signal peptidase II cleavage site; signal peptidase II is an enzyme that is involved in cleavage of signal sequences of prokaryotic membrane-transported proteins (15). In addition, the cysteine residue following this cleavage site is a likely point of attachment of membrane proteins to a lipid moiety (15) and the serine at position two of the cleaved sequence has been shown to be an outer membrane trafficking signal in E. coli (44).

In summary we cloned a gene that encodes an immunogenic protein of H. hepaticus. The protein was expressed as a fusion protein and was used as an antigen in an ELISA to detect antibodies to H. hepaticus in sera from infected mice. This ELISA was compared to an H. hepaticus-specific ELISA that uses a detergent extract of H. hepaticus as the antigen. Both assays performed with an equally high specificity; however, the recombinant-protein-based ELISA was not as sensitive in detecting infected mice. This report provides a foundation for the further development of recombinant-protein-based immunassays for the detection of H. hepaticus infections in mice.

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