Identification and Expression of a *Mycoplasma gallisepticum* Surface Antigen Recognized by a Monoclonal Antibody Capable of Inhibiting Both Growth and Metabolism

SHIGETO YOSHIDA,* AYUMI FUJISAWA,* YOSHINARI TSUZAKI, AND SHUJI SAITOH

Life Science Laboratory, Research and Development Center, Nippon Zeon Co., Ltd., 1-2-1 Yako, Kawasaki-ku, Kawasaki 210-8507, Japan

Received 13 December 1999/Returned for modification 7 February 2000/Accepted 26 February 2000

In order to identify antigenic proteins of *Mycoplasma gallisepticum*, monoclonal antibodies (MAbs) against virulent *M. gallisepticum* R strain were produced in mice. MAb 35A6 was selected for its abilities to inhibit both growth and metabolism of *M. gallisepticum* in vitro. The MAb recognized a membrane protein with an apparent molecular mass of 120 kDa. The corresponding gene, designated the mgc3 gene, was cloned from an *M. gallisepticum* genomic DNA expression library and sequenced. The mgc3 gene is a homologue of the ORF6 gene encoding 130-kDa protein in the P1 operon of *M. pneumoniae* and is localized downstream of the mgc1 gene, a homologue of the P1 gene. To assess the characteristics of MG3 protein, all 10 TGA codons in the mgc3 gene, which encode a tryptophan in the *Mycoplasma* species, were replaced with TGG codons, and recombinant fowlpox viruses (FPV) harboring the altered mgc3 gene were constructed. One of the recombinant FPVs was improved to express MG3 protein on the cell surface in which the signal peptide of MGC3 protein was replaced with one from Marek's disease virus gB. These results should provide the impetus to develop a vaccine based on MGC3 protein which can induce antibodies with both growth inhibition and metabolic-inhibition activities using a recombinant FPV.

*Mycoplasma gallisepticum* is the aetiologic agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys (37). The disease is characterized by nasal discharge, respiratory rales, coughing, and airsacculitis. *M. gallisepticum* infection causes reduced feed conversion and egg production, and the outbreaks remain a persistent cause of severe economic loss for broiler and turkey production firms (36). The best solution for controlling this disease may reside in the development of safe and effective vaccines.

An attenuated strain, the F strain, can induce protective immune responses and subsequently improve egg production in vaccinated chickens. However, the F strain is not completely apathogenic for young chickens (25) and turkeys (20), and it may spread to noninfected chickens and turkey farms. Other attenuated strains, ts11 (40) and 6/85 (7) have been used as vaccines in multi-age layers because of lower virulence. These strains, however, confer somewhat less protection than the F strain (1). On the other hand, for an inactivated vaccine, an experimental immunostimulating complex (ISCOM) vaccine consisting of detergent-solubilized *M. gallisepticum* antigens and Quillaja saponin induced protective immunity and significantly reduced lesion scores in the air sac after challenge (31). The success of the inactivated vaccine using the adjuvant suggests that the isolation of specific immunogens responsible for protective immunity may lead to the development of effective vaccines without the adverse side effects associated with the administration of whole organisms.

We have focused on the identification and structural analysis of *M. gallisepticum* surface antigens which are prominent targets of the chicken immune responses and may influence key host interactions (27). The attachment of *M. gallisepticum* to mucosal epithelium of the respiratory tract of birds is thought to be prerequisite for infection and disease (19). Therefore, a vaccine designed to induce inhibition responses to the attachment and the growth of *M. gallisepticum* in vivo should provide protective immunity to the organism.

The present study describes the production of a mouse monoclonal antibody (MAb) that inhibits both growth and metabolism of *M. gallisepticum* in vitro and the identification of an antigen recognized by the MAb. The antigen, designated MG3, was a 120-kDa membrane protein and a homologue of 130-kDa protein encoded by the ORF6 gene, which is a part of P1 operon of *M. pneumoniae* (30). Recently, the 40- and 90-kDa proteins from 130-kDa protein have been shown to be responsible for the tip structure formation associated with P1 (17). Since we demonstrate for the first time that MG3 protein possesses epitopes recognized by MAbs with growth inhibition and metabolic-inhibition activities, few attempts have so far been made to use the 130-kDa protein or its homologues as vaccine candidates. It is of interest to express the mgc3 gene and to determine whether MG3 protein is important as a potential target of humoral responses in chickens. For these purposes, we used a recombinant fowlpox virus (FPV) expression system which has been established as a live viral vector for use of vaccines against avian viruses such as Newcastle disease virus (13, 24) and Marek's disease virus (MDV) (23, 35, 38) in our laboratory. Based on the recombinant FPV technology, MG3 protein expressed by recombinant FPVs was analyzed in chicken fibroblast embryo (CEF) cells.

MATERIALS AND METHODS

Strains and growth conditions. The sources of *M. gallisepticum* strains R, F, S6, and KP13 have been described elsewhere (10, 16). These *M. gallisepticum*...
strains were grown statically at 37°C for 3 days in Chanock's modified medium (5). *M. gallisepticum* strains were filtered cloned according to the recommendations of the Subcommittee on the Taxonomy of Mollicutes (14, 33) and subsequently frozen (28). CEF cells were maintained in H-1640 (Invitrogen-McCoY medium, Life Technologies, Inc., Rockville, Md.) supplemented with 4% calf serum and antibiotics. A large plaque variant of cell culture-attenuated FPV (22) was used as the parental virus from which recombinants were constructed.

Polyclonal antibody production. Mice were immunized subcutaneously with 100 μg of whole *M. gallisepticum* R strain protein emulsified in Freund's complete adjuvant. Three weeks later, the mice were injected intra-peritoneally with the same antigen concentration in Freund's incomplete adjuvant. The next day, peripheral blood was collected, and spleen cells were cultured in Penassay broth (Melbourn R Technologies, Inc., Rockville, Md.), using an established procedure (4). Hybridoma clones were screened by enzyme-linked immunosorbent assay (ELISA) using the whole *M. gallisepticum* R strain (3). ELISA-positive hybridoma clones were used for preparation of *M. gallisepticum*-specific antibody-producing tumors in pristane (2,6,10,14-tetramethylpentadecane)-primed BALB/c mice. Ascites fluids containing MAbs were clarified by centrifugation at 2,000 × g for 10 min and stored at −20°C. The immunoglobulin concentration of each ascites fluid was measured with a IgG kit (EUSA, Quantitation Kit [Bethyl Laboratories, Inc.]). The immunoglobulin class and subclass were typed with the MonoAB-ID EIK kit (Zymed).

SDS-PAGE and immunoblotting. Whole-cell preparations of *M. gallisepticum* strains used in this study were separately suspended at a protein concentration of 1 mg/ml in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.1% bromophenol blue), heated in a boiling water bath for 3 min, and stored at −20°C until used. Mycoplasma proteins were separated by SDS-PAGE and transferred electrophoretically to Immobilon Transfer Membrane (Millipore, Bedford, Mass.) for immunoblotting. The membrane was treated with MAb 30G8 at 1 mg/ml in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer containing 5 mM dithiothreitol, 200 mM NaCl, and 0.1% SDS. The membrane was blocked with 10% nonfat dry milk and 1% Tween 20 in phosphate-buffered saline (PBS) for 1 h, washed with PBS for 15 min, and then blocked with 4% bovine serum albumin in PBS. The cultures were incubated at 37°C for 3 days. To determine the number of CFU, serial 10-fold dilutions in modified Chanock's medium were performed in duplicate. Aliquots of 10 or 100 μl were plated on modified Chanock's agar medium following gentle passage of the cultures through a 0.45-μm filter. After 6 to 7 days of incubation at 37°C, the number of CFU was counted under a stereomicroscope (Leitz).

Metabolic inhibition assay. The complement-independent inhibition of glucose metabolism by MAbs resulting in the reduction of an acidity shift in the medium was used to identify MAb in microtiter plates (11). Twenty-five microliters of MAbs (5 mg/ml) or sera were serially diluted in a 10-fold dilution series. The samples were incubated at 37°C for 3 days, filtered through a 0.45-μm filter, and diluted to 1.2 × 10^6 CFU/ml. Three hundred CFU of the suspension cultures were seeded in the presence of 50 μg of MAbs or serum dilutions of 1:10 to 1:10^5 in volume of 0.3 ml. The cultures were incubated at 37°C for 3 days. To determine the number of CFU, serial 10-fold dilutions in modified Chanock's medium were performed in duplicate. Aliquots of 10 or 100 μl were plated on modified Chanock's agar medium following gentle passage of the cultures through a 0.45-μm filter. After 6 to 7 days of incubation at 37°C, the number of CFU was counted under a stereomicroscope (Leitz).

Flow cytometric analysis. For analysis of the expression of a 120-kDa protein on the cell surface, 10^6 *M. gallisepticum* cells were incubated either with MAb 35A6 or control MAb 1A8N6 (29) for 45 min on ice. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Pharmining, San Diego, Calif.) for 30 min at RT. After a second washing, the cells were analyzed by FACScan (Becton Dickinson, San Jose, Calif.).

Production of polyclonal antiserum. For the preparation of chicken polyclonal anti-*M. gallisepticum* R strain serum, 5-week-old specific-pathogen-free chickens (line M; Institute of Nippon Biological Science, Tokyo, Japan) were immunized subcutaneously in the right thigh with 100 μg of whole *M. gallisepticum* R strain protein emulsified in Freund's complete adjuvant. Two subsequent boosters of the same antigen concentration in Freund's incomplete adjuvant were administered at monthly intervals. Sera were collected 2 weeks after final booster.

Construction and screening of an *M. gallisepticum* genomic DNA expression library. *M. gallisepticum* genomic DNA was extracted as described previously (15). The genomic DNA of *M. gallisepticum* R strain was partially digested with AluI and size-selected (1 to 3 kb) DNA fragments were ligated to Xgt11 arm DNA (Strategene GmbH, Heidelberg, Germany) and packaged using Gigapack gold packaging extract (Strategene) according to the manufacturer's instructions, resulting in a primary genomic library with a size of 10^6 PFU. The library was immunoscreened with chicken polyclonal anti-*M. gallisepticum* R strain serum. Positive clones were pooled and reimmunoscreened with the mouse polyclonal anti-120-kDa-protein serum.

DNA cloning and sequencing. A Xgt11 phage DNA was extracted from a plaque that reacted with the mouse polyclonal anti-120-kDa-protein serum. An insert DNA fragment was amplified from the phage DNA by PCR. To obtain a full-length gene encoding 120-kDa protein, the PCR products were radiolabeled with [α-33P]dCTP and used as a probe for Southern blot analysis (28). DNA fragments identified by Southern blot analysis were cloned into pUC18 by a standard procedure (2). DNA sequencing was performed on double-stranded plasmid by the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Inc., Foster City, Calif.). DNA sequence data was analyzed with the GENETYX-MAC software package, as well as the GenBank and SwissProt databases, for comparison of the 120-kDa protein predicted amino acid sequence with database entries.

Results of recombinant FPVs. Transfector pNZ29RMC3 was constructed by inserting a 3,228-bp BamHI-SphI fragment from pUC-M3S into the BamHI-SphI site of insertion vector pNZ1829R (24). Transfector vector pNZ29RMGC3-S was constructed by replacing the 5′ terminal encoding the MG3C predicted signal sequence with the 5′ terminal encoding the MDV glycoprotein. The 5′-terminal fragment from MDV gB signal sequence was obtained from plasmid pNZ29RMDgB (35) by PCR using primers pgB-1 (5′-CCCCCGAGTTACCATGCACTATTAGG-3′) and pgB-2 (5′-CCCGAGGACTCTCGGATGTACCACTTTTGGT-3′) (a BamHI site is underlined) and pgB-2 (5′-CCCGAGGACTCTCGGATGTACCACTTTTGGT-3′) (a newly created SacI site is underlined). This set of primers had a restriction site flanked by five additional bases to protect the site and facilitate enzyme digestion. Transfector vector pNZ29RMGC3-S was constructed by replacing a 67-bp BamHI-SacI fragment from pNZ29RMGC3 with a 105-bp BamHI-SacI fragment of the PCR products. The procedure for transfection of FPV-infected cells with the transfector vectors by electroporation and generation of recombinant FPVs was described previously (24, 35). The resulting FPVs were designated recFPV-MG3C-S and recFPV-MG3CS. Two recombinant FPVs, (i) Immunoprecipitation. CEF cells were infected with recombinant FPVs at a multiplicity of infection of 5 PFU/cell. Labeling with [35S]methionine and immunoprecipitation with MAbs 35A6 and 34H1 were performed as described previously (38).

(iii) Immunofluorescence. Indirect immunofluorescence was performed on monolayers of CEF cells grown on eight-chambered tissue culture slides (Miles Scientific, Division of Miles Laboratories, Inc., Naperville, Ill.) infected with recombinant FPVs. At 16 h postinfection, cells were fixed with 100% methanol at −20°C for 10 min or with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and then blocked with 4% bovine serum albumin in PBS. The slides were incubated with MAS 35A6 for 1 h at room temperature. Bound MAb was detected with FITC-conjugated goat anti-mouse IgG (Life Technologies, Inc.) by fluorescence microscopy.

Nucleotide sequence accession number. The nucleotide sequence of the mgc3 gene has been deposited with GenBank under accession number AB023292.
TABLE 1. Effects of MAbs and polyclonal antibodies on M. gallisepticum growth and metabolism

<table>
<thead>
<tr>
<th>Antibody added</th>
<th>Growth inhibition*</th>
<th>Metabolic inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(CFU/ml)</td>
<td>(%)</td>
</tr>
<tr>
<td>MAbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1AN86</td>
<td>$2.5 \times 10^7$</td>
<td>100</td>
</tr>
<tr>
<td>30G8</td>
<td>$9.8 \times 10^7$</td>
<td>$-8.8 \times 10^{-6}$</td>
</tr>
<tr>
<td>35A6</td>
<td>$8.5 \times 10^7$</td>
<td>$-1.4 \times 10^{-7}$</td>
</tr>
<tr>
<td>34H1</td>
<td>$3.0 \times 10^7$</td>
<td>$-3.6 \times 10^{-7}$</td>
</tr>
<tr>
<td>Sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mouse serum</td>
<td>$8.7 \times 10^7$</td>
<td>348</td>
</tr>
<tr>
<td>Mouse anti-M. gallisepticum serum</td>
<td>$7.3 \times 10^7$</td>
<td>$-8.8 \times 10^{-6}$</td>
</tr>
<tr>
<td>Mouse anti-120-kDa (MGC3) serum</td>
<td>$4.6 \times 10^7$</td>
<td>$1.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>Normal chicken serum</td>
<td>$1.6 \times 10^7$</td>
<td>640</td>
</tr>
<tr>
<td>Chicken anti-M. gallisepticum serum</td>
<td>$1.5 \times 10^7$</td>
<td>$1.2 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

* a Samples of 250 µl of M. gallisepticum R strain (1.2 × 10⁷ CFU/ml) were cultured in the presence of 50 µg of MAbs per 50 µl or serum dilutions of 1:10 per 30 µl. Following 96 h of incubation at 37°C, aliquots of serial diluted culture medium were plated on modified Chanok’s agar medium. After 6 to 7 days of incubation at 37°C, the number of CFU was counted under a stereomicroscope.

* b % Growth $=[(A - O)/(C - O)] \times 100$, where A is CFU at 96 h of culture incubated in tested antibodies, C is CFU at 96 h of culture incubated in control MAb 1AN86, and O is initial starting CFU at 0 h.

* c Twenty-five microliters of MAbs (5 µg/µl) or sera was serially diluted in microtiter plate. M. gallisepticum R strain (1 CCM/well) in 150 µl of culture medium containing phenol red were added to each well. After 3 days of incubation at 37°C, metabolism inhibition titers were determined by a color shift of medium.

Strong growth inhibition activities. The percentages of cell growth in the presence of these MAbs compared with control MAb 1AN86 are even less than 0%, indicating that these MAbs inhibit cell multiplication completely for 3 days after incubation. The highest metabolic inhibition was obtained in the presence of MAb 35A6. On the other hand, MAbs 30G8 and 34H1 that strongly inhibited M. gallisepticum growth were less effective in inhibiting metabolism of the organism than MAb 35A6. As a positive control, the mouse polyclonal anti-M. gallisepticum R strain serum displayed a modest level of both growth inhibition and metabolic-inhibition activities, whereas normal mouse serum or a control MAb, 1AN86, did not have growth inhibition and metabolic-inhibition activities. MAb 35A6 is an IgG2a isotype, and it recognizes the multiple bands with molecular sizes ($M_c$) of 105 to 120 kDa from a M. gallisepticum lysate (Fig. 1). The prominent band in the complexity of the binding pattern is a 120-kDa protein. Thus, we found MAb 35A6 to be a valuable tool for the identification of immunogenic antigens of M. gallisepticum.

Cloning of a gene encoding 120-kDa protein. In an attempt to identify a gene encoding a 120-kDa protein, a gt11 genomic DNA library of M. gallisepticum was constructed. In an earlier study, no positive clone was obtained by immunoscreening with MAb 35A6 from the library. Since the most outstanding feature in the codon usage of mycoplasma species is that the TGA stop codon is utilized for tryptophan (34), a gene encoding an epitope recognized by the MAb would never be obtained from the library. Of these clones, a single positive clone ($λ#100$), which contained an insertion of 2.8 kb, was obtained by immunoscreening with the mouse polyclonal anti-120-kDa-protein serum. In fact, the $λ#100$ clone did not react with MAb 35A6.

To obtain a full-length gene encoding the 120-kDa protein, Southern hybridization was performed using the insertion DNA fragment, amplified by PCR, as a probe. Three overlapping fragments (2.8-kb EcoRI, 2.8-kb SacI, and 3.8-kb EcoRV) were cloned into pUC18 and designated M11-1, -2, and 3, respectively (Fig. 2). These fragments were partially sequenced to determine an open reading frame (ORF) encoding 120-kDa protein.

Analysis of deduced amino acid sequence. The nucleotide sequence of a 4-kbp fragment containing the $λ#100$ insert DNA has an ORF which comprises 3,186 bp, and the predicted primary translation products would be a protein of 1,062 amino acids with a predicted size of 115.8 kDa. The gene has 10 TGA codons encoding tryptophan. Hydrophobicity analysis indicates that the protein contains characteristic features of membrane proteins, namely, a 5' hydrophobic signal sequence at the N-terminal end between positions 1 and 22, an external hydrophilic surface domain, a hydrophobic membrane-spanning domain, and a basic, highly charged cytoplasmic domain (data not shown). The predicted size of the gene product following a signal sequence cleavage (113.5 kDa) is in good agreement with the 120-kDa protein as estimated by SDS-PAGE.

A comparison of the predicted amino acid sequence of the gene to other mycoplasma counterparts revealed that the C-terminal region extending from amino acid residues 927 to 1,003 shares significant homologies with the M. pneumoniae 130-kDa protein, which is encoded by the ORF6 gene (12) (70% identity), and the M. genitalium 114-kDa protein, which...
is encoded by the ORF3 gene (9) (75% identity) (Fig. 3). Interestingly, the region also has a striking homology with the C terminus of MGCI (15), which is a homologue of M. pneumoniae P1. We found a partial sequence encoding the C terminus of MGCI upstream of the ORF, which is consistent with the gene arrangement that the ORF6 gene is located downstream of the P1 gene. Since Keeler et al. (15) and Hnatow et al. (11) have identified that the M. gallisepticum mgc1 and mgc2 genes are homologous to the ORF4 and P1 genes, respectively, we have named the gene encoding 120-kDa protein the mgc3 gene based on their genetic nomenclature. In addition, the absence of cysteine residues in the MGCI3 protein sequence is also consistent with the M. pneumoniae 130-kDa and M. genitalium 114-kDa proteins.

Cell surface localization of MGCI3 protein in M. gallisepticum. On the basis of the predicted amino acid sequence of the mgc3 gene, MGCI3 protein was expected to be located on the cell surface as a membrane protein. To address this, the freshly harvested organisms were treated with MAb 35A6 and analyzed for cell surface expression by flow cytometry. Strong immunofluorescent staining was detected when MAb 35A6 was used (Fig. 4). This observation is consistent with the previous report that the ORF6 gene product was a membrane-associated protein expressing a surface-exposed region (18).

Reactivities of MAb 35A6 for different strains. MGCI3 protein was affinity-purified with MAb 35A6 and inoculated into mice to produce a polyclonal antiserum to this protein. These sera were found to have high antibody titers against M. gallisepticum and also inhibited the growth and metabolism of M. gallisepticum in vitro (Table 1). To examine the reactivity of MAB 35A6 with other strains, Western blotting analysis was performed on M. gallisepticum strains R, S6, F, and KP13. M. gallisepticum R, S6, and KP13 possessed the 120-kDa protein as recognized by MAB 35A6, whereas M. gallisepticum F strain, an avirulent vaccine strain, was not recognized (Fig. 5A). In the case of M. pneumoniae, mutant missing 130-kDa protein is avirulent (8). In view of the relationship between the lack of MGCI3 protein and avirulence, it was of interest to examine whether M. gallisepticum F strain possesses MGCI3 protein.

Expression of the mgc3 gene by using recombinant FPVs in CEF cells. Whereas the antigen can be affinity purified using MAB 35A6 from M. gallisepticum strain lysate, only a limited amount of the antigen was obtained by this method. Therefore, an alternative strategy, which we have chosen, was the use of recombinant DNA techniques to determine the gene for the protective immunogen and to generate a recombinant FPV for analysis of the expression. All 10 TGA codons in the mgc3 gene were replaced with TGG codons by PCR using 15 primers. A recombinant FPV, recFPV-MGCI3 encodes the full-length mgc3 gene under the control of a strong synthetic prok promoter (35). Another recombinant FPV, recFPV-MGCI3-S, encodes the mgc3 gene fused to the MDV gB signal sequence in the place of its native signal sequence. The mgc3 gene products were synthesized in CEF cells infected with the recombinant FPVs and were characterized by immunoprecipitation with MAB 35A6 (Fig. 6). A 120-kDa protein was found in CEF cells infected with recFPV-MGCI3 (lane 2), which migrated to the same position as native MGCI3 protein of M. gallisepticum. In contrast, an approximately 50-fold-higher level of expression of a protein of 145 kDa was seen in CEF cells infected with recFPV-MGCI3-S (lane 3). Both 120- and 145-kDa proteins were also recognized by MAB 34H1 with growth inhibition activity (data not shown). No specific band was observed in the parent FPV-infected CEF cells (lane 1). These results clearly demonstrated that the mgc3 gene product possesses growth inhibition and metabolic-inhibiting epitopes recognized by MABS 35A6 and 34H1.

N-linked carbohydrate modification of MGCI3 in recFPV-infected cells. To address the size differences between MGCI3

![FIG. 2. Restriction map and ORF of the mgc3 gene. Plasmid subclones of this region (M11-1, -2, and -3) were obtained by colony hybridization with the λ<sub>100</sub> fragment as a probe. These plasmids were used not only as templates for DNA sequence but also as templates for PCR to construct recombinant transfer vectors, pNZ29RMGC3 and pNZ29RMGC3-S, as described in Materials and Methods. The ORF of the mgc3 gene is shown by a box, with an arrow showing the direction of transcription. The 10 TGA codons in the ORF are indicated by closed circles. The entire region of the M11-2 clone and a portion of the M11-1 and M11-3 regions were sequenced in both strands to determine the ORF of the mgc3 gene. E, EcoR I; EV, EcoRV; P, PstI; S, Sac I.](http://iai.asm.org/3189.00000001_02.jpg)
proteins expressed by recFPV-MGC3 and recFPV-MGC-S, these immunoprecipitated proteins were treated with endoglycosidases. In recFPV-MGC3-infected cells, no mobility shifts was observed in MGC3 protein either after Endo H or PNGase F treatment (Fig. 6, lanes 3 and 4), whereas a mobility shift was observed at from 145- to 120 kDa after Endo H and PNGase F treatments in recFPV-MGC3-S-infected cells (Fig. 6, lanes 6 and 7). This result demonstrated that the size differences were due to the posttranslational modifications, and the 145-kDa protein expressed by recFPV-MGC3-S was the simple high-mannose type. There are 15 potential N-linked glycosylation sites in MGC3 protein. The altered mobility shift indicated that 10 to 13 glycosylation sites of MGC3 protein are N-linked with high-mannose carbohydrates, assuming an increase in size of 1,000 to 2,000 Da per glycosylation. Thus, using FPV expression system, the protective epitopes on MGC3 protein recognized by the MAbs capable of inhibiting both growth and metabolism were unaffected by N-linked glycosylation.

Cell surface localization of MGC3 in recombinant FPV-infected cells. Expression of MGC3 protein on the cell surface might also play an important role in humoral immunity because this protein elicits growth-inhibiting antibodies. To assess whether MGC3 protein expressed by recombinant FPV is transported to the cell surface, an immunofluorescent analysis was performed with MAb 35A6. Strong cell surface immunofluorescence was observed on the recFPV-MGC3-S-infected cells, whereas only a faint signal was detected on the recFPV-MGC3-infected cells (Fig. 7). This result showed that MGC3 protein was transported to the cell surface by replacing with the MDV gB signal peptide.

DISCUSSION

This study describes the identification of a gene encoding an epitope recognized by MAb 35A6 which is capable of inhibiting both the growth and metabolism of M. gallisepticum in vitro. The gene, designated the mgc3 gene, is a homologue of the ORF6 gene in the P1 operon of M. pneumoniae and is located downstream of the mgc1 gene. The two genes of the P1 operon of M. gallisepticum have been identified as the mgc1 and mgc2 genes homologous to the P1 and ORF4 genes, respectively (15, 11). MGC3 protein encoded by the mgc3 gene may function as a cytoadherence-associated molecule because the M. pneumoniae 130-kDa protein has been implicated in the cellular adhesion process (8).

A striking homology at the amino acid level was found in the C terminus among 130-kDa protein homologues, where the regions might function as a membrane spanning and anchoring. This result implies that there is a similarity in membrane structure among the mycoplasmas. On the other hand, the sequence diversity at N-terminal region might reflect differences in mycoplasma receptor proteins found on the surfaces of human and avian epithelial cells. We found a couple of differences with regard to gene expression and organization between M. gallisepticum and M. pneumoniae. The 130-kDa protein is co- or posttranslationally cleaved to 40- and 90-kDa proteins and is located on the cell surface as a membrane-associated protein (18). In addition, the ORF6 gene carries repetitive DNA sequences (RepMP5) which are dispersed as multiple copies on the chromosome, suggesting that gene conversion between the multiple-copy regions results in antigen variation (26). In contrast to the ORF6 gene, the mgc3 gene exists as a single chromosomal copy, and the gene product does not seem to be cleaved.

We have developed a recombinant FPV expression system to examine its potential to protect against avian diseases. For example, immunization with a recombinant FPV expressing the MDV gB gene elicited neutralizing antibodies in chickens, resulting in protection against a lethal MDV challenge (23).
addition, gB antigen expressed by the recombinant FPV was located on the cell surface (39). We therefore used not only this recombinant FPV expression system but also the gB signal sequence for cell surface expression. To assess the characteristics of MGC3 protein in its host cells, all of the TGA codons in the mgc3 gene, which encode a tryptophan in Mycoplasma species, were replaced with TGG codons. Additionally, recombinant FPVs harboring the altered mgc3 gene were constructed. Replacement of the natural signal sequence of MGC3 protein with the MDV gB signal sequence improved not only the expression level of MGC3 protein. Although MGC3 protein expressed by recFPV-MGC3-S underwent N-linked glycosylation and would not be the native form in M. gallisepticum, the protective epitopes recognized by MAbs 35A6 and 34H1 were successfully expressed on the surface of its host cells. It is likely that surface-exposed epitopes which induce a neutralizing immune response should increase its immunogenicity. In addition, the MDV gB signal peptide could provide a good model system to express a gene derived from bacteria such as mycoplasma on the eukaryotic cell surface.

Our findings provide the first demonstration that MGC3 protein contains epitopes which can induce antibodies responsible for growth inhibition and metabolic-inhibition activities and is expressed on the cell surface in the recombinant FPV expression system. It is possible that the induction of antibodies to the epitopes recognized by the MAbs in chickens can result in protective immune responses against M. gallisepticum challenge. We are in the process of investigating recombinant FPV-based vaccines for the protection of chickens against M. gallisepticum infection.

ACKNOWLEDGMENTS

We thank A. Yasuda for DNA sequencing and B. Cowen for critical review of the manuscript. We especially thank K. Kamogawa and N. Yanagida for encouragement and support of this study.

ADDENDUM

The 5′-terminal regions of the mgc3 genes of M. gallisepticum F, S6, and KP13 strains were cloned by PCR and partially sequenced from the ATG start codon to nucleotide position 1,130 downstream of the start codon. An alignment of the mgc3 genes of the four strains revealed that the DNA sequences of M. gallisepticum R and KP13 were identical and that the DNA sequence homologies between strains S6 and S and between strains R and F are 93.1 and 89.7%, respectively. The partial nucleotide sequences of the mgc3 genes of M. gallisepticum F and S6 strains will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB033210 and AB033211, respectively.

REFERENCES


