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Identification and Mapping of an Immunogenic Region of *Mycoplasma hyopneumoniae* p65 Surface Lipoprotein Expressed in *Escherichia coli* from a Cloned Genomic Fragment

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A previously characterized lipid-modified amphiphilic surface protein of *Mycoplasma hyopneumoniae*, p65, has been defined by its reaction with a surface-binding monoclonal antibody (MAb) and by its exclusive partitioning into the detergent phase during Triton X-114 phase fractionation (K. S. Wise and M. F. Kim, J. Bacteriol. 169:5546–5555, 1987). In the current study, polyclonal mouse antibody (PAb) to gel-purified p65 was used to identify recombinant phage plaques expressing p65-related epitopes. Several characteristic partial tryptic fragments of p65 were recognized by both PAb to p65 and MAb to p65, but the PAb population specifically eluted from recombinant phage plaques bound only epitopes restricted to the largest of these fragments. Graded carboxypeptidase-Y digestion of intact *M. hyopneumoniae* generated C terminally truncated peptides that were recognized by PAb to p65 and MAb to p65, indicating that the C terminus and much of the adjoining region of p65 were present and accessible on the external face of the membrane. However, antibody eluted from recombinant phage plaques bound only to the largest truncated polypeptide, suggesting that a recombinant product corresponding to the C-terminal region of p65 was expressed in *Escherichia coli*. A 19-kilodalton recombinant protein (p19), which was recognized by PAb to p65 but not by MAb to p65, was detected in recombinant phage lysates. Serum antibodies from swine taken after, but not before, experimentally induced *M. hyopneumoniae* pneumonia preferentially recognized the native, amphiphilic p65 lipoprotein and also bound specifically to the p19 recombinant product. This confirmed that the p65 lipoprotein is a major immunogen of *M. hyopneumoniae* recognized during disease and identified its C-terminal region as an immunogenic domain.

Mycoplasma hyopneumoniae is the etiologic agent of mycoplasma pneumonia in swine (14, 20). This is a globally prevalent, chronic disease of low mortality but high morbidity and is one of the most economically damaging swine diseases known, mainly because of its abilities to affect feed efficiency, to retard growth of the animals, and to predispose them to bacterial pneumonia (20). Control of the disease has been hampered in part by the lack of effective and practical vaccines for active prophylactic immunization and by the lack of sensitive diagnostic tools for the specific detection of infection by this organism. This latter problem is compounded by the presence of other commensal, antigenically related *Mycoplasma* species in most swine populations (14, 20).

M. hyopneumoniae is one of more than 70 species in the genus *Mycoplasma*, a genetically diverse group of small, wall-less procaryotes in the class *Mollicutes*, that are phylogenetically related to gram-positive eubacteria (23). Although several *Mycoplasma* species are important animal pathogens (19), little is known regarding the actual mechanisms underlying their deleterious effects during disease. However, the surface architecture of these organisms is becoming increasingly appreciated as a key factor in determining their physical interaction with the host, as well as the effectiveness and outcome of host immune recognition. Contributing to the surface structure of mycoplasmas is the presence of prominent, lipid-modified membrane proteins which have emerged from recent studies as a characteristic feature of several mycoplasmal species and related mollicutes (2, 3, 5, 12, 13, 21, 24). Surface lipoproteins have recently been shown to be involved in complex antigenic and structural variation in *Mycoplasma hyorhinis*, one of the common *Mycoplasma* species found in swine (13).

We previously characterized (21) three major membrane lipoproteins of *M. hyopneumoniae* (p65, p50, and p44). These were shown to be exposed at the external face of the single, limiting mycoplasmal membrane by virtue of their reaction with specific surface-binding monoclonal antibodies (MAbs). These lipoproteins were also shown to be structurally distinct, since highly specific hyperimmune mouse polyclonal antibodies (PAbs) to each purified lipoprotein recognized distinctive sets of corresponding tryptic fragments. Characterization and isolation of these components were facilitated by their selective partitioning into the detergent phase during Triton X-114 (TX-114) phase fractionation, which allows their highly efficient separation from the majority of hydrophilic proteins of the organism, including some that comigrate during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antiserum generated in swine by immunization with disrupted *M. hyopneumoniae* (6) recognized these three lipoproteins, but it has not been established whether antibodies in swine convalescing after *M. hyopneumoniae* infection and disease are selectively directed toward epitopes on these specific components.

A report by Young and Ross (27) indicated that convalescent-phase serum antibodies from swine with experimental *M. hyopneumoniae* pneumonia did in fact recognize components (immunogens) with relative mobilities in SDS-PAGE similar to those of p65, p50, and p44 lipoproteins. In partic-

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ular, a 64-kilodalton (kDa) immunogen was identified which had the additional (and unique) feature of not reacting with antisera to other common commensal swine mycoplasmas, *M. hyorhinis* and *Mycoplasma flocculare*.

Determining the identity, structural characteristics, surface orientation, and immunogenic regions of *M. hyopneumoniae* surface proteins selectively and specifically recognized during infection may be critical for designing immunogenic and diagnostic reagents capable of detecting and ultimately controlling mycoplasmal pneumonia in swine. In an initial attempt to address this problem, we confirmed in this study the recognition of the p65 lipoprotein as a major immunogen of *M. hyopneumoniae*, identified a cloned gene of *M. hyopneumoniae* expressing an immunogenic portion of the p65 surface antigen in *Escherichia coli*, and used antibodies binding to this recombinant product and to other regions of p65 to assess the orientation of this lipoprotein on the *M. hyopneumoniae* membrane.

MATERIALS AND METHODS

***Mycoplasma* stocks.** *M. hyopneumoniae* (*suipneumoniae*) J (ATCC 25934) was propagated in broth culture as described elsewhere (21).

Antibodies. MAbs to *M. hyopneumoniae* antigens p65 (F188C42A), p66 (F177C21A), and p82 (F187C55A) and PAbs to gel-purified p65, p50, and p44 (and corresponding preimmune sera) are described in detail elsewhere (21). Convalescent-phase and corresponding preimmune sera from two separate experimental series of swine inoculated intratracheally with *M. hyopneumoniae* 194 or 232 (the latter derived from strain VPP11) (15, 27) were provided by R. F. Ross, Iowa State University, Ames, Iowa. The convalescent-phase sera were collected 24 to 28 days after inoculation.

Genomic library construction. A recombinant phage library containing random *M. hyopneumoniae* genomic DNA fragments was prepared by procedures described previously (18). Briefly, *M. hyopneumoniae* genomic DNA was partially digested with *Eco*RI restriction endonuclease. Fragments of 8 to 22 kilobases were isolated from agarose gels and used to replace internal *Eco*RI fragments of phage λ Charon 4A. Viable phage particles were produced by in vitro packaging of recombinant phage DNA by use of a commercial packaging mixture (Promega Corp., Madison, Wis.). Phage plaques were generated in *E. coli* strain DP50*supF* as described previously (18) on plates overlaid with NZCYM medium (10) containing 0.6% (wt/vol) agarose (International Biotechnologies, Inc., New Haven, Conn.). The library was determined to represent over 99.8% recombinant phage by assessing the ratio of phage with or without β-galactosidase activity (18).

Immunoscreening of phage library. Immunostaining of the phage library was performed essentially as described elsewhere (18). Briefly, 60-mm plates containing approximately 1,000 fresh plaques were overlaid with nitrocellulose filters for 10 min. The filters were removed, blocked with TS buffer (150 mM NaCl, 10 mM Tris [pH 7.4]) containing 3% (wt/vol) bovine serum albumin (Fisher Scientific Co., Fair Lawn, N.J.), and incubated in primary antibody for 16 h at 4°C. Primary mouse and swine antisera were used directly or after absorption with sonically disrupted *E. coli* (18), at a final dilution of 1:200 in TS buffer containing 10% (vol/vol) fetal bovine serum (Hazelton Dutchland, Denver, Pa.). MAb was used as undiluted hybridoma culture supernatant (2). Filters were rinsed, incubated with the respective horseradish peroxidase-conjugated secondary antibody to mouse or swine immunoglobulin G (Cooper Biomedical, Inc., Malvern, Pa.), and developed with the substrate *o*-dianisidine as described previously (22).

Phage purification and preparation of lysates. To purify phage encoding expressed antigens, plaques selected by immunoscreening were picked, replated at low density, and again immunoscreened. Two rounds of plaque purification were used to prepare phage isolates. To prepare lysates from isolated phage, top agarose from fresh plates containing confluent plaques was removed and centrifuged at 10,000 × *g* for 15 min. The supernatant was transferred, and methanol was added to a final concentration of 90% (vol/vol). After incubation at −70°C for 16 h, the resulting precipitate was sedimented at 10,000 × *g* for 10 min at 4°C, and the pellet was dissolved in SDS-PAGE sample buffer (2% [vol/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 62.5 mM Tris [pH 8.6]) and heated for 5 min at 100°C. Methanol-precipitated material from four 60-mm plates was loaded into a 15-mm-wide gel channel for SDS-PAGE and immunoblot analysis.

Elution of antibodies from phage plaques. Filter blots of recombinant phage isolates were prepared from 60-mm plates seeded to near confluence with approximately 2,000 PFU. Filters were blocked and incubated with primary antibody as described above. The blots were rinsed extensively with TS buffer, and a small portion of each filter was removed for subsequent immunostaining. The remaining filter was treated immediately with 3 ml of elution buffer (50 mM glycine, 0.1% [wt/vol] BSA, 500 mM NaCl [pH 2.2]) for 10 min at room temperature with gentle agitation. The eluate was collected and immediately adjusted to pH 7.5 with 3 M Tris (pH 8.0). Fetal bovine serum was added to a final concentration of 10% (vol/vol), and the eluted antibody solution was sterile filtered and stored at 4°C for use as a primary antibody on immunoblots of SDS-PAGE-separated *Mycoplasma* proteins. In order to monitor the effectiveness of elution, eluted filters were rinsed with TS buffer and developed with secondary antibody and substrate in parallel with portions of filters removed prior to elution.

Detergent-phase fractionation and trypsin digestion of *Mycoplasma* proteins. TX-114 detergent-phase fractionation of *Mycoplasma* proteins was performed as described in detail elsewhere (21). Detergent-phase proteins were partially digested with varying amounts of acetylated trypsin (Sigma Chemical Co., St. Louis, Mo.) as previously described (21). Products generated from individual digestions were pooled and heated in SDS-PAGE sample buffer at 100°C for 5 min. A sample of TX-114 detergent-phase protein not digested with trypsin was heated in SDS-PAGE sample buffer and added to the pooled trypsin digestion products to insure the presence of intact protein in the mixture.

Carboxypeptidase treatment of intact mycoplasmas. Treatment of intact organisms with carboxypeptidase Y has been described elsewhere (13). Briefly, mycoplasmas were harvested by centrifugation from mid-logarithmic-phase cultures and rinsed in phosphate-buffered saline. Cells from approximately 0.2 ml of original culture were incubated at 37°C for 1 h in 15 μl of phosphate-buffered saline (pH 6.0) containing 0, 75, or 250 μg of carboxypeptidase Y (Pierce Chemical Co., Rockford, Ill.) per ml. SDS-PAGE sample buffer was then added, and samples were heated at 100°C for 5 min and loaded into 8-mm-wide gel channels for SDS-PAGE and immunoblot analysis.

Electrophoresis and immunostaining of blots. SDS-PAGE, electrophoretic transfer to nitrocellulose, and immunostain-

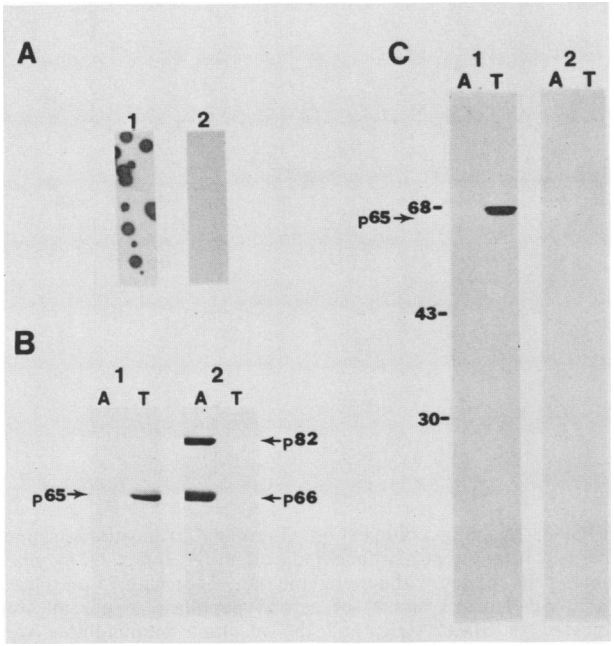

FIG. 1. Immunological detection and confirmation of a p65-related recombinant product expressed in *E. coli*. (A) Approximately equal numbers of phage plaques representing purified populations of λMhpJ35 (filter 1) or wild-type λ Charon 4A (filter 2) were transferred to nitrocellulose filters and stained with anti-p65 PAb as described in Materials and Methods. (B and C) TX-114 phase-fractionated *M. hyopneumoniae* proteins were prepared and the TX-114 detergent-phase (T) or aqueous-phase (A) proteins were separated by SDS-PAGE and immunoblotted as described in Materials and Methods. Blots of paired channels in panel B were stained with MAb to the amphiphilic p65 lipoprotein (lanes 1) or with a combination of MAbs to hydrophilic proteins p66 and p82 (lanes 2). Blots in panel C were stained with antibody eluted from plaque blots of λMhpJ35 (lanes 1) or λ Charon 4A (lanes 2) after reaction of the plaque blots with anti-p65 PAb (as described in Materials and Methods). Arrows indicate the positions and relative molecular masses of immunostained proteins, which were based on molecular-mass standards (68, 43, and 30 kDa) run on the same gels. The doublet form of p65 is apparent in this figure.

ing were performed as previously described (21). Primary and secondary antibodies were used as described above (for immunoscreening of phage library) at dilutions indicated in the figures. All samples for electrophoresis were treated under reducing conditions (21).

RESULTS

Immunological detection of recombinant phage expressing p65-related epitopes. The PAb to purified p65 (anti-p65 PAb) recognizing multiple epitopes on the p65 lipoprotein (21) was used to screen the phage λ Charon 4A *M. hyopneumoniae* genomic library. Of approximately 1,000 plaques screened, 20 showed strong, specific staining with this PAb, clearly distinguishable from the very low background observed with the rest of the screened population (data not shown). Phage isolated by two cycles of plaque purification maintained this selective staining (Fig. 1A). These phage isolates failed to stain with several antibodies previously described (21), including preimmune serum corresponding to anti-p65 PAb, hyperimmune PAbs to the purified p50 and p44 lipoproteins of *M. hyopneumoniae*, MAb to p65, or MAbs to *M. hyopneumoniae* proteins p82, p66, p50, p44, or p41 (data not shown). All of these antibodies also failed to detect any recombinant phage in the original library.

Although several positive phages were isolated by anti-p65 PAb, one phage, λMhpJ35, was used for the subsequent studies reported here. To confirm that the antibodies binding to recombinant products were in fact directed to epitopes shared by and specific for the authentic mycoplasmal p65 lipoprotein, antibodies were eluted from λMhpJ35 or wild-type λ Charon 4A plaque blots after primary reaction with the anti-p65 PAb and were used to immunostain TX-114 phase-fractionated *M. hyopneumoniae* proteins. The efficient partitioning of amphiphilic and hydrophilic mycoplasmal proteins by this procedure is shown in Fig. 1B, where p65 was identified exclusively in the detergent phase by staining with MAb (Fig. 1B, lane 1T). In contrast, the comigrating hydrophilic p66 protein and another hydrophilic protein, p82, were both exclusively detected (with corresponding MAbs) in the aqueous phase (Fig. 1B, lane 2A). When using this same technique, antibody eluted from λMhpJ35 plaque blots after reaction with anti-p65 PAb recognized the characteristic amphiphilic p65 protein (Fig. 1C, lane 1T), whereas an identically processed control preparation eluted from wild-type phage plaque blots after reaction with anti-p65 PAb failed to stain any component (Fig. 1C, lanes 2). This result insured that the epitopes recognized on the recombinant product were not directed to some unrelated protein inadvertently present in the immunizing preparation used to generate anti-p65 PAb and that the epitopes did not fortuitously cross-react with this PAb.

Recognition of a restricted set of p65 epitopes expressed on the recombinant product. We have previously shown that anti-p65 PAb recognizes a highly characteristic set of partial tryptic peptides from p65 that are identified in graded tryptic digestion products of *M. hyopneumoniae* TX-114 detergent-phase proteins (21). In order to determine whether gene products expressed by phage λMhpJ35 contained the full spectrum of epitopes represented on these p65 partial tryptic fragments, replicate immunoblots of a digestion mixture were stained with anti-p65 PAb or with antibody eluted from λMhpJ35 plaque blots after their reaction with this PAb (Fig. 2). Several partial digestion products stained with anti-p65 PAb, ranging in size from about 13 to 43 kDa (Fig. 2, lane 1). However, only one large (43-kDa) fragment was recognized by antibody eluted from the recombinant phage (Fig. 2, lane 2). This directly indicated that the epitopes recognized on the recombinant product represented a restricted subset of those present on the native p65 molecule. The absence of binding of the eluted antibody to several smaller fragments, including one approximately 40 kDa in size, further suggested that epitopes present on a large portion of the p65 protein were not represented in the recombinant product. That the smaller tryptic fragments were indeed related to p65 was confirmed by the restaining of a blot with MAb to p65 after initial staining with plaque-eluted antibody (Fig. 2, lane 3). The MAb recognized most of these smaller fragments, which confirmed their origin from the p65 protein and showed the difference in location of epitopes recognized by the MAb from those identified by plaque-eluted antibodies.

Mapping epitopes expressed on the recombinant product to the C-terminal region of p65. Because antibodies eluted from recombinant products appeared to recognize a restricted portion of the p65 protein which was distinct from that recognized by the surface-reactive MAb to p65, these two antibodies were used to investigate the orientation of p65 on the mycoplasmal surface. This was accomplished by using a recently developed procedure that truncates proteins on the

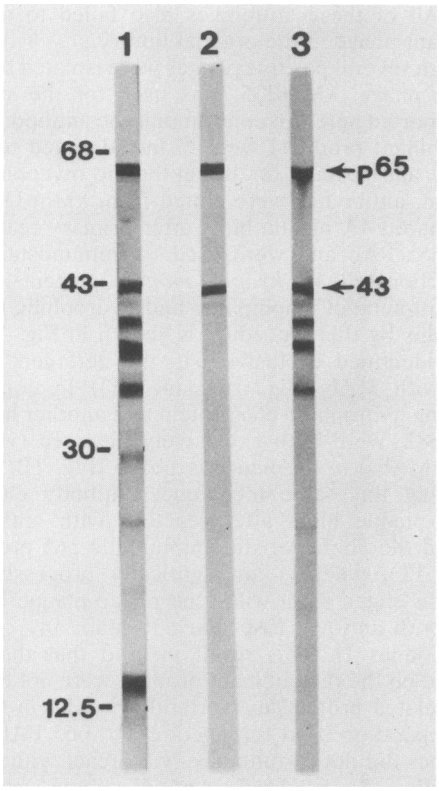

FIG. 2. Identification of a restricted set of epitopes on the p65-related recombinant product expressed in *E. coli*. A sample containing untreated and pooled, trypsin-treated *M. hyopneumoniae* TX-114 detergent-phase proteins was separated by SDS-PAGE, and the subsequent immunoblot was stained as described in Materials and Methods. Adjacent strips of the blot were stained with anti-p65 PAb (lane 1), antibody eluted from a plaque blot of λMhpJ35 phage after reaction of the plaque blot with anti-p65 PAb (lane 2), or the same plaque-eluted antibody as in lane 2 followed by a second immunostain with MAb to p65 (lane 3). The positions of intact p65 and the large 43-kDa digestion product are indicated by arrows. Molecular mass markers are indicated (in kilodaltons) as in Fig. 1.

surface of intact mycoplasmas by graded digestion with carboxypeptidase Y (13). Results of the analysis of p65 are shown in Fig. 3. Intact *M. hyopneumoniae* organisms were treated with graded concentrations of carboxypeptidase Y, and each digestion reaction mixture was subjected to SDS-PAGE gel and immunoblotted. MAb to p65 recognized intact p65 in untreated organisms (Fig. 3A, lane 1), a set of three additional C terminally truncated intermediates (61, 50, and 46 kDa in size) in partially digested preparations (Fig. 3A, lane 2), and no products in more extensively digested preparations (Fig. 3A, lane 3). The presence of the undegraded, internal p66 protein in the extensively digested preparation (Fig. 3A, lane 4) indicated that cells remained intact during treatment with enzyme. This was further confirmed by a third round of staining, with MAb to the aqueous-phase p82 protein, which showed that this protein also was unaffected by external enzymatic digestion (data not shown). Proteins p66 and p82 were susceptible to digestion in aqueous-phase preparations (data not shown). These results suggested that the C terminus of p65 is accessible on the surface and that the epitope recognized by the MAb is present on an N-terminal portion of the molecule with a maximum size of 46 kDa.

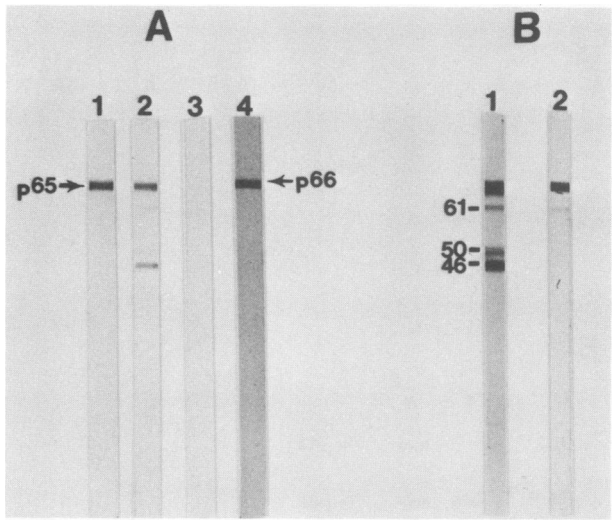

FIG. 3. Mapping epitopes on C-terminal truncation products generated from the p65 surface lipoprotein. (A) Intact *M. hyopneumoniae* cells treated without enzyme (lane 1) or with 75 μg (lane 2) or 250 μg (lanes 3 and 4) of carboxypeptidase Y per ml were subjected to SDS-PAGE, and the resulting immunoblots were stained with MAb to p65 (lanes 1 through 3) or with MAb to p66 (lane 4). The positions of p65 and p66 are indicated by arrows. (B) Strips of a blot adjacent to the one shown in lane 2 of panel A were immunostained with anti-p65 PAb (lane 1) or with antibody eluted from a plaque blot of λMhpJ35 after reaction of the plaque blot with anti-p65 PAb (lane 2). The sizes (in kilodaltons) of immunostained digestion products are indicated in panel B.

The same three partially truncated intermediates were stained with anti-p65 PAb (Fig. 3B, lane 1); however, antibody eluted from λMhpJ35 phage plaques after their reaction with this PAb recognized the largest (61 kDa) intermediate but none of the smaller, more prominent, truncated products (Fig. 3B, lane 2). This suggested that the restricted set of epitopes represented and recognized on the recombinant phage product were associated with a region close to the C terminus of p65.

Direct identification of the p19 recombinant phage product. The protein expressed from recombinant phage λMhpJ35 was identified by immunostaining SDS-PAGE-separated proteins from *E. coli* phage lysates (Fig. 4). Lysate prepared from λMhpJ35 contained a 19-kDa protein that was stained with anti-p65 PAb but not with corresponding preimmune serum (Fig. 4A, lanes 1 and 2, respectively). A faintly stained protein of approximately 40 kDa was occasionally recognized by the PAb in some lysate preparations, but it is not represented in Fig. 4. MAb to p65 did not stain any component in the recombinant phage lysate (not shown). Neither anti-p65 PAb nor the corresponding preimmune serum stained any component in identically prepared lysate generated with wild-type λ Charon 4A phage (Fig. 4B, lanes 1 and 2, respectively). This directly identified the p19 protein as the expressed recombinant phage protein related to the p65 lipoprotein. Antibody eluted from the immunoblotted recombinant protein recognized the authentic amphiphilic p65 protein when the procedure described in Fig. 1 was used (data not shown).

Selective binding of p65 and p19 recombinant protein by serum antibodies from convalescent swine. Sera from individual swine taken prior to (preimmune) or after (convalescent phase) experimental infection with *M. hyopneumoniae* and resultant pneumonia were diluted and used to immunoblot

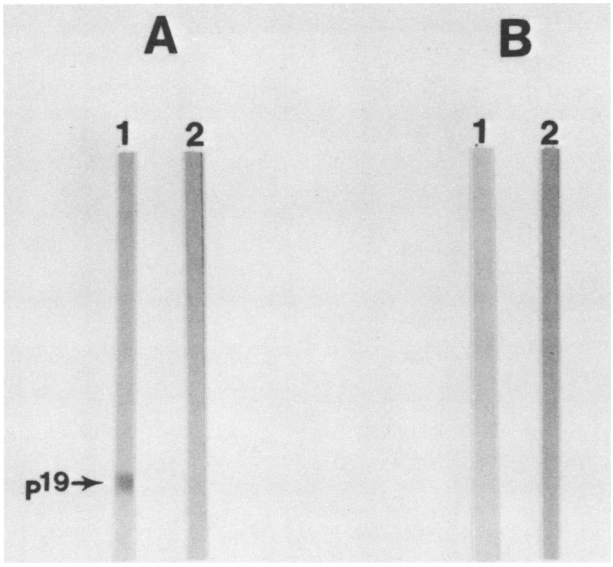

FIG. 4. Identification of the p65-related recombinant protein expressed in *E. coli*. Equivalent phage lysates were prepared from λMhpJ35 (A) and λ Charon 4A (B) and analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods. Blot strips were stained with anti-p65 PAb (lane 1) or with corresponding preimmune serum (lane 2). The position of p19, the 19-kDa component immunostained in the recombinant phage lysate, is indicated by an arrow.

TX-114 detergent-phase proteins of *M. hyopneumoniae* (Fig. 5A and B). Preimmune sera (Fig. 5A and B, lanes 1) bound some components of *M. hyopneumoniae*, restricted mostly to the aqueous-phase proteins (Fig. 5A and B, lanes 1A). A low-level nonspecific reaction was also seen with the TX-114 detergent-phase p44 lipoprotein (lanes 1T). In contrast, the corresponding convalescent-phase sera from two separate series inoculated with *M. hyopneumoniae* 194 or 232 (Fig. 5A and B, lanes 2, respectively) showed strong and preferential staining of the p65 lipoprotein present in the TX-114 detergent-phase proteins (Fig. 5A and B, lanes 2T). These convalescent-phase sera showed relatively weak staining of aqueous-phase proteins (notably, p66) comigrating with p65 in SDS-PAGE (Fig. 5A and B, lanes 2A).

Analysis of convalescent-phase sera of six individual swine from a series inoculated with *M. hyopneumoniae* 232 showed that all animals developed strong preferential antibody reactions to the amphiphilic p65 lipoprotein (Fig. 5C, lanes 2 through 7), compared to preimmune serum (lane 1), thereby indicating that this surface component is a major immunogen of *M. hyopneumoniae* recognized during infection and disease.

To determine whether epitopes present on the recombinant protein p19 were also recognized by serum antibodies in convalescent swine, immunoblots of λMhpJ35 lysate were stained with the same sera represented in Fig. 5C. Because all swine sera (preimmune or convalescent phase) showed strong background staining of *E. coli* components present in phage plaque blots, immunoblotting of SDS-PAGE-separated phage lysates was required to detect specific recognition of the p19 product. Figure 5D shows that convalescent-phase swine sera (lanes 2 through 7), but not preimmune serum (lane 1), specifically recognized the recombinant phage protein. This demonstrated that epitopes present on p19, and by inference near the accessible C-terminal region of p65, were among those recognized in swine during infection with *M. hyopneumoniae* and subsequent disease.

DISCUSSION

Our analysis of convalescent-phase swine sera binding to TX-114 phase-fractionated *M. hyopneumoniae* components confirms the observation of Young and Ross (27) that a component of approximately 65 kDa is preferentially recognized by serum antibodies during *M. hyopneumoniae* infection and disease and extends these results by identifying the amphiphilic surface lipoprotein p65 (and not comigrating hydrophilic components) as the major target of this response. Other lipoproteins recognized in the current study by convalescent-phase sera included the previously described p50 and p44 surface antigens (21), which also corresponded to components identified by that group. Notably, the 41-kDa component defined in a different gel system by those investigators (27) corresponds to our p44 (21).

The surface orientation and processing of *M. hyopneumoniae* surface lipoproteins are critical aspects of these prevalent membrane components. Several features were revealed by proteolytic analysis of the integral membrane surface antigen p65. Carboxypeptidase Y successively cleaves amino acids from accessible C termini of proteins exposed on the outer surface of the single mycoplasmal membrane (13). Since it hydrolyzes Lys, Arg, and His residues at a greatly reduced relative rate (7), sequences rich in these residues (or otherwise sterically less accessible) cause "pausing" of the enzyme, yielding discrete digestion intermediates that can be identified by separation by SDS-PAGE and subsequent immunoblotting with proper antibodies. The identification of C-terminal truncation products of the protein generated by enzymatic treatment of intact cells indicated that a substantial, continuous C-terminal region of the protein is present outside the single limiting plasma membrane of the organism. Although a 46-kDa product was shown in this report to be the smallest intermediate recognized by both MAb and PAb to p65, these antibodies could in fact recognize a product of 30 kDa, generated by a different degree of digestion (unpublished observation). While it is possible that p65 contains membrane-spanning domains further toward the N terminus of the molecule, these results also support an alternative the prototype model of procaryotic lipoprotein membrane anchorage, which is mediated solely through fatty acid moieties covalently bound to the amino terminus (25). This model for p65 orientation is consistent with that proposed for lipoproteins of other mycoplasmas (2, 13) as well as other procaryotic pathogens, including species of *Borrelia* (1) and *Treponema* (16, 17). Surface lipoproteins are also major immunogens of these organisms and are recognized during the course of disease caused by these respective agents.

An interesting feature of the p65 protein is its occurrence as a doublet during SDS-PAGE. This has been observed previously in radiolabeled samples (21) and is also apparent in the immunostained samples in Fig. 1B of this study. This doublet has been observed in other strains of *M. hyopneumoniae* that show different ratios of the two components (unpublished results). Although this could represent the differential processing of a typical lipoprotein signal peptide (25), the absence of similar doublets of lipoproteins p50 and p44 expressed from the same organism raises the possibility that other posttranslational events may selectively occur with p65. Two possibilities include (i) modification by addition of other moieties and (ii) partial suppression of transla-

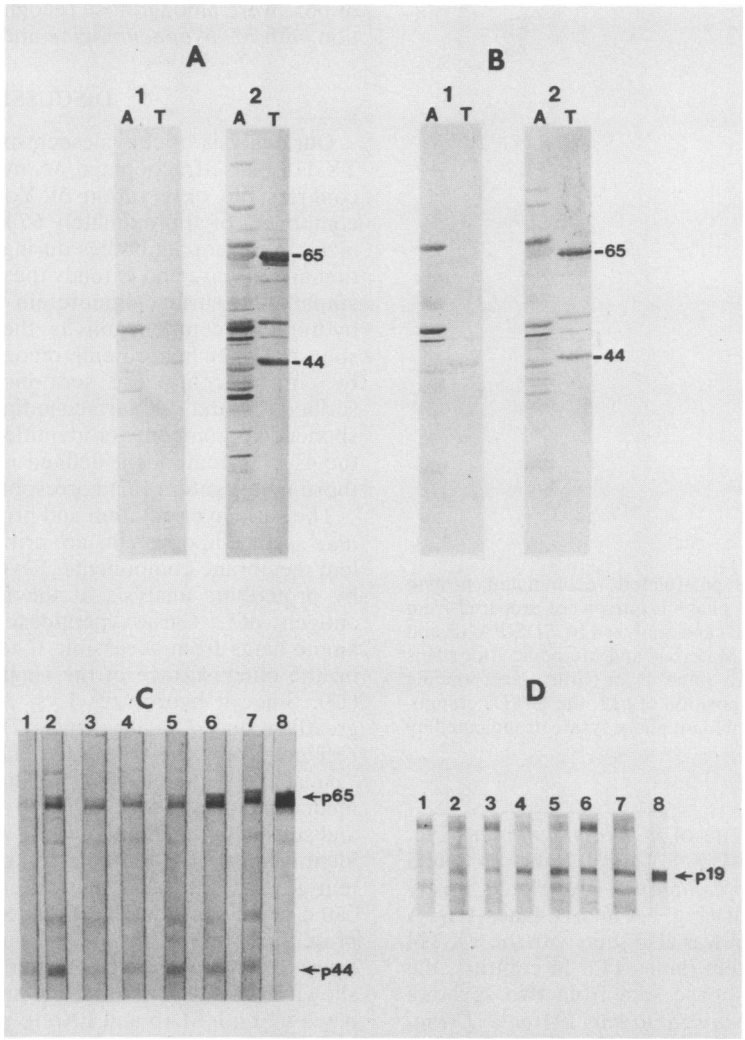

FIG. 5. Selective recognition of native p65 and recombinant protein p19 by serum antibodies from convalescent swine. (A and B) Immunoblots of paired TX-114 detergent-phase (T) and aqueous-phase (A) proteins were prepared and immunostained as in Fig. 1. Panels A and B were stained with preimmune serum (lanes 1) or convalescent-phase serum (lanes 2) from swine after experimental infection with *M. hyopneumoniae*. The two panels were stained with serum from two separate series in which different swine and different strains of *M. hyopneumoniae* were used for inoculation. (C and D) A representative preimmune serum (lane 1) or convalescent-phase sera (lanes 2 through 7) from individual swine from a series of animals with experimentally induced *M. hyopneumoniae* pneumonia were used to immunoblot SDS-PAGE-separated TX-114 detergent-phase proteins of *M. hyopneumoniae* (C) or phage lysate proteins generated with λMhpJ35 (D). Control strips stained with MAb to p65 (panel C, lane 8) or anti-p65 PAb (panel D, lane 8) were included to mark the position of native p65 protein and the p19 recombinant protein, respectively. Panels C and D show only relevant portions of immunoblots. Swine sera shown in this figure were all used at a 1:200 dilution.

tion termination codons to generate an extension of the protein sequence at the C terminus. The latter mechanism has been proposed for an acylated surface immunogen of *Treponema pallidum* that shows a similar doublet structure (4, 8). Posttranslational modifications or differential processing could have a major influence on expression of antigenic regions of p65. Analyzing the processing and modification of this component in *M. hyopneumoniae* or as a cloned gene product in other bacterial hosts will be important in elucidating these features.

The identification and isolation of the *M. hyopneumoniae* genomic region encoding the p65 protein is an important outcome of this study. This will allow direct determination of its primary structure and identification of key features regarding its expression, modification, processing, and association with the membrane. The expression in *E. coli* of a 19-kDa protein synthesized from recombinant phage deserves special note, particularly in light of experiments immunologically relating it to the C-terminal portion of p65. Of several recombinant phages recognized in our library by anti-p65 PAb, some were found to represent overlapping genomic regions, and yet plaque-eluted antibodies from each isolate identified the native p65 protein and showed the same staining pattern on tryptic digestion products (indicating a restricted set of epitopes). Moreover, all of these recombinant phage produced the p19 protein. The occurrence of certain truncated products is expected from *Mycoplasma* genes expressed in *E. coli*, since *Mycoplasma* species (26) including *M. hyopneumoniae* (9) are known to use the translation termination codon UGA to code for tryptophan. However, products truncated in this manner would generally represent N-terminal portions of authentic proteins normally

expressed in mycoplasmas. The immunological identification of p19 as a markedly truncated product corresponding to C-terminal regions of the much larger p65 protein requires an alternative explanation. This may be found in the recently documented expression of multiple translational products expressed in *E. coli* from *Mycoplasma* genomic sequences, which have been shown to arise from promiscuous translation initiation at multiple sites within the coding region of the *Mycoplasma* gene (11). Such products share C-terminal regions with the authentic protein and can be synthesized from regions 3′ of UGA codons. We consider this a likely explanation for the expression of the p19 product and its relationship to the C-terminal region of p65. This effect could also explain the occasional immunological detection of a faint 40-kDa product also synthesized from the recombinant phage.

Several practical applications arise from the ability to express p65 sequences in *E. coli*, including (i) the engineering of products representing specific antigenic domains of the protein, (ii) expression of recombinant constructs lacking lipid modification sites, thereby overcoming the major problems of solubility that hamper manipulations with the native amphiphilic protein, and (iii) overexpression of quantities of this protein for evaluation as a potentially useful immunogen in vaccine development or as a target for immunological detection of infection. Future analysis of this component may have a practical benefit in disease detection and control and may substantially enhance our understanding of several features that are possibly shared by a number of membrane lipoproteins of mycoplasmas and other procaryotes.

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