Biofunctional Domains of the *Mycoplasma pneumoniae* P30 Adhesin

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The P30 adhesin genes of spontaneous, hemadsorption-negative (HA⁻) class II *Mycoplasma pneumoniae* mutants that displayed P30 adhesin-deficient protein profiles were analyzed. One subclass of P30-deficient mutants possessed the entire *p30* structural gene without alterations (825 nucleotides, encoding 275 amino acids with a predicted molecular mass of 29,743 Da [S. F. Dallo, A. Chavoya, and J. B. Baseman, *Infect. Immun.* 58:4163–4165, 1990]). However, the second mutant subclass contained a deletion in *p30* resulting in the expression of a 25-kDa peptide (681 nucleotides, encoding 227 amino acids with a calculated molecular mass of 24,823 Da). This P25-truncated peptide lacked 8 of the 13 proline-rich amino acid repeat sequences at the carboxy terminus. Whole-cell radioimmunoprecipitation of *M. pneumoniae* with antibodies directed against the proline-rich repeat sequences located in the carboxy terminus demonstrated their surface accessibility. In contrast, antibodies generated against N-terminal amino acid sequences upstream of the repeats did not bind to intact mycoplasmas. The amino acid sequence homologies exhibited by the P30 adhesin and eucaryotic structural proteins were corroborated by cross-reactive epitopes shared between the P30 adhesin and fibrinogen, keratin, and myosin. These data reinforce the importance of the P30 protein in cytodherence and virulence and provide a molecular basis for postinfectious autoimmunity associated with *M. pneumoniae*-mediated pathologies.

Cytodherence of *Mycoplasma pneumoniae* to the respiratory epithelium is an initial and essential step in tissue colonization and subsequent disease pathogenesis. Two surface proteins, the 170-kDa protein designated P1 and the 30-kDa protein designated P30, have been identified as adhesins on the basis of biochemical, immunological, ultrastructural, and genetic data (2–4, 6, 10, 11, 14, 18–24, 38, 39). Both adhesins are clustered at the differentiated tip organelle of virulent mycoplasmas, which provides polarity to the cytodherence event. Also, these adhesins elicit a strong immunological response in convalescent-phase sera from humans and experimentally infected hamsters, and antiadhesin monoclonal antibodies (MAbs) block cytodherence (31). The existence of distinct, spontaneously arising, hemadsorption-negative (HA⁻), avirulent mycoplasmas that lack the P1 and P30 adhesins and fail to adhere to respiratory epithelium (2, 23), along with the isolation of spontaneous hemadsorption-positive (HA⁺) revertants that resynthesize these proteins and regain hemadsorption, cytodherence, and virulence capabilities (2, 4, 22), established the critical importance of each adhesin. These observations are supported by the fact that the P1 and P30 adhesins are encoded by two distinct genetic loci, consistent with the isolation of spontaneous, noncytadhering phenotypes that lack either, but not both, of the adhesins (2, 7, 42).

Further characterization of the *M. pneumoniae* HA⁻, P30-deficient, spontaneous mutants revealed two subclasses, one incapable of synthesizing detectable P30-related peptides and the other capable of synthesizing a P30-truncated 25-kDa peptide (i.e., P25 [2]). Since both mutant subclasses synthesized all other proteins previously implicated in cytodherence, including the P1 adhesin and the cytodherence accessory proteins (2, 7),

the P30 deletion mutant in particular provided an opportunity to identify a unique P30 adhesin domain essential for cytodherence. In this study, each mutant subclass was examined for DNA sequence and protein profile differences from wild-type *M. pneumoniae*. Furthermore, the predicted homologies shared between the P30 adhesin and mammalian structural proteins were addressed by the demonstration of cross-reactive epitopes, implicating molecular mimicry as a basis for mycoplasma-mediated postinfectious autoimmunity.

MATERIALS AND METHODS

Organisms and culture conditions. Wild-type HA⁺ *M. pneumoniae* M129-B16 and P30-deficient HA⁻ class II mutants 3, 7, and 15 (2, 7, 23) were grown in 32-oz (ca. 950-ml) glass prescription bottles in 70 ml of SP-4 medium (10) at 37°C for 3 days (HA⁺ *M. pneumoniae*) or 4 days (HA⁻ mutants). Glass-adherent wild-type mycoplasmas were washed four times with phosphate-buffered saline (PBS) (pH 7.2) and collected by centrifugation (9,500 × g, 20 min). Since mutants 3, 7, and 15 adhered poorly to glass, broth cultures were processed by centrifugation at 9,500 × g for 20 min and mycoplasma pellets were washed in PBS.

DNA extraction and analysis of P30-deficient mutants. Extraction of genomic DNAs from wild-type and mutant *M. pneumoniae* cells has already been described (10, 11, 39). To clone the *p30* genes of the HA⁻ mutants, the 4.6-kb *Pst*I DNA fragment, previously identified in the characterization of the *p30* gene of wild-type *M. pneumoniae* (10), was obtained from each mutant and ligated to the *Pst*I site of pUC19. Individual DNA samples were used to transform library-efficient, competent cells of *Escherichia coli* DH5α in accordance with the instructions of the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). Transformants were selected on LB agar plates containing 50 μg of ampicillin (Sigma, St. Louis, Mo.) per ml (27), and plasmid DNA was isolated from overnight cultures by the alkaline lysis method (27). The restriction endonuclease-digested plasmid DNAs were separated on agarose gels, blotted onto nitrocellulose filters (Schleicher & Schuell, Keene, N.H.), and hybridized to the ³²P-labeled (DuPont NEN, Boston, Mass.) wild-type *M. pneumoniae p30* gene (10). The *Pst*I DNA fragment thus identified from each mutant was mapped by using restriction endonucleases and compared to the wild-type *M. pneumoniae* 4.6-kb *Pst*I insert DNA. The *Hinc*II and *Hind*III restriction fragments from the *Pst*I DNA of the mutants were subcloned into M13 phage vectors, and single-stranded DNAs purified from the recombinant phages were used as sequencing templates (29).

DNA sequences were determined by the dideoxy-chain termination method of Sanger et al. (10, 35). M13 sequencing kits were purchased from Bethesda

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Research Laboratories, and the reactions were performed in accordance with the instructions of the manufacturer, except for the replacement of dGTP by deoxy-7-deaza-GTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (30). Both strands of the entire P30-related structural genes of the mutants were sequenced. Analyses of nucleic acids and proteins were performed with the Microgenie Data Banks (Intelligenetics, Inc., Mountain View, Calif.). DNA and deduced protein sequences were compared with recent releases of the National Institutes of Health GenBank DNA sequence database and the National Biomedical Research Foundation protein sequence database, respectively.

Protease sensitivity assay. To examine the proteolytic digestion patterns of wild-type and mutant P30-related proteins, specific proteases and protease inhibitors were purchased from Sigma. The proteases were trypsin type III (from bovine pancreas), papainase type IV (from papaya latex), α -chymotrypsin type VII (from bovine pancreas), and *Streptomyces griseus* protease type XIV. The protease inhibitors were TPCK (L-1-tosylamide-2-phenylmethyl chloromethyl ketone), TLCK (*N* α -p-tosyl-L-lysine chloromethyl ketone), phenylmethylsulfonyl fluoride, and trypsin inhibitor type II-T (from turkey egg white). Briefly, intact mycoplasma cells (400 μ g of total protein) in 500 μ l of PBS were added to microcentrifuge tubes and each sample was incubated with 50 μ g of a specific protease for 30 min at 37°C. Protease inhibitors at concentrations twofold greater than that of the protease were added to interrupt proteolytic digestion, and sample incubation was continued at 0°C for 10 min (32). Samples were then centrifuged at 14,000 $\times$ g, washed once with cold PBS, and solubilized in SP buffer. The solubilized preparations were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose for immunoblotting with anti-P30 MAbs (31, 40).

Antibody reagents. Two synthetic peptides derived from the proline-rich carboxy-terminal repeat regions of adhesin P30 (amino acids PGMAPRPGMPPH [repeats A and B] and PGMAPRPGFPPCA [repeats A and C], i.e., amino acids 171 to 269 [10]) and one peptide upstream from these repeats (RLLEEKERQ EQLAEQ [amino acids 106 to 120]) were purchased from Peninsula Laboratories (Belmont, Calif.) and coupled to keyhole limpet hemocyanin by the procedure supplied by Pierce (Rockford, Ill.). Eucaryotic structural proteins and goat antibodies to human fibrinogen were purchased from Sigma. MAbs reactive against the P30 adhesin of *M. pneumoniae* were previously described (31). Rabbit monospecific antibody reagents were generated by The University of Texas Health Science Center Institutional Immunology Facility. In this case, 2.5-kg male New Zealand White rabbits were immunized with 0.5 mg of the keyhole limpet hemocyanin-coupled P30 synthetic peptide or a keyhole limpet hemocyanin-irrelevant peptide emulsified in Freund's incomplete adjuvant. On days 24, 43, and 59, rabbits received boosters of 300 μ g, 400 μ g, and 3.5 mg, respectively, of coupled peptides resuspended in Freund's incomplete adjuvant. All immunizations were administered subcutaneously at multiple sites.

Whole-cell radioimmunoprecipitation (RIP) assay. To determine antibody-accessible epitopes of the P30 adhesin on the surface of wild-type and mutant mycoplasmas, whole-cell RIP was performed with [³⁵S]methionine-labeled mycoplasmas as described previously (6, 31). Briefly, metabolically [³⁵S]methionine-labeled mycoplasmas were washed and resuspended in PBS. Aliquots of 0.125 ml of mycoplasma cells (10⁸ cells) were placed in microcentrifuge tubes along with 0.04 ml of the monospecific antibody reagent or a prebleeding serum control. Individual tubes were incubated for 90 min at 4°C on a rocker. Mycoplasmas were then washed once with PBS, gently lysed with 1.0 ml of TDSET (10 mM Tris [pH 7.8], 0.2% sodium deoxycholate, 0.1% SDS, 10 mM tetrasodium EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, and incubated at 37°C for 60 min. The samples were centrifuged, and the supernatants (0.9 ml) were treated with 0.25 ml of insoluble protein A (Sigma catalog no. P7155), incubated 90 min at 4°C, and centrifuged. The pellets were washed four times with TDSET buffer, resuspended in solubilization buffer, and processed by SDS-PAGE and autoradiography (31).

RESULTS

Protein characterization of P30-deficient mutants. MAbs reactive against the P30 adhesin of *M. pneumoniae* were used to characterize the class II hemadsorption-negative, avirulent P30 mutants designated 3, 7, and 15 (2). Immunoblots of wild-type *M. pneumoniae* and mutants are shown in Fig. 1. As expected, a 30-kDa peptide was detected in wild-type HA⁺ *M. pneumoniae* (lane 1). In contrast, a 25-kDa peptide (P25) appeared in mutant 7 (lane 4) and no P30-related cross-reacting peptides were detected in mutant 3 (lane 7). Mutant 15 displayed characteristics identical to those of mutant 3. To further characterize the P30-deficient mutants, intact cells of wild-type *M. pneumoniae* and mutants were treated with proteases. After proteolytic digestion of surface proteins, mycoplasma preparations were analyzed by SDS-10% PAGE and immunoblotted

FIG. 1. Immunoblot analysis of the P30 adhesin-related regions in wild-type HA⁺ *M. pneumoniae* and HA⁻ mutants. Intact mycoplasmas were incubated with 0 or 50 μ g of proteases prior to analysis by SDS-10% PAGE. Gels were immunoblotted and probed with an anti-P30 MAb (H13 4F4 [31]). Lanes: 1, 2, and 3, wild-type HA⁺ *M. pneumoniae*; 4, 5, and 6, class II HA⁻ mutant 7; 7, 8, and 9, class II HA⁻ mutant 3; 1, 4, and 7, untreated mycoplasmas; 2, 5, and 8, mycoplasmas treated with 50 μ g of *S. griseus* protease; 3, 6, and 9, mycoplasmas treated with 50 μ g of trypsin. The numbers to the left are molecular masses in kilodaltons.

with anti-P30 MAbs. Exposure to 50 μ g of *S. griseus* protease resulted in cleavage of the wild-type P30 protein to a 29-kDa peptide (lane 2) and the mutant 7 P25 peptide to a 24-kDa peptide (lane 5). No detectable peptides were observed in mutant 3 (lane 7). Trypsin had no proteolytic effect on P30-related peptides in the wild type and the mutants (lanes 3, 6, and 9). Also, no proteolytic digestion of P30-related peptides by 50 μ g of papainase or chymotrypsin was observed (data not shown).

p30 gene characterization in P30-deficient mutants. Since mutants 3 and 15 lacked P30-related peptides and mutant 7 possessed the truncated P30 peptide (i.e., P25), we examined the genomic DNAs from these mutants by Southern hybridization by using the wild-type 0.9-kb *Hind*III fragment of *p30* as the probe (10). The wild type and mutants 3 and 15 showed identical hybridization patterns (20-kb *Bam*HI, 9.4-kb *Eco*RI, 0.9-kb *Hind*III, and 4.6-kb *Pst*I fragments, Fig. 2). Comparison of the mutant 7 and wild-type hybridization patterns revealed no apparent differences in the molecular weights of *Bam*HI, *Eco*RI, and *Pst*I fragments. The only obvious distinction occurred between the *Hind*III hybridized fragments (wild type, 0.9 kb; mutant 7, 0.7 kb). To further characterize this difference, the *Pst*I DNA fragments that contained the *p30* structural genes from wild-type and mutant *M. pneumoniae* were purified and digested with *Hinc*II and *Hind*III for hybridization with the 0.9-kb *Hind*III *p30* gene fragment. The fragments that contained the *p30* gene are indicated by arrows in Fig. 3. In the wild type and mutants 3 and 15, the sizes of these fragments were identical (1.4-kb *Hinc*II and 0.9-kb *Hind*III fragments). However, in mutant 7, the fragments were 1.2-kb *Hinc*II and 0.7-kb *Hind*III fragments. Further characterization of the mutant *p30* structural genes was accomplished by sequence anal-

FIG. 2. Hybridization of ^{32}P -labeled wild-type HA^+ *M. pneumoniae* P30 structural gene (900-bp *Hind*III fragment) to wild-type HA^+ *M. pneumoniae* and class II HA^- mutant 3, 7, and 15 genomic DNAs digested with *Bam*HI (B), *Eco*RI (E), *Hind*III (H), and *Pst*I (P). Molecular sizes (in kilobases) are shown at the left. Note the difference between the *Hind*III fragments of the wild type and mutants 3 and 15 (900 bp) and mutant 7 (700 bp).

ysis of both strands of mutants 3, 7, and 15 and comparison to the wild-type *p30* gene sequence. The *p30* genes of mutants 3 and 15 revealed no sequence alterations compared to wild-type *p30*, although these mutants were incapable of synthesizing

P30-related peptides. A 1.0-kb DNA fragment upstream of the starting codon of the *p30* structural genes of mutants 3 and 15 was also sequenced, and no differences were observed. Sequence analysis of the mutant 7 *p30* gene, however, revealed an open reading frame of 681 nucleotides encoding a peptide with a deduced 227-amino-acid sequence and a calculated molecular mass of 24,823 Da (P25). Figure 4 compares the nucleotide and deduced amino acid sequences of the wild-type P30 adhesin and the P25-truncated peptide of mutant 7.

Comparison of proline-rich repeats at the carboxy end of the wild-type P30 and mutant 7 P25 proteins. One of the unusual properties of the P30 adhesin of wild-type *M. pneumoniae* is the presence of three different types of proline-rich, 6-amino-acid repeats at the carboxy terminus (10). Figure 5A

FIG. 3. Comparison of the P30-containing *Pst*I fragments of wild-type HA^+ and HA^- mutant mycoplasmas. The *Pst*I fragments were digested with *Hinc*II (left) and *Hind*III (right). The arrows indicate the positive bands that hybridized to the wild-type HA^+ P30 structural gene. Molecular sizes (in kilobase pairs) are shown at the left.

<i>p30</i> , reading frame 1			
529	CCCGGTATGGCGCCTCGTCCAGGTATGCCGCCACCCCGGTATGGCTCCAAGA	582	
177	P G M A P R P G M P P H P G M A P R	194	
	A B A		
583	CCTGGTTTCCACCTCAACCCGGTATGGCGCCTCGT*CCAGGTATGGCGCCACAC	636	
195	P G F P P Q P G M A P R P G M P P H	212	
	C A B		
637	CCCGGTATGGCTCCAAGACCTGGTTTCCACCTCAACCCGGTATGGCGCCTCGT	690	
213	P G M A P R P G F P P Q P G M A P R	230	
	A C A		
691	CCAGGCATGCCGCCACCCCGGTATGGCTCCAAGACCTGGTTTCCACCTCAA	744	
231	P G M P P H P G M A P R P G F P P Q	248	
	B A C		
745	CCCGGTATGGCGCCTCGT*CCCGGAATGCAACCACACGCTCTGGCATGCCACCC	798	
249	P G M A P R P G M Q P P R P G M P P	266	
	A		
799	CAACCCGGTTTCCACCAAAACGCTAA[TTAGTGCTTCAAG ATGACAGATAA]	850	
267	Q P G F P P K R - <i>hmv3</i> , reading frame3 M T D K	274	

FIG. 4. Nucleotide and amino acid analyses of *M. pneumoniae* wild-type P30 adhesin and the truncated P25-related peptide of mutant 7 (10). Only the C-terminal, proline-rich repeat region beginning with nucleotide position 529 in *p30* is shown. The numbers on the left represent the beginning of the nucleotide (top) and the amino acid residue (bottom), and the numbers on the right represent the end of the nucleotide (top) and the amino acid residue (bottom). Proline-rich repeats are underlined, and identical repeats are designated A, B, or C as in Fig. 5A. The asterisk denotes deletion of nucleotides 619 to 762 with corresponding loss of amino acids 207 to 254 in mutant 7. The 13-bp intervening sequence and the start of *hmv3* are bracketed.

FIG. 5. (A) Schematic representation of the wild-type P30 adhesin and mutant P25-related peptide of *M. pneumoniae*. The repeat domains are indicated by the letters A (amino acids PGMAPR), B (PGMPPH), and C (PGFPPQ). Synthetic peptides composed of domains A and B and A and C were used to generate rabbit monospecific antibody reagents (see Materials and Methods). The asterisk indicates a nonrepetitive, non-proline-rich upstream sequence (amino acids 106 to 120 [RLLEEKERQEQLAEQ]) against which antisera were also raised. (B) Hydrophilicity plot of the deduced amino acid sequences of the functional P30 adhesin and its nonfunctional truncated 25-kDa counterpart (P25) determined by the method of Hopp and Woods (17). On the abscissa, positive numbers represent hydrophilic domains of the protein and negative numbers indicate hydrophobic domains; ordinate values are amino acid residues.

schematically contrasts the wild-type P30 adhesin, with its proline-rich 13-repeat units, with the mutant 7 truncated P25-related peptide. This latter deletion is very much apparent in the hydrophilicity plots of the wild-type and mutant 7 P30-related peptides (Fig. 5B). The truncated 25-kDa peptide of mutant 7 also contained each of the three different proline-rich repeats. However, repeat A (PGMAPR) was present seven times in the wild type and only three times in mutant 7; repeats B (PGMPPH) and C (PGFPPQ) were present three times in the wild type and only once in mutant 7. Interestingly, the deletion of the 144-bp comprising the eight repeat units in mutant 7 was in frame and did not affect the downstream reading frame of the cytoadherence accessory *hmv-3* gene (Fig. 4).

Surface localization of the P30 proline-rich repeat domain. Whole-cell mycoplasma RIP was performed with antibodies generated against the proline-rich A-B and A-C repeat regions and an upstream non-proline-rich, hydrophilic region (Fig. 5A). As shown in Fig. 6B, antibodies reactive against the proline-rich A-B repeat region precipitated a 30-kDa protein from wild-type *M. pneumoniae*, a 25-kDa protein from mutant 7, and no peptides from mutant 15. An identical pattern was observed when antibodies generated against the A-C repeat region were

used (data not shown). Antibodies generated against the upstream non-proline-rich region and prebleeding control sera detected no surface-accessible epitopes from either the wild-type or mutant mycoplasmas (Fig. 6A). Consistent with the RIP analysis (Fig. 6), the non-proline-rich, hydrophilic region (amino acids 106 to 120) is located between two membrane-spanning domains (amino acids 72 to 97 and 122 to 150), suggesting an intracytoplasmic orientation.

Cross-reactive epitopes shared between the P30 adhesin and mammalian proteins. Many regions of the P30 adhesin exhibit significant amino acid homology to eucaryotic structural proteins (2, 7, 10). For example, the A-B-C proline-rich, surface-exposed repeat domains and the upstream, surface-inaccessible, highly hydrophilic region depicted in Fig. 5A and B exhibit 42 to 56% amino acid identity with keratin, myosin, and fibrinogen. As described in Materials and Methods, rabbit monospecific antibodies directed against these P30 regions were generated (Fig. 5A), and these sera were pooled and incubated with human keratin, myosin, and fibrinogen. Figure 7 demonstrates the observed immunologic cross-reactivity between these P30 epitopes and human structural proteins. Individually, antibodies generated to the A-B proline-rich repeats reacted strongly with human keratin, myosin, and fibrinogen; antibodies to the A-C proline-rich region reacted strongly with fibrinogen; and antibodies to the upstream, nonrepetitive, non-proline-rich region reacted strongly with keratin, myosin, actin, and troponin (data not shown). Also, goat antibodies generated against human fibrinogen cross-reacted with the wild-type P30 adhesin and the P25-truncated peptide of mutant 7 and not with mutant 3 peptides (Fig. 8). Furthermore, representative sera from patients with confirmed *M. pneumoniae*-mediated respiratory infections exhibited increased titers of antibodies to human structural proteins (41; unpublished data).

FIG. 6. Whole-cell RIP of intact wild-type and mutant *M. pneumoniae* with rabbit anti-P30 monospecific antibodies raised against synthetic peptides described in the legend to Fig. 5A. (A) Immunoblot with an antibody reactive against the upstream, non-proline-rich, N-terminal sequence. (B) Immunoblot with an antibody reactive against the A-B proline-rich, carboxy-terminal repeat region. Results identical to those in panel B were obtained with an antiserum to the A-C region. Pb, prebleeding serum; Im, immune serum. The numbers to the left are molecular sizes in kilodaltons. The asterisk indicates the position of the P30 (30-kDa) adhesin of *M. pneumoniae*.

FIG. 7. Immunoblot of wild-type *M. pneumoniae* proteins and human structural proteins with anti-P30-monospecific antibody reagents (see the legend to Fig. 5A, and Materials and Methods). The three anti-P30 rabbit sera described in the legend to Fig. 5A were pooled at a final dilution of 1:1,000. Ponceau staining of the human structural proteins revealed similar patterns. Pooled pre-immune rabbit sera failed to cross-react with the human structural proteins or *M. pneumoniae* proteins. The numbers at the left are the molecular weights (in thousands) of the proteins used as standards.

DISCUSSION

The tip-mediated adherence of *M. pneumoniae* to eucaryotic target cells is complex (2, 4, 7). It appears that mycoplasma adhesins and cytodherence accessory proteins interact cooperatively to provide a structural network that permits the proper folding and functional integrity of the adhesins, which cluster at the differentiated tip organelle, and mediate mycoplasma colonization of the respiratory epithelium and other tissue sites. Distinct mutant classes that lack these proteins display a noncytadhering, avirulent phenotype and abnormal tip structures (2–4, 7, 22, 23, 38). Other evidence further reinforces the complexity of mycoplasma cytodherence, including the existence of multiple operons and distinct genetic loci that encode the cytodherence-related proteins, the requirement for intact and metabolically active mycoplasmas for parasitism of host cell surfaces, the role of mycoplasmal cyclophilins in growth and host cell-mycoplasma interactions, and the ability of mycoplasmas to enter the intracellular spaces of host cells follow-

ing signal transduction events (2, 5, 7, 8, 15, 20–23, 31, 33, 34, 36, 37).

The detection of spontaneously arising, noncytadhering mutants of *M. pneumoniae* that were deficient only in the expression of the intact P30 adhesin reinforced the essential role of P30 in cytodherence and virulence. Two subclasses of P30-deficient mutants were identified, one lacking P30 or P30-related peptides (mutants 3 and 15) and the other displaying a truncated P30 peptide, i.e., P25 (25 kDa, mutant 7) (Fig. 1, 4, and 5). Sequence analysis of the *p30* gene in mutants 3 and 15, which lacked P30 or P30-related peptides, revealed an intact *p30* gene without sequence alterations, including adjacent upstream and downstream regions (Fig. 2 and 3 and unpublished data). However, similar analysis of mutant 7, which expressed the truncated P25 mutant peptide, revealed the deletion of 144 nucleotides corresponding to the region which encoded 8 of 13 proline-rich repeat sequences (Fig. 2 to 5).

Other evidence indicates that the *p30* gene, like the *p1* adhesin gene of *M. pneumoniae* (7, 16, 42) and the *p140* and *p32* adhesin genes of *M. genitalium* (20, 32), is part of a multiple-gene operon. The fact that we detected cytodherence accessory proteins HMW3 and HMW1 in the P30-deficient mutants (2, 7) suggests that mutations associated with expression of the *p30* gene in mutants 3 and 15, or in the region encoding the carboxy-terminal domain of mutant 7, did not affect the expression of the HMW proteins. Possibly, the transcription start site for *hmw-3* lies in the carboxy-terminal region of *p30* and an in-frame deletion upstream of the start site, as in mutant 7, may not alter the expression of the downstream *hmw-3* gene. Ribosomal bypassing, which leads to the expression of downstream genes on different reading frames of a multigene operon, has been demonstrated in procaryotes (1, 13, 20, 32). Consistent with this possibility is the existence of a potential stem-loop structure at the 3' end of the *p30* gene between nucleotides 806 and 820, which may permit the ribosomal bypassing of specific nucleotides and the expression of HMW3 on a different reading frame (Fig. 4). Also, the expression of the *hmw-1* gene, which is located 4.3 kbp downstream of *hmw-3*, may be unrelated to P30 (12). Furthermore, regulatory sequences located either upstream or downstream of the *p30* operon may be involved in HMW expression, consistent with evidence that cytodherence regulatory regions exist that are distinct from the loci containing specific adhesins and cytodherence accessory proteins (16, 34). A recent report of a spontaneous, hemadsorption-negative *M. pneumoniae* mutant that exhibited the P25 phenotype and lacked HMW1 was likely the result of two different mutations (25).

The isolation and characterization of spontaneous P30-deficient, noncytadhering mutants of *M. pneumoniae* clearly reinforced the critical dependence of cytodherence on the P30 adhesin. Furthermore, the essential role of the proline-rich carboxy-terminal 6-amino-acid repeats in the structural and functional properties of P30 was established. Interestingly, this repeat region shares substantial (55 to 67%) homology with the carboxy terminus of the P1 adhesin of *M. pneumoniae* (2, 10). Since both the P1 and P30 proteins densely cluster at the tip organelle of virulent *M. pneumoniae* and are essential for cytodherence (2, 3, 6, 7, 24), their structural and functional cooperativity appears to be critical to the successful outcome of mycoplasma cytodherence. The special role of prolines in protein folding and the location of proline pairs (i.e., X-Pro-Pro-X), which are associated with β -coil regions of proteins and other chain conformations (26), in and near the COOH terminus of P30 (i.e., 11 of 12 proline pairs occur in the carboxy-terminal half of P30) strongly suggest that these proline-rich domains and their spatial organization control the func-

FIG. 8. Immunoblot of *M. pneumoniae* wild-type and mutant 3 and 7 proteins reacted with goat anti-fibrinogen serum diluted 1:100 and an anti-P30 MAb diluted 1:1,000. Lanes: 1 and 4, wild type; 2 and 5, mutant 7; 3 and 6, mutant 3. Preimmune goat sera failed to cross-react with *M. pneumoniae* proteins.

tionality of adhesins P30 and P1. MAbs reactive against P1 or P30 inhibit mycoplasma cytodherence, further implicating the anatomical interdependency of these proteins (2, 7, 24). Additional information suggests that these adhesins require several other accessory proteins to anchor and orient themselves in the membrane (2, 3, 7, 37). Recently, we described the possible role in cytodherence of peptidyl-prolyl isomerases, i.e., cyclophilins, that catalyze *cis-trans* interconversions and folding of proline-rich proteins, such as the cytodherence-related proteins of mycoplasmas (33). In the latter report, we implicated these prolyl isomerases in key regulatory mechanisms that influence mycoplasma cytodherence, virulence, and growth. It is also possible that mycoplasma cyclophilins are incorporated directly into the cytodherence-related protein complex for stabilization and activation. Therefore, the cooperation of this multiprotein network dictates spatial order, which in turn defines the operative tip organelle. Ultimately, the interaction of P1, P30, and the cytodherence accessory proteins, plus the possible function of cyclophilins, may lead to the proper folding and orientation of the proline-rich proteins at the mycoplasma tip. The P25 deletion that results in the loss of 8 of the 13 proline-rich repeats may disrupt or destabilize the ordered protein topography and prevent cyclophilin interactions. It appears that the proline-rich repeat domains at the carboxy terminus of the P30 adhesin have a global impact on mycoplasma tip structure and function and subsequent tissue colonization. Clearly, the conservation of the entire complement of P30 proline-rich repeats is essential for cytodherence and spontaneous partial loss of these repeats leads to a non-cytadhering phenotype.

Both adhesins P1 and P30 share homology with eucaryotic structural proteins like fibrinogen, keratin, myosin, and actin (2, 7, 8, 10, 39, 41). The possibility exists that these mycoplasma proteins, along with other cytodherence-related proteins, make up a primitive cytoskeleton-like network that functions to maintain the biological and morphological integrity of the differentiated tip organelle, confer unidirectional motility, and permit successful parasitism of host cells via receptor-mediated mechanisms and signal transduction events (2, 5, 7, 15, 28). The conservation of proline-rich repeats among the adhesins of pathogenic mycoplasmas provides convincing evidence of their biological and evolutionary significance (7, 11, 20, 32). Recently, we described a mechanism by which postinfectious autoimmunity may result from the molecular mimicry that exists between mycoplasma adhesins and eucaryotic matrix components (7, 8, 41). Consistent with these observations, we showed that monospecific antibodies generated against specific domains of the P30 adhesin cross-reacted with human fibrinogen, keratin, and myosin (Fig. 7). Further evidence of this shared immunoreactivity was established by the use of antifibrinogen antibodies, which bound to the wild-type P30 adhesin and mutant P25 peptide (Fig. 8). These results reinforce the clinical association between autoimmune disorders and mycoplasma infections (7–9, 41). Further analysis of the domains of mycoplasma adhesins and cytodherence accessory proteins should provide insights into protein-protein interactions mediated by critical proline-rich regions and lead to a fundamental understanding of the mechanisms by which mycoplasma proteins regulate cytodherence and the subsequent steps associated with disease pathogenesis.

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