Adherence epitopes of *Mycoplasma genitalium* adhesin

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(Received 18 February 1992; revised 11 May 1992; accepted 22 May 1992)

The adherence-mediating sites of the 153 kDa adhesin of *Mycoplasma genitalium* (MgPa-protein) were characterized at the amino acid sequence level using six monoclonal anti-MgPa antibodies which showed adherence-inhibiting activity. For characterization of the regions to which antibody bound, three segments of the adhesin (N-terminal region, a D1-domain located approximately in the middle of the molecule and a D2-domain located near to the C-terminus) were synthesized as overlapping octapeptides. These regions were chosen in analogy to the three domains of *Mycoplasma pneumoniae* that are involved in the adhesion process. Whereas two monoclonal antibodies (mAb 5B11 and mAb 6F3) bound exclusively to an epitope in the N-region, mAb 3B7 and mAb 6A2 reacted with two distinct epitopes of the D2-domain only. Binding to short synthetic peptides of different regions was analysed for mAb 3A12 (N-region and D1-region) and mAb 2B6 (N-region and D2-region). Close proximity of the N-region and the D2-region in the native MgPa-protein of *M. genitalium* was indicated in a competitive ELISA test, using freshly harvested *M. genitalium* cells. Epitope mapping and competition experiments with monoclonal anti-MgPa antibodies revealed interesting differences in the adherence-mediating sites of MgPa and the adhesin (P1-protein) of *M. pneumoniae*. Whereas a three-dimensional arrangement of protein loops is suggested for both native adhesins, the MgPa-protein and the P1-protein adherence-mediating epitopes are located in non-homologous regions of these two related proteins. Thus, the two mycoplasma species appear adapted to different host epithelial cells, i.e. to those of the human urogenital tract as the main binding site for *M. genitalium* and to those of the respiratory tract as the binding site for *M. pneumoniae*.

Introduction

Adhesion to host cells of the mycoplasma species *M. genitalium* and *M. pneumoniae* is a prerequisite for colonization of epithelia and for further multiplication. The lack of a cell wall and therefore of any of the structures normally responsible for attachment of prokaryotes suggests that mycoplasmal adhesins are components of their cytoplasmic membranes. Recent studies on adhesins of *M. pneumoniae* and *M. genitalium* revealed species-specific differences, but common protein molecules which are the attachment sites of mycoplasmas to host cells (Razin & Jacobs, 1992). Interestingly, these two different protein adhesins are both concentrated in specialized tip structures of adherent filamentous mycoplasma cells (Feldner *et al.*, 1982; Hu *et al.*, 1987).

A comparison of the molecular properties of the adhesin of *M. genitalium* (MgPa-protein) with the

P1-adhesin of *M. pneumoniae* reveals: (i) the MgPa-protein, with a relative molecular mass of 153 kDa, is smaller than the P1-adhesin (170 kDa) (Mader *et al.*, 1991); (ii) both proteins contain no cysteine residues indicating a flexible structure and (iii) both proteins possess a proline-rich C-terminus indicating a rigid structure for the C-terminal region; (iv) both proteins are immunodominant antigens during natural infections (Jacobs *et al.*, 1991); and (v) matching the amino acid sequences of the MgPa-protein of *M. genitalium* and of the P1-protein of *M. pneumoniae* for maximum amino acid homology reveals 747 amino acids in common, which are clustered in extended regions (Mader *et al.*, 1991). Inserts of single amino acids or even of protein loops of several amino acids (up to 13 amino acids in length) of the P1-protein are lacking in the smaller MgPa-protein sequence; and (vi) moreover, structural similarities were found using computer-based predictions of membrane-anchoring helices according to Eisenberg *et al.* (1984). These membrane-associated domains of both proteins are located in corresponding regions of the primary sequences (Mader *et al.*, 1991).

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Abbreviations: mAb, monoclonal antibody; pin, solid-phase carrier.

Fig. 1. The binding sites (arrowheads) of adherence-inhibiting monoclonal antibodies within the N-region, and the D1- and D2-domains of the MgPa-protein of *M. genitalium* and the P1-protein of *M. pneumoniae* (Gerstenecker & Jacobs, 1990). Anti-MgPa mAbs bound to non-homologous regions of the *M. genitalium* MgPa sequence compared to the *M. pneumoniae* P1 amino acid sequence. Homologous amino acids within the N-region, D1- and D2-domains of both proteins are connected and additional amino acids of the larger P1-protein are shown as spikes of length proportional to the number of amino acids involved. Almost all of the anti-P1-protein mAbs bound to these extra amino acid residues or to polypeptides with no corresponding sequence of the related *M. genitalium* adhesin sequence. The areas M₁–M₆ indicate predicted membrane-associated helices of both proteins according to Eisenberg *et al.* (1984).

In a recent study it was shown that the topographical arrangement of the P1-adherence-mediating complex is composed of three different protein loops. These contain adherence-mediating sites which are juxtaposed by protein folding to form the tertiary structure of the native P1-adhesin (Gerstenecker & Jacobs, 1990).

Since the P1-protein and the MgPa-protein show close similarities, it was of interest to examine whether the functional adherent sites of the MgPa-protein of *M. genitalium* are comparable with the known sites of the P1-protein of *M. pneumoniae*. By analogy to the strategy employed for P1-analysis, anti-MgPa adherence-inhibiting mAbs were established. The binding sites of these mAbs were characterized using synthetic octapeptides of three domains of the adhesin sequence of *M. genitalium*. Furthermore, the topology of defined epitopes in the native MgPa-protein was characterized using a competitive enzyme immunoassay with viable *M. genitalium* cells.

Methods

Peptide synthesis. *N*^α-(9-fluorenylmethyloxycarbonyl) (Fmoc) amino acid chemistry (Dryland & Sheppard, 1986; Sheppard, 1986) was used for the preparation of synthetic octapeptides as antigens. Peptides with an overlapping sequence of seven amino acids were synthesized on to solid phase carriers (pins) using the epitope scanning kit (CRB, Cambridge) according to the method of Geysen *et al.* (1984).

For analysis of the mAb binding sites, three different regions of the MgPa-protein were synthesized which corresponded to the adherence domains of the P1-protein of *M. pneumoniae* (Fig. 1): the N-region

(amino acids 200 to 384), the D1-region (769 to 954) and the D2-region (1123 to 1360) (Dallo *et al.*, 1989; Gerstenecker & Jacobs, 1990).

Adherence-inhibiting mAbs. Two different antigen preparations were used for the immunization of 3-month old Balb/c mice. For the first injection freshly harvested glass-adherent *M. genitalium* cells (strain G 37) cultured in Hayflick's modification of Edward's medium (Hayflick, 1965) were injected intraperitoneally (1 mg total protein per injection). After 1 month two further intraperitoneal injections of the purified MgPa-protein (100 µg per injection) (Mader *et al.*, 1991) were applied at weekly intervals. All preparations were used without any adjuvant. Four days after the final MgPa injection spleen cells were removed for fusion with X63-Ag8.653 myeloma cells (de St. Groth & Scheidegger, 1980). Viable hybridoma clones were recovered from four fusion experiments.

The secreted antibodies were screened for the following activities: (i) binding to purified MgPa-protein or to sonicated whole mycoplasma cells, in enzyme immunoassays (Jacobs *et al.*, 1991); (ii) binding to MgPa-protein in immunoblots of *M. genitalium* protein (Jacobs *et al.*, 1986); (iii) adherence inhibition of sheep red blood cells to viable *M. genitalium* cells (Jacobs *et al.*, 1985); and (iv) positive immunofluorescence activity directed to the distinct tip structure of *M. genitalium* cells using glass-adherent mycoplasmas (Feldner *et al.*, 1982).

The appropriate hybridomas were subcloned using a limiting dilution technique and cultured *in vitro*. Monoclonal antibodies were precipitated from the culture supernatants, dialysed and stored at 4 °C (Gerstenecker & Jacobs, 1990).

The immunoglobulin subclasses of the different mAbs were determined using rabbit anti-mouse reagents according to the manufacturer's instructions (ICN-Immunobiologicals).

Epitope scanning. Supernatants from the different hybridoma clones were enriched with blocking buffer [1% (w/v) BSA, 1% (w/v) ovalbumin and 1% (v/v) Tween 20 in PBS (0.14 M-NaCl, 0.01 M-sodium phosphate, pH 7.2)] and incubated overnight with the solid-phase (pin)-immobilized octapeptides at 4 °C. The pins were washed with PBS/Tween 20 and incubated with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulins, Dianova)

diluted in blocking buffer. After final washing, the pins were incubated in substrate solution (10 mg *p*-nitrophenyl phosphate ml⁻¹ in diethanolamine buffer, pH 9.6) for 1 h at 37 °C (Gerstenecker & Jacobs, 1990), and phosphatase activity was measured as the increase in *A*₄₀₅ (Titertek Multiscan, Flow Laboratories).

mAb competition experiments. Glass-adherent *M. genitalium* cells were scraped off and washed with PBS. The freshly harvested cell suspension was adjusted to a protein concentration of 100 µg ml⁻¹. Aliquots of the cell suspension (100 µl) were incubated on microtitre plates (pretreated with poly(L-lysine) (50 µg per well) and glutaraldehyde (100 µl of a 20%, v/v, solution in deionized water) for 2 h at 37 °C under gentle rotation (Gerstenecker & Jacobs, 1990). The plates were washed with pre-warmed PBS. Remaining aldehyde groups were blocked with 3% BSA/PBS for 1 h at 37 °C.

Unlabelled mAb (100 µl per well) was added and incubated for 2 h at 37 °C under maximum antigen saturation conditions. Biotin-labelled mAb (500 ng in 20 µl per well) was then added and incubated for 1 h at 37 °C. The plates were washed three times with PBS/0.05% Tween-20. The binding of the biotin-labelled mAb was detected with streptavidin/peroxidase complexes (200 ng per well, Sigma). Substrate (H₂O₂/tetramethylbenzidine) was added and colour development (*A*₄₅₀) measured after incubation for 30 min at room temperature.

Results

Four fusion experiments revealed 181 hybridoma clones which produced antibodies reacting with the sonicated *M. genitalium* whole cell antigen preparation in an enzyme immunoassay. Sixty-four of these supernatants also recognized the purified MgPa-protein used as a defined antigen. Only six of these latter antibodies inhibited the adherence of sheep erythrocytes to viable *M. genitalium* cells in an adherence inhibition assay (Table 1). Additionally, these adherence inhibiting antibodies showed a specific binding to the MgPa-protein in Western immunoblots and a distinct positive signal in the immunofluorescence test (results not shown). Five of these mAbs belonged to the IgG₁ κ immunoglobulin subclass; mAb 5B11 is an IgM antibody (Table 1).

For epitope analysis the six adherence-inhibiting mAbs were tested for binding to overlapping octapeptides representing three MgPa regions analogous to the adherence-mediating sites of the P1-protein of *M. pneumoniae* (Fig. 1). The first region (N-region) is located near the N-terminus of the mature MgPa-protein, in front of two predicted membrane-associated helices (M₁ and M₂ in Fig. 1). The D1-domain (D1) is located almost in the middle of the MgPa-amino acid sequence, whereas the D2-domain (D2) is located next to the hydrophobic C-terminal M₆-sequence.

Binding of mAbs to overlapping immobilized octapeptides was distinct and specific (Fig. 2). mAb 6F3 exclusively recognized an octapeptide [NH₂-(245)-P-V-K-D-S-S-K-Q] located in the N-region (Table 1). Antibody 5B11 also bound to an N-region sequence

Table 1. Different anti-MgPa monoclonal antibodies (mAb) with positive adherence inhibition in the adherence-inhibiting assay (AIA) and their activity to synthetic overlapping solid-phase octapeptides of the N-region, D1- and D2-domains

mAb	Ig-sub-classes	AIA	First amino acid of the octapeptide in:*		
			N-region (200–384)	D1-domain (769–954)	D2-domain (1123–1360)
6F3	IgG ₁	+	245		
3A12	IgG ₁	+	259	782	
5B11	IgM	+	274		
2B6	IgG ₁	+	349		1183
3B7	IgG ₁	+			1183
6A2	IgG ₁	+			1323

* Amino acids are numbered from the N-terminus.

[NH₂-(274)-A-K-A-L-K-V-E-V] located near to the mAb 6F3 binding site (Table 1). Similarly, mAb 6A2 [NH₂-(1323)-P-Q-T-Q-Q-F-I-P] and mAb 3B7 [NH₂-(1183)-S-E-G-L-K-E-E-T] bound to different octapeptides, but located in the D2-domain. In contrast, each of the mAbs 3A12 and 2B6 reacted with two distinct octapeptides. mAb 2B6 bound to an octapeptide [NH₂-(349)-S-E-N-H-T-A-F-G] of the N-region and to an octapeptide of the D2-domain [NH₂-(1183)-S-E-G-L-K-E-E-T]. The latter binding site was also the binding site of mAb 3B7. mAb 3A12 bound to an amino acid sequence within the N-region [NH₂-(259)-T-T-A-S-S-M-S-S] and to the D1-domain sequence [NH₂-(782)-S-P-S-T-S-A-S-S]. To exclude the possible presence of two different antibody-producing hybridoma cell lines within clones mAb 2B6 and mAb 3A12, these were further subcloned and retested. The dual binding sites of both mAbs were confirmed.

For investigation of the topographical arrangement of the different mAb binding sites in the native MgPa-protein, the adherence-inhibiting mAbs were tested in a competitive immunoassay using intact *M. genitalium* cells as antigen. Unlabelled mAbs were preincubated with *M. genitalium* cells under antigen saturation conditions. In a second incubation step biotinylated mAbs competing with the unlabelled mAb for MgPa-binding sites were applied (Table 2 and Fig. 3a). It was found that: (i) preincubation with mAb 2B6 (dual binding sites in the N-region and the D2-domain) completely inhibited binding of mAbs 6A2 (D2-domain) and 3B7 (D2-domain), and partially inhibited binding of mAb 5B11 (N-region); (ii) the IgM antibody mAb 5B11 (N-region) blocked the binding of all other adherence-inhibiting mAbs; (iii) mAb 6A2 (D2-domain) inhibited the binding of mAbs 2B6, 3B7, 5B11 and 6F3 (Fig. 3); (iv) preincubation of *M. genitalium* cells with mAb 3B7 (only one binding site in the D2-domain which is also

Fig. 2. ELISA activity of mAb 6F3 culture supernatants with synthetic, overlapping pin-immobilized octapeptides of the MgPa amino acid sequence (amino acids 200 to 280) of the N-region. Each bar represents the activity to a single octapeptide of this sequence.

Table 2. Competition-ELISA for topological mapping of the MgPA-protein adhesin structure with viable *M. genitalium* cells and adherence-inhibiting mAbs

Inhibition (I) was defined arbitrarily as follows: $I \leq 20\%$, no inhibition (0); $I = 21-50\%$, partial inhibition (+/-); $I > 50\%$, inhibition (+). Undisturbed binding of each mAb was used as a non-competition control and is defined as having 100% binding efficiency and producing 0% inhibition. The test included self-competition controls.

Preincubation with mAb	Inhibition of probed biotin-labelled mAb:*					
	5B11 (N)	6F3 (N)	3A12 (N/D1)	2B6 (N/D2)	3B7 (D2)	6A2 (D2)
5B11	+	+	+	+	+	+
6F3	+/-	+	+/-	0	0	+/-
3A12	+/-	+	+	0	+/-	+/-
2B6	+/-	+	0	+	+	+
3B7	+	+/-	0	+/-	+	+
6A2	+	+	+/-	+	+	+

* N, N-region; D1, D1-domain; D2, D2-domain.

recognized by mAb 2B6) partially inhibited binding of mAb 2B6 and completely inhibited binding of mAbs 6A2 and 5B11; (v) preincubation with mAb 6F3 resulted in no or only partial inhibition of the binding of other mAbs. On the other hand, binding of mAb 6F3 was completely inhibited by all other mAbs, indicating that its binding site is probably located on a protein segment that is obscured under *in vivo* conditions by other, more surface-exposed, binding sites; and (vi) mAb 3A12 whose N-region binding site is located between those of

Fig. 3. The binding sites of the different adherence inhibiting mAbs on the N-region, the D1- and D2-domains and the possible topographical arrangement of these positions according to the analysis of the results of competitive ELISA (Table 2) in (a) a two-dimensional and (b) a three-dimensional cartoon. The dual binding sites of mAbs 2B6 and 3A12 are connected in (a).

mAb 5B11 (N-region) and mAb 6F3 (N-region) on the linear amino acid sequence, showed a more efficient blocking of mAb 6F3 than mAb 5B11. Since mAb 3A12 has a further binding site within the D1-domain, it was of interest to examine whether its binding could be blocked by the D2-binding mAbs (6A2, 2B6 and 3B7). However, none of these were able to block binding completely. In contrast, two of the mAbs with binding sites in the N-region (mAb 5B11 and mAb 6F3) did block mAb 3A12 binding, whereas mAb 2B6, which bound on the C-terminal end of the N-region, did not.

Discussion

In this study six adherence-inhibiting monoclonal anti-MgPa antibodies were used for epitope mapping and topological studies of adherence sites of the MgPa-adhesin of *M. genitalium*. At least two domains, the

N-terminal region and the D2-domain, which is located near the C-terminal end, seem to be involved in the adherence process. mAbs 2B6 and 5B11, both with N-region binding sites, and mAbs 6A2 and 2B6 (and 3B7) with D2-domain binding sites recognize epitopes which cover the 'corners' of the still hypothetical proposed adherence complex (Fig. 3b). Moreover, with this arrangement the binding sites for the adherence-inhibiting mAbs might be brought into close contact by protein folding. This hypothesis is supported by the results of competition experiments, which showed maximum reciprocal inhibition between mAbs with binding sites in the N-region and the D2-domain. The mapping experiments with epitopes are helpful for ready identification and further characterization of molecular structures with particular biological functions, i.e. adherence sites. Mapping results with antibodies recognizing more than one epitope may be interpreted as follows. (i) Since antibodies are mainly directed to discontinuous binding sites (van Regenmortel, 1989), the antibody response may be directed to more than one amino acid sequence if the avidity of the mAb is high enough to allow firm binding to the different fragments. These fragments may be located on different protein loops in close proximity, each contributing short amino acid sequences to the complete epitope recognized by the mAb. (ii) A second reasonable explanation may be that both binding sites contain closely related or identical amino acid sequences. With respect to mAb 2B6, conformational binding of the antibody would support the hypothesis of close contact between the N-region and the D2-domain. However, it cannot be excluded that the first two identical N-terminal amino acid residues of the octapeptides [i.e. serine and glutamic acid, underlined within the octapeptide sequences (see Results)] may be sufficient for recognition by mAb 2B6. This would indicate binding to two similar epitopes in the MgPa-protein that are located at different sites in this polypeptide. In a first replacement experiment in which the negatively charged amino acid NH_2 -(1184) glutamic acid was exchanged by the non-polar amino acid alanine in both peptides, it was shown that the antibody completely lost the capacity to react with the modified epitopes (results not shown). This may indicate that the negatively charged amino acid in this position is an essential contact residue in the mAb 2B6 recognition site. Since both modified peptides totally lost their antigenicity, it seems to be more likely that mAb 2B6 is directed towards a very short amino acid sequence, rather than towards a discontinuous epitope, composed of different amino acids from both octapeptides. Since the signal intensity of this mAb versus the N-region peptide was about one-third higher than that for the D2-domain peptide (N-region $A_{405} = 3.0$ compared to

D2-domain $A_{405} = 2.1$), it has to be shown whether further serine-glutamic acid-attached amino acid residues may contribute to the elevated N-region signal.

Epitope mapping revealed an unexpected result: mAb 3B7 bound to the NH_2 -(1183)-octapeptide which was also one of the binding sites of mAb 2B6. The inhibition experiments with these two mAbs showed that mAb 2B6 was able to block the binding of mAb 3B7 completely. On the contrary, preincubation with mAb 3B7 resulted only in a partial inhibition of mAb 2B6 binding, also indicating a strong affinity of mAb 2B6 for the N-region binding site under *in vivo* conditions. More interestingly, since mAb 3B7 was only partially able to block mAb 2B6 binding, it seems reasonable to assume that the binding site of mAb 3B7 is not directed to the first two amino acids of this octapeptide (NH_2 -S-E). mAb 3B7 might therefore be able to block mAb 2B6's D2-binding site sterically.

In contrast to the close proximity of the N-region and the D2-domain, one can only speculate about the location of the D1-domain in respect to the other two domains. One mAb (3A12) could be established with binding sites in the N- and in the D1-domain. Comparing the amino acid sequences of these octapeptides [NH_2 -(782)-S-P-S-T-S-A-S-S] and [NH_2 -(259)-T-T-A-S-S-M-S-S], the A-S-S residues were present in both peptides. The activity of this mAb to these two peptides seemed similar ($A_{405} = 3.0$), indicating that mAb 3A12 could not discriminate between the two different epitopes. The competition experiments showed that mAb 3A12 was not able to block the binding of mAb 2B6 and could only partially inhibit binding of mAbs 6A2, 3B7 and 5B11. If the binding site of mAb 3A12 is not located within the main adherence complex, this would also imply that the D1-domain (second binding site of mAb 3A12) does not contribute to the adherence complex. This adhesin complex may therefore differ from the adherence complex of the P1-protein of *M. pneumoniae* (Fig. 1). Furthermore, comparing the binding specificities of the anti-MgPa mAbs with those recently published for adherence-inhibiting anti-P1 mAbs, established against *M. pneumoniae* (Gerstenecker & Jacobs, 1990), it was found that: (i) all the defined binding sites of the different *M. genitalium*-inhibiting mAbs were located in non-homologous regions, in comparison to the amino acid sequences of the P1-protein; and (ii) that all different binding sites of adherence-inhibiting mAbs which were established against *M. pneumoniae* are located on non-homologous regions or even additional protein loops of the larger P1-protein of *M. pneumoniae* (Fig. 1). Moreover, cross-reaction experiments using anti-adherence mAbs, established against *M. genitalium*, showed no adherence inhibition activity of *M. pneumoniae* to erythrocytes. One non-adherence-

inhibiting anti-MgPa mAb showed a cross-reactivity with the P1-protein of *M. pneumoniae* in a Western immunoblot and a strong *M. pneumoniae* adherence-inhibiting activity (results not shown).

One may therefore speculate that the two species selected a common 'ancestor' class of proteins for adherence purposes when colonizing different compartments of the host, i.e. epithelial cells of the urogenital tract and of the respiratory tract by *M. genitalium* and *M. pneumoniae* respectively. Host receptor structures might be quite different, so that differing adaptation of adhesin binding sites was necessary. Whereas the receptor sites of *M. pneumoniae* are known to be glycoproteins and glycolipids, the uroepithelial receptor sites of *M. genitalium* are still undefined (for detailed information see the review of Razin & Jacobs, 1992). Such an adaptation to different epithelial cells might be enhanced by a flexible adherence complex, composed of different surface-exposed loops.

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