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Organization of multi-binding to host proteins: The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *Mycoplasma pneumoniae*

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ABSTRACT

Mycoplasma pneumoniae is a frequent cause of community-acquired infections of the human respiratory tract. During the evolutionary adaptation of the bacteria to the host, the genome of the pathogen is strongly reduced resulting in the loss of cell wall, limited metabolic pathways and a relatively small number of virulence factors. As interacting with host proteins, surface-exposed proteins with a primary function in cytosol-located processes of metabolism and regulation such as glycolytic enzymes, heat-shock proteins and chaperones have been considered as contributing to pathogenesis. Among these moonlighting proteins, some members are confirmed as binding to several host components. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *M. pneumoniae* is a typical example of such multi-binding proteins. To investigate the organization of these interactions, GAPDH was divided into four parts. Recombinant proteins were successfully expressed in *Escherichia coli* and polyclonal antisera were produced. Binding of full length and parts of GAPDH to human A549 cells was proven. Furthermore, interactions with human plasminogen, vitronectin, fibronectin and fibrinogen were demonstrated for nearly all recombinant GAPDH proteins. In the presence of these proteins, plasminogen can be activated to the protease plasmin. In contrast, the localization on the surface of bacterial cell was confirmed for the C-terminal part of GAPDH only. By using overlapping peptides covering this region, binding of the investigated host components to the sequence ^{326}QLVRVVNYCAKL^{337} was found. The results of the study suggest a prominent role of the surface-localized C-terminal part of GAPDH in associations with different human proteins indicating its importance for host-pathogen-interactions.

1. Introduction

Mycoplasma pneumoniae is a member of the bacterial class of mollicutes characterized by small genomes and lack of a classical cell wall. The species is a common cause of a broad range of infections of the human respiratory tract up to severe cases of interstitial pneumonia. In epidemic periods (every 3–7 years), between 20 and 40% of all community-acquired pneumonia infections can be attributed to *M. pneumoniae* (Waites et al., 2017). In addition, multi-facetted extrapulmonary manifestations have been described in the last years (Narita, 2016). With a genome size of 814 kb and 688 open reading frames, *M. pneumoniae* possesses limited metabolic capabilities (Kühner et al., 2009). However, the bacteria are perfectly adapted to the infection of the epithelium of the upper human respiratory tract and a parasitic, in many cases long-term, lifestyle in this niche exclusively. Known virulence factors include the complex adhesion apparatus accomplishing the adherence of bacteria to the target cells as first step of colonization (Hasselbring et al., 2006) and the expression of cell-damaging substances like the unique CARDS toxin as well hydrogen peroxides (Kannan and Baseman, 2006; Hames et al., 2009).

Recently, an increasing number of studies described bacterial proteins with primarily cytosol-located functions as occurring also on the surface of the cells. In this location, the proteins are able to bind to host components. This phenomenon was found not only in phylogenetically very different bacterial species (Gram-positives and -negatives) with and without pathogenic potential but also in protozoan parasites (Lama et al., 2009) and helminthes (González-Miguel et al., 2015) suggesting a common aspect of interaction between microorganisms and their hosts. Enzymes of metabolism (*e.g.* glycolytic enzymes) and proteins with function in cellular regulation (chaperones, elongation factors) are typical members of the so-called moonlighting proteins (Henderson, 2014). In bacterial species infecting humans, a broad spectrum of host factors binding to these proteins are demonstrated including plasminogen (plg), components of the extracellular matrix (ECM) like

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Interaction with human				References
Plasminogen	Fibrinogen	Fibronectin	Vitronectin	
+[a]	++	++	++	Gründel et al., 2016a; 2016b

[a] - + medium, ++ strong (based on comperative ELISA experiments)

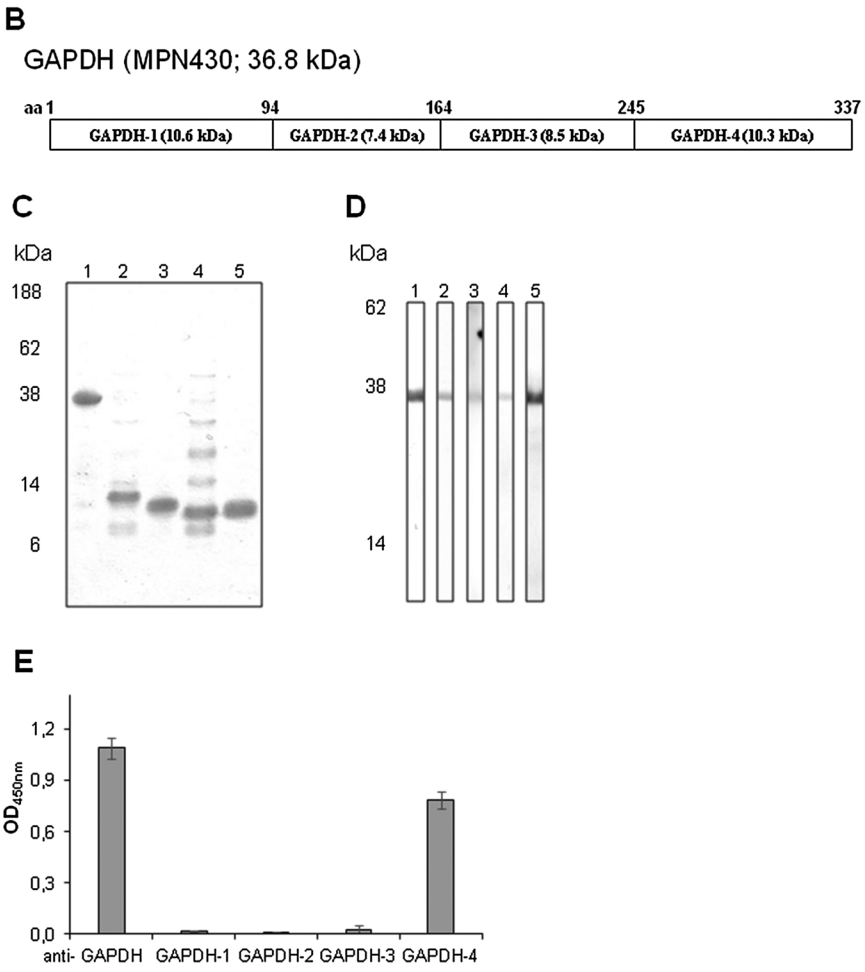

Fig. 1. General characterization of GAPDH of *M. pneumoniae* and production of recombinant proteins and polyclonal antisera. (A) Summary of known interactions of recombinant full-length GAPDH with selected host proteins. (B) Schematic illustration of recombinant protein parts of GAPDH investigated in the study. (C) Coomassie-stained polyacrylamide gel after electrophoretic separation of recombinant proteins (1 - full-length GAPDH, 2 - GAPDH-1, 3 - GAPDH-2, 4 - GAPDH-3, 5 - GAPDH-4). (D) Reactivity of guinea pig antisera to recombinant proteins against whole antigen of *M. pneumoniae* in immunoblot (1 - anti-GAPDH, 2 - anti-GAPDH-1, 3 - anti-GAPDH-2, 4 - anti-GAPDH-3, 5 - anti-GAPDH-4). (E) ELISA reactivity of antisera to whole antigen of *M. pneumoniae*. Means and standard deviations of eight replicates.

fibrinogen (fg), fibronectin (fc), vitronectin (vc), and factors of the complement system (Chagnot et al., 2012; Henderson and Martin, 2011; Henderson et al., 2011; Sanderson-Smith et al., 2012; Zipfel et al., 2013). In general, the complex interplay between the moonlighting proteins of bacteria and host factors are considered as contributing to successful colonization of hosts by influencing such important processes as adherence, immune escape and nutrient supply of microorganisms (Bhattacharya et al., 2012; Singh et al., 2010, 2012; Morgenthau et al., 2013). In the last years, moonlighting proteins have been confirmed also among mollicutes indicating that a classical cell wall is not a prerequisite for anchoring the proteins in surface structures of microorganisms. In *M. pneumoniae*, eight glycolytic enzymes (Gründel et al., 2016b), the chaperones DnaK and GroEL (Hagemann et al., 2017) and the elongation factor Tu (Widjaja et al., 2017) are characterized as surface-localized and able to interact with different host factors. Among these mycoplasmal proteins, several ones were found as interacting with more than a single host component. A representive member of this class of proteins is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showing binding to plg, fg, fc and vc (Gründel et al., 2016a, 2016b, Fig. 1A). Firstly described in streptococci (Pancholi and Fischetti, 1992), multi-binding of GAPDH was not only found in *M. pneumoniae* but also in different other species, like *Escherichia coli* (Egea et al., 2007), *Streptococcus suis* (Li et al., 2015), *Francisella tularensis* (Pavkova et al., 2017) and *Candida albicans* (Gozalbo et al., 1998), respectively. However, the molecular organization of multi-binding is poorly understood as the comparative binding characteristics to the different host factors, the defined binding sites of the bacterial protein and their accessibilities on the surface of bacteria are in most cases unknown. In the present study, we investigated different parts of GAPDH of *M. pneumoniae* with respect to their localization and association with human components to contribute to further understanding of the role of a typical moonlighting protein in host-pathogen interaction.

2. Materials and methods

2.1. Bacterial strains and human cells

M. pneumoniae strain M129 (ATCC 29,342) and *E. coli* strains NovaBlue and BL21(DE3) (Novagen) as well as A549 cells (human lung carcinoma cell line, ATCC CCL-185) were grown in cell culture flasks as described (Gründel et al., 2016b).

Table 1
Oligonucleotides for amplification of parts of GAPDH of *M. pneumoniae* (underlined: vector-specific sequence).

Oligonucleotide	Sequence (5' → 3')
MPgapVf	GAC GAC GAC AAG ATG CTA GCA AAG AGT AAG ACT ATC
Mpgap1Vr	GAG GAG AAG CCC GGT TTA ATT GTG TTC TGC CCA TGG C
Mpgap2Vf	GAC GAC GAC AAG ATT GAT ATT GTG GTT GAA TCC AC
Mpgap2Vr	GAG GAG AAG CCC GGT TTA TGG TGC TAA GCA ATT GGT GG
Mpgap3Vf	GAC GAC GAC AAG ATG GTA CAC GTC TTG GAA AAG AAC
Mpgap3Vr	GAG GAG AAG CCC GGT TTA CGA ACC AGT TAG TAC GGG AAC ACG
Mpgap4Vf	GAC GAC GAC AAG ATC GTT GAA CTT TGT GTA GCC C
MPgapVr	GAG GAG AAG CCC GGT TTA AAG CTT GGC ACA ATA GTT AAC

2.2. Recombinant production of full-length and parts of GAPDH of M. pneumoniae and polyclonal antibodies

Construction of the pET-30/LIC vector (Novagen) containing the full-length *gapdh* gene (*mpn430*) with the mutation of the single TGA codon and transformation of *E. coli* strains NovaBlue and BL21(DE3) were described in a previous report (Dumke et al., 2011). Freshly grown *E. coli* BL21(DE3) cells were harvested and the plasmid was prepared with the QIAprep spin miniprep kit (Qiagen) as recommended by the manufacturer. For expression of four recombinant proteins covering the complete *gapdh* gene (Fig. 1B), the plasmid was used as target to amplify the *gapdh* parts with the primer pairs summarized in Table 1. Determination of these protein parts was arbitrary and independent from the NAD binding and C-terminal domain of GAPDH separated by the CTTNC motif (amino acids157-161). Purification of PCR products, cloning into pET30/LIC vector, transformation of *E. coli* strains NovaBlue and BL21(DE3), expression and purification of the N-terminal 6xHis-tagged recombinant proteins were performed as reported (Dumke et al., 2011). The recombinant proteins were concentrated by vivaspin 6 centrifugal columns (MWCO 5000; Vivascience) and the protein concentration was measured with the BCA protein assay kit (Pierce). To confirm the quality of recombinant protein expression, the concentrated eluates were separated by SDS-PAGE (Novagen) and Coomassie staining (Merck) according to standard procedures. For immunoblotting, the separated recombinant proteins (100 µg/ml) were transferred to nitrocellulose membranes (Novagen) as recommended by the manufacturer.

For production of polyclonal antisera, guinea pigs (Charles River) were subcutaneously immunized with the recombinant proteins using Freund's adjuvant (Sigma). Animal experiments were proved by the ethical board of Landesdirektion Sachsen, Dresden, Germany (no. 24-9168.25-1/2011-1). Booster immunizations and serum collection were done as described previously (Dumke et al., 2011). To test the specificity of polyclonal antisera, separated and blotted proteins of *M. pneumoniae* whole cells (100 µg) were incubated with guinea pig antisera (1:10 or 1:500) and detection was performed with HRP-conjugated secondary antibody specific for guinea pig Ig (Dako; 1:2000). Quantitative reactivity of the polyclonal sera was tested in ELISA experiments as described recently (Gründel et al., 2016a). Briefly, cavities of 96-well plates (Greiner) were coated with *M. pneumoniae* total proteins (10 µg/ml carbonate buffer) and blocked with 10% fetal calf serum (Gibco). Wells were incubated with sera to recombinant full-length GAPDH and four GAPDH parts (1:250 each). For detection, peroxidase-conjugated anti-guinea pig Ig antibody (1:750) was used. Reaction of substrate (TMB super slow; Sigma) was stopped with 1 M HCl and absorbance was measured at 450 nm.

2.3. Localization of M. pneumoniae GAPDH parts

Surface-association is the most important pre-condition for the relevance of the interaction of a bacterial protein with host factors requiring the reliable determination of the localization by different techniques.

To assess surface accessibilities of GAPDH regions, mild proteolysis of *M. pneumoniae* cells was carried out. Freshly grown *M. pneumoniae* cells (200 µg/ml) were incubated with PBS and increasing concentrations of chymotrypsin (Sigma) as described (Gründel et al., 2016b). After separation of proteins by SDS-PAGE and blotting, membranes were treated with antisera (1:10 to 1:500) to GAPDH, GAPDH parts, the cytosolic NADH oxidase (nox; negative control; Pollack et al., 1997) and to the surface-located C-terminal part of the main P1 adhesin of *M. pneumoniae* (P12; positive control; Schurwanz et al., 2009), respectively. Detection of proteins and their degradation products was done with peroxidase-labelled anti-guinea pig Ig (1:500).

For colony blotting, freshly grown *M. pneumoniae* cells were harvested, diluted with PBS and plated on PPLO agar (Millipore). After incubation for nine days at 37 °C, surface proteins of colonies were blotted onto nitrocellulose membranes (Schleicher and Schuell). Membranes were incubated with antisera (1:500) to GAPDH, to four GAPDH parts, to the cytosolic nox (negative control) and to P12 (positive control), respectively. Detection was done with HRP-conjugated anti-guinea pig Ig (1:750).

Alternatively, cell surface localization of GAPDH parts was investigated by fluorescence microscopy as described (Dumke et al., 2011). Briefly, mycoplasmas were grown in chamber slides (Nunc), fixed and incubated with a mixture of guinea pig antiserum against a GAPDH part and rabbit antiserum to the Triton X 100-insoluble fraction (representing the fraction of membrane-associated proteins; Regula et al., 2001) of *M. pneumoniae* total proteins (1:250 each). The antiserum to the TX 100-insoluble fraction serves as control and overall staining of bacterial cells. A mixture of FITC-labeled anti-guinea pig IgG (Sigma) and TRITC-labeled anti-rabbit IgG (Sigma, 1:500 each) antibodies was added for detection. Fluorescence signals of the cells were checked using a fluorescence microscope SP5 (Leica).

2.4. Binding of recombinant proteins to human plg, selected ECM components and to human A549 cells

The interaction of whole proteins of *M. pneumoniae* and of recombinant proteins with plg, fc, fg and vc was analyzed as described previously (Gründel et al., 2016a, 2016b). Briefly, wells of 96-well plates were coated with recombinant proteins GAPDH (positive control), GAPDH-1 to -4 and BSA (negative control), respectively. Based on the results of preliminary experiments, the following concentrations of human proteins (Sigma) were added: plg (0, 0.1, 0.5, 1.0, 3.0 and 5.0 µg/ml PBS), fc (0, 15, 30, 45, 65 and 85 µg/ml PBS), fg (0, 5, 10, 15, 25 and 35 µg/ml PBS) and vc (0, 0.05, 0.1, 0.25, 0.5 and 1.0 µg/ml PBS). After incubation (2 h; 37 °C), bound human proteins were detected by using the corresponding antisera (Sigma; rabbit anti-plg: 1:2500; rabbit anti-fc 1:1500; goat anti-fg: 1:1000; rabbit anti-vc: 1:5000) and peroxidase-conjugated anti-rabbit IgG or anti-goat IgG (both Dako; 1:2000). Dissociation constants (K_D) for binding affinity of full-length GAPDH and the surface-displayed part GAPDH-4 to plg and the three ECM proteins were estimated as described recently (Gründel et al., 2016a). Briefly, ECM proteins (10 µg/ml) were immobilized in wells of ELISA plates and incubated with increasing concentrations of recombinant protein (0, 0.1, 0.25, 0.5, 1, 2, 3, 4, 5 µM). Bound proteins were detected with the corresponding guinea pig antisera (1:1000) and peroxidase-conjugated anti-guinea pig IgG (1:1000). Using the ELISA data (means of eight replicates per concentration), K_D values were calculated.

To further confirm binding of human plg, fc, fg and vc to surface-located GAPDH-4, a ligand immunoblot assay was used (Thomas et al., 2013). Briefly, preparation of recombinant GAPDH-4 was separated by SDS-PAGE and transferred to nitrocellulose membranes as described before. Sliced membranes were incubated with human plg and ECM

proteins (50 µg/ml) and in parallel with PBS (negative control) for 12 h at 4 °C. Detection of host proteins was carried out with corresponding antisera (1:200) and conjugate sera (1:1000). For localization of GAPDH-4, a membrane was incubated with guinea pig anti-GAPDH-4 as described.

Binding of recombinant GAPDH parts to human A549 cells were analyzed by ELISA and immunofluorescence as reported (Gründel et al., 2016a). For ELISA, cells were harvested and used to coat wells of ELISA plates (10 µg/ml). In parallel, recombinant full-length GAPDH and GAPDH-1 to -4 (10 µg/ml) were immobilized. Recombinant proteins P12 (involved in cytadherence) and P8 (part of the central region of the P1 protein which is not able to interact with cells; Schurwanz et al., 2009) served as positive and negative controls. After blocking, the above-mentioned recombinant proteins (10 µg/ml) were added to the cell-coated wells (2 h, 37 °C) whereas wells with recombinant proteins were incubated with PBS under the same experimental conditions. Detection of recombinant proteins was carried out in all wells with corresponding antisera to the proteins (1:800) and HRP-conjugated anti-guinea pig Ig (1:1000). For immunofluorescence experiments, A549 cells were grown in chamber slides to nearly confluent layers. After blocking, cells were incubated with recombinant GAPDH (positive control), GAPDH-1 to -4, and P8 (negative control) in a concentration of 15 µg/ml (2 h, 37 °C). After washing, corresponding antibodies were added (1:250, 1 h) followed by incubation with Alexa Fluor488-labelled anti-guinea pig IgG antibody (Thermo Scientific, 1:1000, 45 min). Membranes of A459 cells were stained with CellMask Deep Orange (Thermo Scientific, 1:1000) as recommended by the manufacturer. Signals of the cells were detected at 519 nm (Alexa Fluor488) and 567 nm (CellMask Deep Orange), respectively.

2.5. Characterization of binding region of surface-displayed C-terminal part of GAPDH with plg and ECM components

The sequence of the surface-located recombinant protein GAPDH-4 showing interactions with human plg, fc, fg and vc was divided into 10 peptides, each consisting of 12 amino acids with an overlap of three amino acids and synthesized (Peptide Specialty Laboratories). The peptides (1 mg/ml) were spotted onto two nitrocellulose membranes. Recombinant GAPDH-4 and BSA were used as positive and negative controls. The membranes were dried for 20 min at room temperature. After washing, 20 µg/ml of plg, fc, fg or vc was added to one membrane and PBS to the other. The membranes were incubated for 2 h at room temperature on a shaker and washed. Bound host proteins were detected with corresponding antisera (1:250) and peroxidase-conjugated anti-rabbit or anti-goat Ig (1:1000).

Influence of peptide 10 on interaction of recombinant GAPDH-4 with human proteins was investigated by ELISA. Plg, fg, fc and vc (15 µg/ml) were immobilized in the wells of an ELISA plate and blocked. Recombinant GAPDH-4 was incubated with PBS, peptide 3 (negative control) and peptide 10 (2 h, room temperature), and added to cavities of the plate (2 h, 37 °C). Bound GAPDH-4 was detected with anti-GAPDH-4 serum (1:750) and peroxidase-conjugated anti-guinea pig Ig (1:1000).

2.6. Activation of human plg in the presence of recombinant GAPDH proteins

The activation of plg to the protease plasmin in the presence of recombinant GAPDH and parts of GAPDH was quantified by an assay described recently (Hagemann et al., 2017). Briefly, cavities of 96-well plates were coated with whole proteins of *M. pneumoniae* (positive control), recombinant proteins GAPDH and GAPDH-1 to -4, peptide 10 and BSA (negative control), respectively. After washing and blocking, human plg (10 µg/ml), urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA; both Millipore; 5 ng/well) and the plasmin-specific substrate D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (0.75 mM; Sigma) were added and incubated at 37 °C over night. Controls without plg or activator were prepared in parallel. After incubation, optical density (OD) was measured at 405 nm.

3. Results

3.1. Expression of recombinant proteins and reactivity of corresponding antisera

After successful amplification of the four parts covering the complete *gapdh* gene of *M. pneumoniae* (Fig. 1B, Table 1), PCR products were cloned into the pET-30/LIC vector that was used to transform *E. coli* Novablue and BL21(DE3) cells. Overexpressed proteins were purified, concentrated and analyzed by SDS-PAGE. Coomassie staining of the gels demonstrated prominent protein bands with the expected molecular mass (Fig. 1C). Recombinant proteins were used to produce monospecific polyclonal antisera in guinea pigs. The specificity of the antibodies was tested with total proteins of *M. pneumoniae* as antigen in immunoblotting and resulted in exclusive bands matching the predicted mass of GAPDH (Fig. 1D). It should be noted, that sera to GAPDH-2, -3 and -4 were used in a 1:10 dilution whereas the further sera were diluted 1:500. However, the specificity of all antisera could be confirmed successfully. In a comparative ELISA, the reactivities of antisera against total proteins of *M. pneumoniae* were quantified (Fig. 1E) and resulted in high OD values of antisera to complete GAPDH and to the protein part GAPDH-4. In accordance with the results of immunoblotting, the other antisera react very weak indicating strong differences in overall antigenicity of the GAPDH parts. In view of possible cross-reactions in binding studies (identity between *M. pneumoniae* and human GAPDH: 44.4%), Western blot analysis of immobilized A549 cells incubated with mycoplasma anti-GAPDH sera resulted in missing signals at the predicted molecular weight of human GAPDH of 36.0 kDa (data not shown).

3.2. Surface localization of GAPDH parts

Surface accessibility of GAPDH parts was investigated by mild proteolysis of *M. pneumoniae* cells, colony blotting and immunofluorescence of fixed bacteria. With all approaches, a surface localization of GAPDH-1 to -3 could not be confirmed (data not shown). In contrast, degradation products were detected after treatment of bacteria with increasing concentrations of chymotrypsin and detection with anti-GAPDH (data not shown) and anti-GAPDH-4 (Fig. 2A). It should be noted that complete proteolysis of GAPDH could not be expected as a relevant proportion of protein is cytoplasmic. Additionally, strong signals were demonstrated in colony blot and immunofluorescence after incubation of the membrane and the fixed cells with the antiserum to recombinant GAPDH-4 (Fig. 2B and C). Control experiments with intracellular NADH oxidase remained negative in colony blot as well as in immunofluorescence and no degradation products were detected after protease treatment of *M. pneumoniae* cells. In contrast, the C-terminal part of P1 adhesin (positive control) was degradable and staining of colonies and strong immunofluorescence of *M. pneumoniae* cells were demonstrated after incubation with the corresponding antiserum, confirming the surface localization of this protein. Results of experiments with guinea pig pre-immune serum remained negative in all approaches, excluding an influence of nonspecific cross-reactions on the results (data not shown). Summarizing the findings, surface exposure of C-terminal part of GAPDH on the *M. pneumoniae* cells is assumed.

3.3. Binding of recombinant proteins to human A549 cells, plg and ECM proteins

Recombinant proteins derived from full-length GAPDH and the different parts of GAPDH were analyzed for adherence to human A549

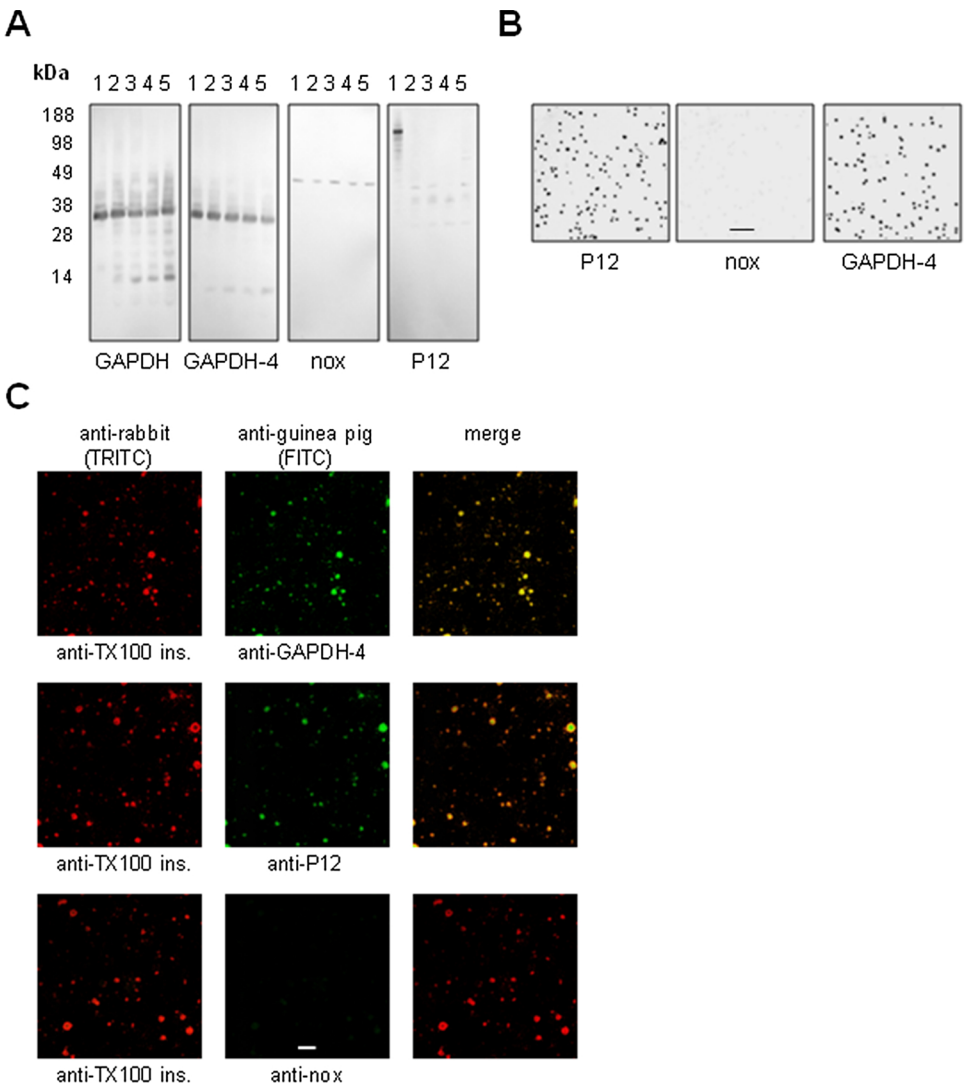

Fig. 2. Surface localization of GAPDH-4. (A) Results of chymotrypsin digestion of freshly grown *M. pneumoniae* cells. Untreated (lane 1) and chymotrypsin-treated (lane 2: 10 µg; lane 3: 20 µg; lane 4: 40 µg; lane 5: 100 µg chymotrypsin/ml) cells were blotted and incubated with the guinea pig antisera to GAPDH, GAPDH-4, NADH-oxidase (nox, negative control for a cytosolic protein) and P12 (surface-located C-terminal part of the P1 adhesin, positive control) followed by anti-guinea pig Ig conjugate. (B) Reactivity of nine days-old colonies of *M. pneumoniae* with anti-P12 serum (positive control), anti-nox serum (negative control) and anti-GAPDH-4, respectively. Bar: 200 µm. (C) Reactivity of anti-GAPDH-4 in immunofluorescence. Fixed mycoplasma cells were probed with a mixture of guinea pig anti-GAPDH-4 and rabbit anti-TX-100 insoluble proteins of *M. pneumoniae* (positive control) serum (first row), a mixture of guinea pig anti-P12 and rabbit anti-TX-100 insoluble proteins (middle row), and a mixture of guinea pig anti-nox and rabbit anti-TX-100 insoluble proteins serum (upper row). For detection, cells were incubated with a mixture of FITC-conjugated anti-guinea pig IgG and TRITC-conjugated anti-rabbit IgG. Bar: 10 µm.

cells. Wells of ELISA plates coated with recombinant protein and incubated with PBS were compared with wells coated with A549 cells pre-incubated with the same recombinant protein under comparable conditions. Binding of recombinant proteins was demonstrated with the corresponding guinea pig antiserum. Using GAPDH as control for a protein with confirmed interaction with A549 cells (Gründel et al., 2016b), a mean OD value of 1.3 was obtained for wells with immobilized recombinant protein. Parallel treated wells with A549 cells incubated with recombinant GAPDH resulted in a mean OD value of 0.7 (56% of OD value obtained from wells with recombinant protein alone), confirming the association of the protein with A549 cells (Fig. 3A). As negative control, cell binding of protein P8 as part of P1 protein not involved in adhesion remains low (mean OD value: 0.05). Using the protein parts GAPDH-1 and -2, the reactivities of recombinant proteins interacting with human cells are reduced to 26% (GAPDH-1) and 12% (GAPDH-2) compared to OD values of wells coated with recombinant proteins. In contrast, OD values of GAPDH-3 and -4 in wells with human cells reached 70% and 73% of those with recombinant proteins confirming a relatively strong interaction with surface components of A549 cells. These data are supported by the results of immunofluorescence experiments of fixed cells (Fig. 3B). With the exception of protein P8 (negative control), incubation with recombinant GAPDH and GAPDH-1 to -4 demonstrated differently distinct but in all cases detectable signals.

To test specific interactions between recombinant parts of GAPDH and increasing concentrations of selected human proteins, binding studies were carried out by ELISA (Fig. 4A). In all experiments, the negative control BSA was not able to interact with any concentration of human fg, fc, plg and vc, respectively (OD values ≤ 0.1). In contrast, nearly all recombinant proteins bind to the human components. Setting an OD value of 0.2 as limit for measurable reactivity, only the interactions of GAPDH-2 with fc and vc remain below this threshold. As found in previous studies, the concentrations of human proteins needed to demonstrate an increasing binding are strongly different (fc > fg > plg > vc). As expected, measurement of interaction of full-length recombinant GAPDH with host factors resulted in higher OD values in comparison with most recombinant GAPDH parts (exception: GAPDH-3 and vc). Depending on the concentration of host factor, comparative binding of surface-localized GAPDH-4 was relatively strong to plg and fc but weaker to the other human ECM proteins. According to estimation of binding affinities of full-length GAPDH and GAPDH-4 (Table 2), the following order of K_D values was found: fg > vc > fg > plg. Regarding the human proteins tested, higher binding affinities of recombinant GAPDH in comparison with GAPDH-4 were measured (factors of mean K_D values between 4.4 and 5.2) which is in accordance with the results of concentration-dependent binding experiments (Fig. 4A).

Since the confirmation of specific interactions of the surface-localized protein GAPDH-4 with host factors is of particular interest, a ligand immunoblot assay was carried out additionally (Fig. 4B). In all

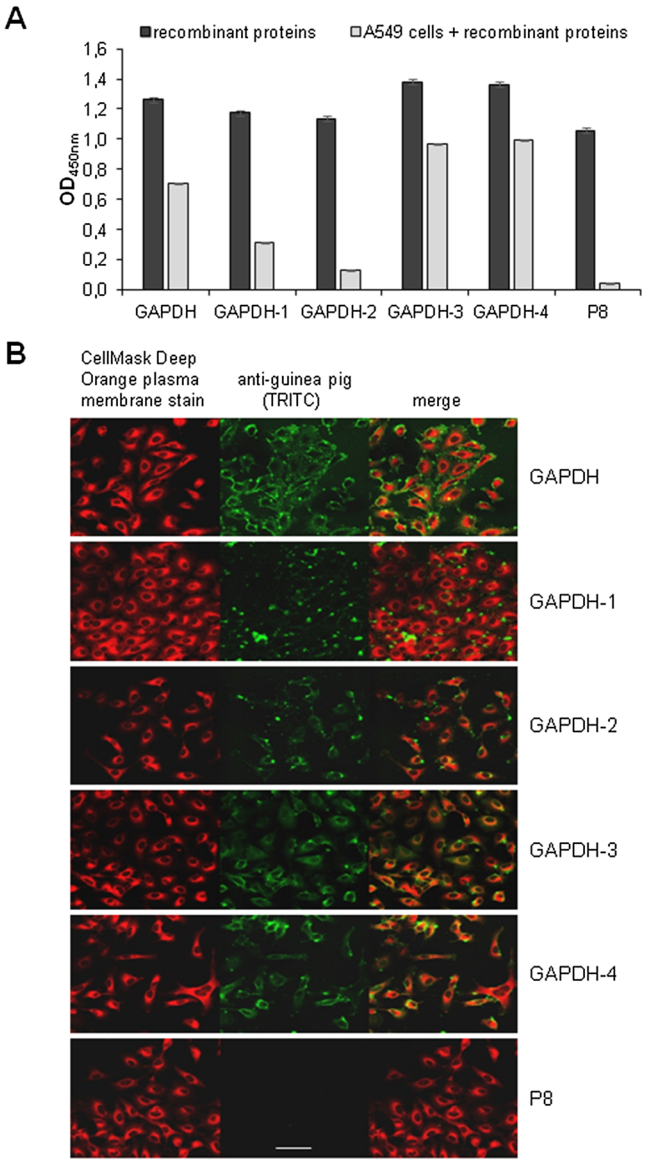

Fig. 3. Binding of recombinant GAPDH parts to human A549 cells. (A) ELISA reactivity of immobilized recombinant proteins and immobilized A549 cells pre-incubated with recombinant proteins. Recombinant full-length GAPDH and a part of the central region of the P1 adhesin of *M. pneumoniae* (P8) served as controls for confirmed cell-binding and non-binding proteins. Means and standard deviations of eight replicates. (B) Immunofluorescence of fixed A549 cells after incubation with recombinant proteins and the corresponding guinea pig antisera. Detection of signals was carried out with TRITC-labeled anti-guinea pig IgG. Human cells were visualized by treatment with CellMask Deep orange plasma stain. Bar: 10 µm.

cases, binding of human components was associated with a protein/proteins showing the molecular mass of GAPDH-4.

3.4. Characterization of binding regions of surface-displayed part of GAPDH to human plg and ECM proteins

According to the results of localization experiments, protein region GAPDH-4 was confirmed as surface-localized. To define the binding sites of the C-terminal part of GAPDH for host proteins, GAPDH-4 was divided into ten overlapping peptides (Fig. 5A). Dot blot testing of the synthetic peptides resulted in reactivity of peptide P10 with human plg as well the ECM components fg, fc and vc, respectively (Fig. 5A). In addition, peptide P7 reacted with plg and fg, and peptide P8 weakly with plg. Strong signals were measured with recombinant GAPDH-4 (positive control) whereas no reactivity was detected for non-binding protein BSA (negative control). Analyzing the control membrane (same antigens and immune reaction but incubation with PBS instead of plg, fg, fc or vc, respectively), no signals were obtained for any peptide or control excluding the occurrence of nonspecific cross-reactions (data not shown).

In comparison with non-binding control peptide P3 and PBS, pre-incubation with peptide 10 reduces the binding of recombinant protein GAPDH-4 to immobilized human ECM proteins significantly (Fig. 5B). Based on mean OD values, a decrease between 57% (vc) and 85% (fc) can be calculated. In contrast, binding of GAPDH-4 to plg was similar after pre-incubation with peptides P3, P10 and PBS, respectively. It should be noted that in previous ELISA experiments testing the reactivity of the peptides with anti-GAPDH-4 serum, higher OD values (> 0.2) were measured for peptides P1 and P6 only (data not shown).

3.5. Generation of plasmin in the presence of recombinant proteins

Activation of proteolytically inactive plg to plasmin plays an important role in host processes. After confirmation of binding of recombinant GAPDH and GAPDH parts to human plg (Fig. 4), it remains to clear if plasmin is generated in the presence of these bacterial proteins or peptide 10 and host activators uPA or tPA. Activation of plg was analyzed in ELISA experiments. To avoid the influence of different binding characteristics on the results, all components of reaction (plg, activator, plasmin-specific substrate) were added together to the wells with immobilized recombinant proteins, peptide 10 or whole proteins of *M. pneumoniae*, respectively. No differences in OD values were obtained between both human activators as well between recombinant proteins, peptide 10, the control BSA (none-plg binding protein) and whole proteins of *M. pneumoniae* (Fig. 6). In parallels without Plg or activator (negative controls), measured OD values after the incubation period are below 0.1 (data not shown).

4. Discussion

via their surfaces, microbes interact in diverse ways with the host. To surface-displayed bacterial structures belong not only adhesins or lipoproteins but also primary regulatory or metabolic proteins, like glycolytic enzymes, transported by a hitherto unknown mechanism from cytosol to the cell surface. These moonlighting proteins are recently described in many bacterial species and, especially in pathogenic microorganisms, their role in the complex processes of colonization and pathogenesis is of increasing interest. Further research efforts in this direction should not only include the identification of further interaction partners on both the microbe and host side but also the characterization of the molecular organization of binding. Investigations of species of genus Mycoplasma show that the occurrence of moonlighting proteins on the cell surface is not necessarily linked to a classical cell wall but can also be realized by their association with the unique cell membrane of these species (Daubenspeck et al., 2016). However, the limited repertoire of metabolic and regulatory proteins in combination with their pathogenic potential in both humans and animals makes the members of Mollicutes to excellent model organisms for study of proteins with multiple functions.

In the present report, we investigated the GAPDH of *M. pneumoniae* as typical example for a moonlighting glycolytic enzyme which was previously confirmed as interacting with many host factors (Gründel et al., 2016a, 2016b). As demonstrated for cultivated A549 cells *in vitro* (Gründel et al., 2016a), it can be assumed that all tested human proteins showing interactions with GAPDH co-occur at the exclusive colonization site of *M. pneumoniae*, the respiratory epithelium. Thus, the potential multi-binding of bacterial proteins allows a complex interplay between human extracellular proteins and pathogen *in vivo*. Question remains how the bacteria organize associations to several host factors.

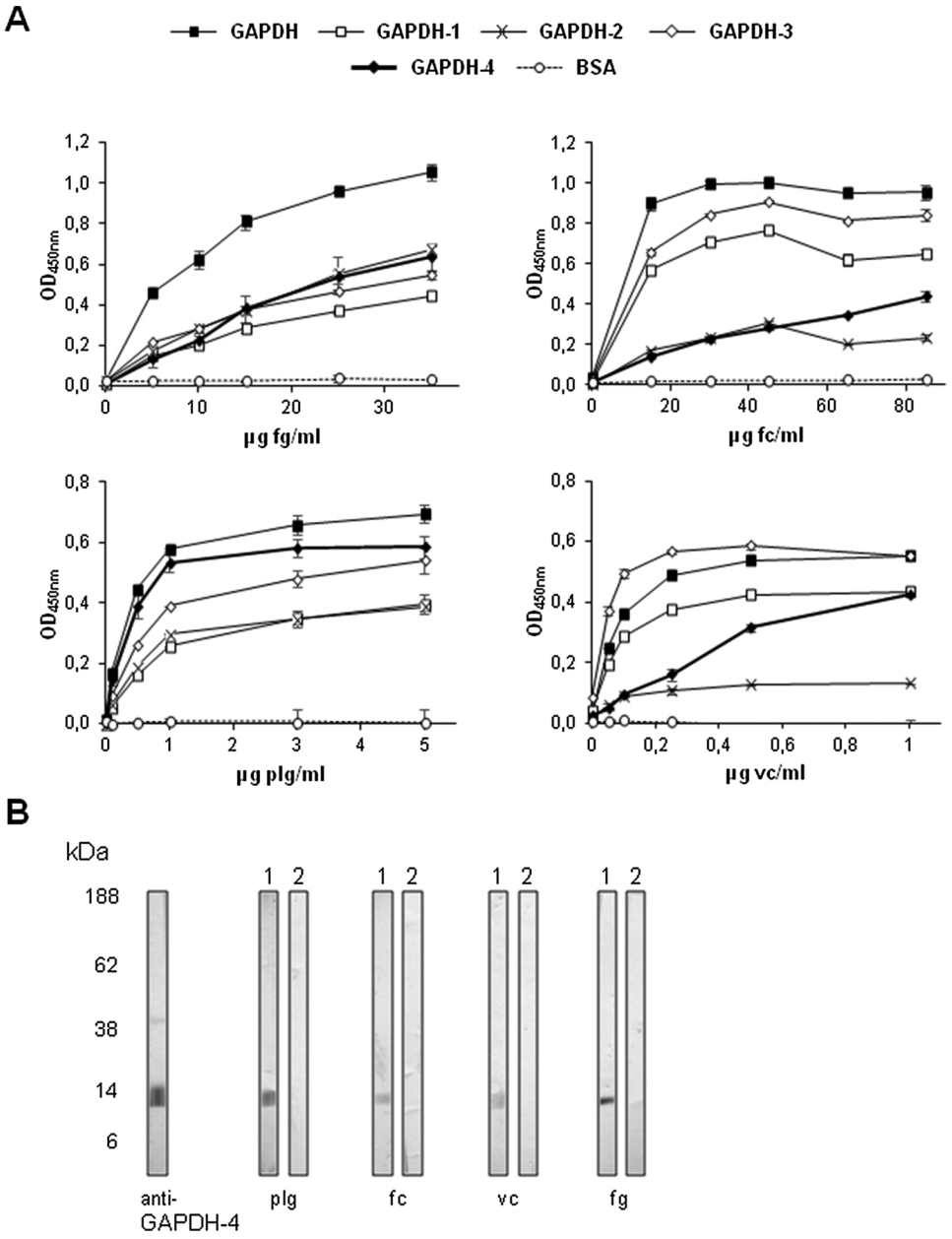

Fig. 4. Interactions of recombinant GAPDH proteins with selected host factors. (A) Concentration-dependent binding of human fibrinogen (fg), fibronectin (fc), plasminogen (plg) and vitronectin (vc) to recombinant GAPDH or GAPDH parts as measured by ELISA. BSA was used as negative control. Means and standard deviations of eight replicates. (B) Reaction of blotted recombinant protein GAPDH-4 after incubation with plg, fc, vc, fg (lanes 1) and with PBS (negative control, lanes 2). Reaction with anti-GAPDH-4 demonstrates the localization of protein GAPDH-4.

In this regard, variable strategies are described like use of distinct sites for different functions, of same residues for different functions, proteins with different structural conformations for diverse functions or post-translational modifications (Das et al., 2017; Daubenspeck et al., 2016; Sirover, 2017). Here, the characterization of four regions covering the complete GAPDH protein showed that nearly all recombinant proteins are able to interact with human fc, fg, plg and vc, respectively. This finding suggests the occurrence of many binding sites on differing parts of the protein and confirmed the results of other reports investigating GAPDH or other glycolytic enzymes of different bacterial species (Henderson et al., 2011; Kainulainen and Korhonen, 2014). In addition, the localization of interacting protein regions on the surface of the

Table 2
Binding affinities (mean K_D values ± standard deviations, nM) of recombinant GAPDH and GAPDH-4 to selected host proteins.

Recombinant protein	Human protein			
	Plasminogen	Fibrinogen	Fibronectin	Vitronectin
GAPDH	3.4 ± 2.2	133.4 ± 85.6	60.7 ± 28.8	91.7 ± 39.5
GAPDH-4	15.1 ± 7.4	696.2 ± 276.2	278.4 ± 118.8	456.4 ± 182.9

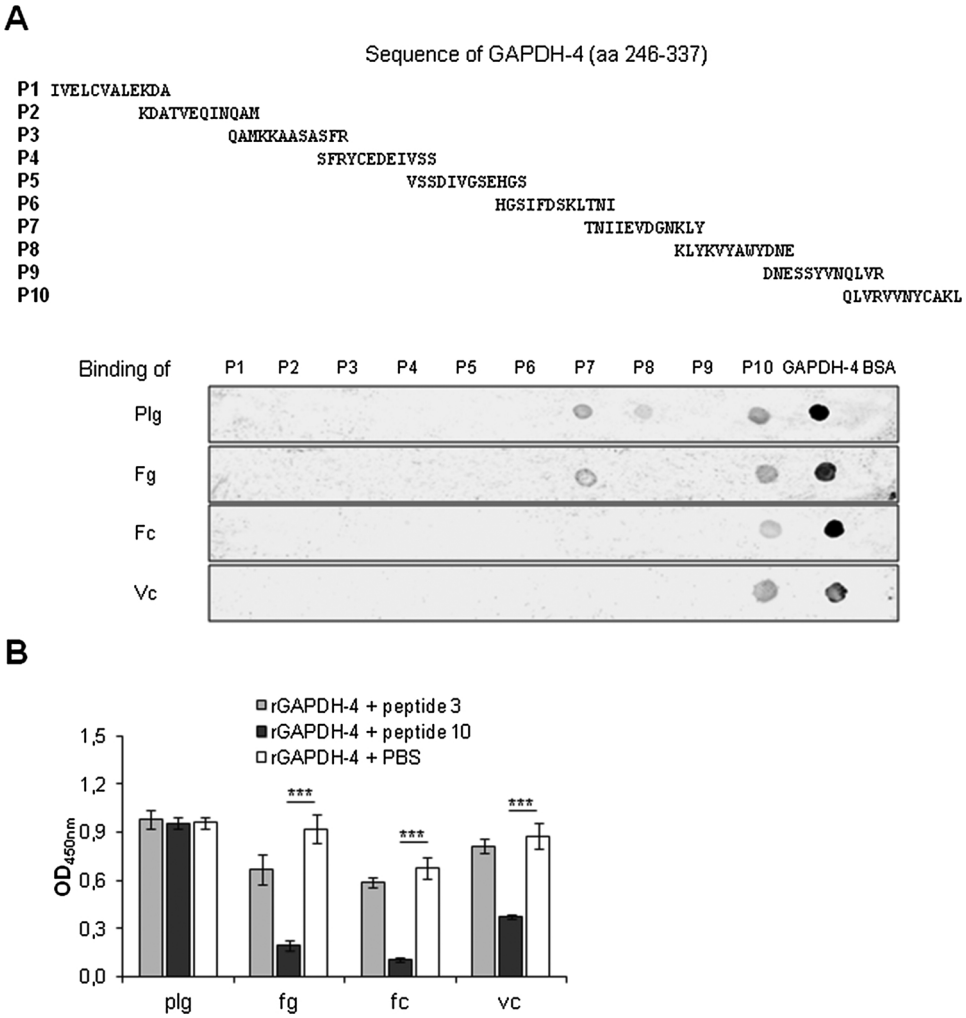

Fig. 5. Identification of binding region to host factors of GAPDH-4 with peptides overlapping the C-terminal part between amino acids 246 and 337 of *M. pneumoniae* GAPDH. (A) Schematic illustration of the ten peptides used and reaction of nitrocellulose membrane spotted with peptides, recombinant GAPDH-4 (positive control) and BSA (negative control) with human proteins plasminogen (plg), fibrinogen (fg), fibronectin (fc) and vitronectin (vc), respectively. Detection was done with the corresponding antisera to host factors and peroxidase-labeled anti-rabbit or anti-goat Ig. (B) ELISA detection of binding of immobilized GAPDH-4 to plg, fg, fc and vc after pre-incubation with peptide 3 (negative control), peptide 10 and PBS, respectively. Data represent the mean and standard deviation of eight replicates. t-test: $***P \leq 0.001$.

mycoplasmal cell is a further crucial aspect for the *in vivo* relevance of an association confirmed *in vitro*. It should be noted that the low reactivities of guinea pig antisera to GAPDH-1 to -3 reduce the significance of the study since the surface-localization of further parts of GAPDH cannot be excluded certainly. However, surface-display of GAPDH-4 was confirmed with all approaches used and the binding of the GAPDH region represented by this recombinant protein to host factors is supposed as important for host-pathogen interaction.

From the data of the present study, it can be suggested that the interaction of human plg with GAPDH is preferred among the host proteins tested. Measurement of binding characteristics (Table 2) resulted in approximately fivefold higher affinities of full-length GAPDH in comparison with GAPDH-4. Despite the fact that interactions of nearly all host factors with GAPDH-1 to -3 were detected (Fig. 4), an influence of incorrectly folded recombinant proteins and of disruption of binding sites by the arbitrary determination of GAPDH fragments on the quantitative association between proteins GAPDH/GAPDH-4 and human components cannot be excluded. However, binding affinity of plg to complete GAPDH and surface-localized GAPDH-4 is much higher in comparison with fc, fg and vc, respectively. Furthermore, results of

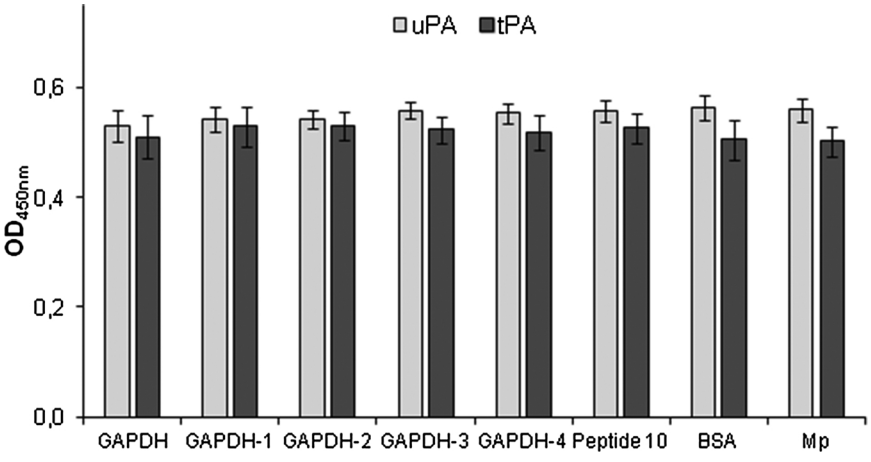

Fig. 6. Activation of human plasminogen in the presence of recombinant GAPDH parts and peptide 10 after incubation with plasminogen, human urokinase-type plasminogen activator (uPA) or tissue plasminogen activator (tPA) and the plasmin-specific substrate D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride, respectively. Full-length recombinant GAPDH and whole protein of *M. pneumoniae* (Mp) were used as positive controls. BSA served as negative control. Plasmin generation was detected by measurement of optical density at 405 nm. Errors represent standard deviations of eight replicates.

Table 3
Confirmed interaction of C-terminal sequences of GAPDH of different bacterial species with host factors: comparison with GAPDH of *M. pneumoniae* (plg - plasminogen, fc - fibronectin, fg - fibrinogen, vc - vitronectin).

Species (number of amino acids)	C-terminal sequence	Interaction with host protein	Reference
M. pneumoniae (338)	326QLVRVVNYCAKL338	plg, fc, fg, vc	this study
Mycoplasma hyopneumoniae (335)	325QFVRVIRDFVQK335	? (predicted protein-protein interaction sites)	Berry et al., 2017
Streptococcus pneumoniae (359)	346QLVRTLEYFAKIAK359	plg	Bergmann and Hammerschmidt, 2007

binding experiments demonstrated not only associations of plg with all four parts of GAPDH but also the occurrence of binding to different peptides covering the C-terminus of the enzyme. In contrast to human fc, fg and vc, blocking of binding site by pre-incubation of plg with peptide 10 showed limited influence on amount of plg bound to recombinant GAPDH-4. An explanation for the particular importance of plg might be the degradation of different host factors after generation of the proteolytically active serine protease plasmin. A previous study demonstrated the activation of plg in the presence of GAPDH (Gründel et al., 2016b) and the degradation of human fg as well vc (Gründel et al., 2016a, 2016b). Here, the production of plasmin was confirmed but was not significantly different between the tested reaction partners indicating that the presence of GAPDH-4 or peptide 10 causes neither an increase nor an inhibition of this process and a comparable influence on degradation of host factors can be expected. As stated in many reports, binding of plg in combination with degradation of components of ECM not only in the infected areal of human respiratory tract but also in other tissues seems favorable for further colonization by pathogenic bacteria (Bhattacharya et al., 2012; Peetermans et al., 2016).

Interestingly, narrowing of the interaction site of GAPDH using overlapping peptides led to the C-terminal sequence ^{326}QLVRVVNYCAKL^{337} which was able to interact with at least four host proteins. To our best knowledge, this is the first description of confirmed binding of a protein region strongly truncated to twelve amino acids with more than a single host factor. To estimate the surface-exposition of the C-terminus, Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2), I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and SWISS-MODEL (https://swissmodel.expasy.org/) server were used to predict the 3D structure of *M. pneumoniae* GAPDH. Identities of amino acid sequences of templates with known related structures (GAPDH of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Thermotoga maritima*, respectively) for building the top models were 55 and 57%. All proposed models predict the helical structure and surface exposition of C-terminal sequence as precondition for interactions with host factors (data not shown). The importance of the C-terminal part of moonlighting proteins for association with human components was not only confirmed for proteins of *M. pneumoniae* (Widjaja et al., 2015) but also for molecules of other species (Fulde et al., 2013). Regarding GAPDH, interactions of the C-terminus were demonstrated or predicted for *Mycoplasma hyopneumoniae* and *Streptococcus pneumoniae* (Table 3). For the latter species, an actual study questioned the importance of the C-terminus of GAPDH (Moreau et al., 2017). Hence, further studies are needed to confirm that the involvement of the C-terminal part of GAPDH of different species in binding to human factors is a common aspect of host-pathogen interaction. While limited information regarding the molecular basis of association of moonlighting proteins with human fc, fn and vc is available, interaction with plg has been more extensively assessed. Importance of lysine residues of bacterial protein regions for plg-binding was reported (Bhattacharya et al., 2012) but peptides missing lysine are also able to interact with plg as confirmed for pyruvate dehydrogenase B (PDHB) of *M. pneumoniae* (Thomas et al., 2013). Moreover, this peptide shows functional similarity but limited homology to a putative plg-binding region of *E. coli* lipoprotein (Gonzalez et al., 2015). Interestingly, PDHB is a further example of a glycolytic enzyme of *M. pneumoniae* with remarkable multi-binding properties (Gründel et al., 2016a, b). However, sequence of PDHB shows only 11% identity to GAPDH indicating the importance of differing amino acid motifs for binding to host proteins. In general, the surface-localization of small and non-immunogenic parts of moonlighting proteins seems advantageous for successful interactions with host components circulating in the respiratory epithelium as the process will not be interfered by the humoral immune response of the host. This is supported by the facts that the production of specific antibodies against GAPDH in sera of guinea pigs intranasally infected with freshly grown *M. pneumoniae* cells is missing (Gründel et al., 2016b) and that binding peptide 10 demonstrated only weak reactivity (OD values < 0.2) with anti-GAPDH and anti-GAPDH-4 sera in ELISA experiments (data not shown).

In conclusion, the present study combines results of surface-location with binding characteristics of different parts of a classical moonlighting protein featuring the ability for multi-binding to host factors. Besides the importance of these interactions for the mechanisms contributing to the successful colonization of the human respiratory tract by the common pathogen *M. pneumoniae*, the findings may lead to a further understanding of organization of the complex interplay between bacterial proteins with dual functions and different host factors.

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References

Bergmann, S., Hammerschmidt, S., 2007. Fibrinolysis and host response in bacterial infections. Thromb. Haemost. 98, 512–520.
Berry, I.J., Jarocki, V.M., Tacchi, J.L., Raymond, B.B.A., Widjaja, M., Padula, M.P., Djordjevic, S.P., 2017. N-terminomics identifies widespread endoproteolysis and novel methionine excision in a genome-reduced bacterial pathogen. Sci. Rep. 7, 11063.
Bhattacharya, S., Ploplis, V.A., Castellino, F.J., 2012. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J. Biomed. Biotechnol. 482096.
Chagnot, C., Listrat, A., Astruc, T., Desvaux, M., 2012. Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components. Cell. Microbiol. 14, 1687–1696.
Das, S., Khan, I., Kihara, D., Orengo, C., 2017. Exploring structure-function relationships in moonlighting proteins. In: Henderson, B. (Ed.), Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections. John Wiley & Sons, Hoboken, pp. 21–43.
Daubenspeck, J.M., Liu, R., Dybvig, K., 2016. Rhamnose links moonlighting proteins to membrane phospholipid in mycoplasmas. PLoS One 11, e0162505.
Dumke, R., Hausner, M., Jacobs, E., 2011. Role of *Mycoplasma pneumoniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mediating interactions with the human extracellular matrix. Microbiology 157, 2328–2338.
Egea, L., Aguilera, L., Gimenez, R., Sorolla, M.A., Aguilar, J., Badia, J., Baldoma, L., 2007. Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic *Escherichia coli*: interaction of the extracellular enzyme with human plasminogen and fibrinogen. Int. J. Biochem. Cell Biol. 39, 1190–1203.
Fulde, M., Steinert, M., Bergmann, S., 2013. Interaction of streptococcal plasminogen binding proteins with the host fibrinolytic system. Front. Cell. Infect. Microbiol. 3, 85.
Gonzalez, T., Gaultney, R.A., Floden, A.M., Brissette, C.A., 2015. *Escherichia coli* lipoprotein binds human plasminogen via an intramolecular domain. Front. Microbiol. 6, 1095.
González-Miguel, J., Morchón, R., Siles-Lucas, M., Oleaga, A., Simón, F., 2015. Surface-

displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host. Acta Trop. 145, 8–16.
Gozalbo, D., Gil-Navarro, I., Azorin, I., Renau-Piqueras, J., Martinez, J.P., Gil, M.L., 1998. The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibrinogen and laminin binding protein. Infect. Immun. 66, 2052–2059.
Gründel, A., Jacobs, E., Dumke, R., 2016a. Surface-displayed glycolytic enzymes of *Mycoplasma pneumoniae* are able to interact with different components of the human extracellular matrix. Int. J. Med. Microbiol. 306, 675–685.
Gründel, A., Pfeiffer, M., Jacobs, E., Dumke, R., 2016b. Network of surface-displayed glycolytic enzymes in *Mycoplasma pneumoniae* and their interactions with human plasminogen. Infect. Immun. 84, 666–676.
Hagemann, L., Gründel, A., Jacobs, E., Dumke, R., 2017. The surface-displayed chaperones GroEL and DnaK of *Mycoplasma pneumoniae*interact with human plasminogen and components of the extracellular matrix. Pathog. Dis. 75, ftx017.
Hames, C., Halbedel, S., Hoppert, M., Frey, J., Stülke, J., 2009. Glycerol metabolism is important for cytotoxicity of Mycoplasma pneumoniae. J. Bacteriol. 191, 747–753.
Hasselbring, B.M., Jordan, J.L., Krause, R.W., Krause, D.C., 2006. Terminal organelle development in the cell wall-less bacterium Mycoplasma pneumoniae. Proc. Natl. Acad. Sci. U. S. A. 103, 16478–16483.
Henderson, B., 2014. An overview of protein moonlighting in bacterial infection. Biochem. Soc. Trans. 42, 1720–1727.
Henderson, B., Martin, A., 2011. Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. Infect. Immun. 79, 3476–3491.
Henderson, B., Nair, S., Pallas, J., Williams, M.A., 2011. Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. FEMS Microbiol. Rev. 35, 147–200.
Kainulainen, V., Korhonen, T.K., 2014. Dancing to another tune – adhesive moonlighting proteins in bacteria. Biology. 3, 178–204.
Kannan, T.R., Baseman, J.B., 2006. ADP-ribosylating and vacuolating cytotoxin of *Mycoplasma pneumoniae* represents unique virulence determinant among bacterial pathogens. Proc. Natl. Acad. Sci. U. S. A. 103, 6724–6729.
Kühner, S., van Noort, V., Betts, M.J., Leo-Macias, A., Batisse, C., Rode, M., Yamada, T., Maier, T., Bader, S., Beltran-Alvarez, P., Castaño-Diez, D., Chen, W.H., Devos, D., Güell, M., Norambuena, T., Racke, I., Rybin, V., Schmidt, A., Yus, E., Aebersold, R., Herrmann, R., Böttcher, B., Frangakis, A.S., Russell, R.B., Serrano, L., Bork, P., Gavin, A.C., 2009. Proteome organization in a genome-reduced bacterium. Science 326, 1235–1240.
Lama, A., Kucknoor, A., Mundodi, V., Alderete, J.F., 2009. Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated, fibronectin-binding protein of Trichomonas vaginalis. Infect. Immun. 77, 2703–2711.
Li, Q., Liu, H., Du, D., Yu, Y., Ma, C., Jiao, F., Yao, H., Lu, C., Zhang, W., 2015. Identification of novel laminin- and fibronectin-binding proteins by far-western blot: capturing the adhesins of *Streptococcus suis* type 2. Front. Cell. Infect. Microbiol. 5, 82.
Moreau, C., Terrasse, R., Thielens, N.M., Vernet, T., Gaboriaud, C., Di Guilmi, A.M., 2017. Deciphering key residues involved in the virulence-promoting interactions between *Streptococcus pneumoniae* and human plasminogen. J. Biol. Chem. 292, 2217–2225.
Morgenthau, A., Pogoutse, A., Adamiak, P., Moraes, T.F., Schryvers, A.B., 2013. Bacterial receptors for host transferrin and lactoferrin: molecular mechanisms and role in host-microbe interactions. Future Microbiol. 8, 1575–1585.
Narita, M., 2016. Classification of extrapulmonary manifestations due to *Mycoplasma pneumoniae* infection on the basis of possible pathogenesis. Front. Microbiol. 7, 23.
Pancholi, V., Fischetti, V.A., 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. J. Exp. Med. 176, 415–426.
Pavkova, I., Kopeckova, M., Klimentova, J., Schmidt, M., Sheshko, V., Sobol, M., Zakova, J., Hozak, P., Stulik, J., 2017. The multiple localized glyceraldehyde-3-phosphate dehydrogenase contributes to the attenuation of the *Francisella tularensis* dsbA deletion mutant. Front. Cell. Infect. Microbiol. 11, 503.
Peetermans, M., Vanassche, T., Liesenborghs, L., Lijnen, R.H., Verhamme, P., 2016. Bacterial pathogens activate plasminogen to breach tissue barriers and escape from innate immunity. Crit. Rev. Microbiol. 42, 866–882.
Pollack, J.D., Williams, M.V., McElhaney, R.N., 1997. The comparative metabolism of the mollicutes (*Mycoplasmas*): the utility for taxonomic classification and the relationship of putative gene annotation and phylogeny to enzymatic function in the smallest free-living cells. Crit. Rev. Microbiol. 23, 269–354.
Regula, J.T., Boguth, G., Görg, A., Hegermann, J., Mayer, F., Frank, R., Herrmann, R., 2001. Defining the mycoplasma "cytoskeleton": the protein composition of the Triton X-100 insoluble fraction of the bacterium *Mycoplasma pneumoniae* determined by 2-D gel electrophoresis and mass spectrometry. Microbiology 147, 1045–1057.
Sanderson-Smith, M.L., De Oliveira, D.M.P., Ranson, M., McArthur, J.D., 2012. Bacterial plasminogen receptors: mediators of a multifaceted relationship. J. Biomed. Biotechnol. 272148.
Schurwanz, N., Jacobs, E., Dumke, R., 2009. A strategy to create chimeric proteins derived from functional adhesin regions of *Mycoplasma pneumoniae* for vaccine development. Infect. Immun. 77, 5007–5015.
Singh, B., Su, Y.C., Riesbeck, K., 2010. Vitronectin in bacterial pathogenesis: a host protein used in complement escape and cellular invasion. Mol. Microbiol. 78, 545–560.
Singh, B., Fleury, C., Jalalvand, F., Riesbeck, K., 2012. Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. FEMS Microbiol. Rev. 36, 1122–1180.
Sirover, M.A., 2017. GAPDH: a multifunctional moonlighting protein in eukaryotes and prokaryotes. In: Henderson, B. (Ed.), Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections. John Wiley & Sons, Hoboken, pp. 149–167.
Thomas, C., Jacobs, E., Dumke, R., 2013. Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in Mycoplasma pneumoniae. Microbiology 159, 352–365.
Waites, K.B., Xiao, L., Liu, Y., Balish, M.F., Atkinson, T.P., 2017. *Mycoplasma pneumoniae* from the respiratory tract and beyond. Crit. Microbiol. Rev. 30, 747–809.
Widjaja, M., Berry, I.J., Pont, E.J., Padula, M.P., Djordjevic, S.P., 2015. P40 and P90 from mpn142 are targets of multiple processing events on the surface of Mycoplasma pneumoniae. Proteomes 16, 512–537.
Widjaja, M., Harvey, K.L., Hagemann, L., Berry, I.J., Jarocki, V.M., Raymond, B.B.A., Tacchi, J.L., Gründel, A., Steele, J.R., Padula, M.P., Charles, I.G., Dumke, R., Djordjevic, S.P., 2017. Elongation factor Tu is a multifunctional and processed moonlighting protein. Sci. Rep. 7, 11227.
Zipfel, P.F., Hallström, T., Riesbeck, K., 2013. Human complement control and complement evasion by pathogenic microbes-tipping the balance. Mol. Immunol. 56, 152–160.