Human and Pneumococcal Cell Surface Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) Proteins Are Both Ligands of Human C1q Protein*

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Background: C1q is a major molecule of the immune innate system. GAPDH exposed at the surface of cells is associated with the virulence of pathogens.

Results: C1q binds GAPDH on human apoptotic and on pneumococcal cells.

Conclusion: GAPDH is a C1q ligand. Pneumococcal GAPDH-C1q interaction leads to complement activation.

Significance: This might bring new insights into host defense against pathogens.

C1q, a key component of the classical complement pathway, is a major player in the response to microbial infection and has been shown to detect noxious altered-self substances such as apoptotic cells. In this work, using complementary experimental approaches, we identified the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a C1q partner when exposed at the surface of human pathogenic bacteria Streptococcus pneumoniae and human apoptotic cells. The membrane-associated GAPDH on HeLa cells bound the globular regions of C1q as demonstrated by pulldown and cell surface co-localization experiments. Pneumococcal strains deficient in surface-exposed GAPDH harbored a decreased level of C1q recognition when compared with the wild-type strains. Both recombinant human and pneumococcal GAPDHs interacted avidly with C1q as measured by surface plasmon resonance experiments (K_D = 0.34–2.17 nM). In addition, GAPDH-C1q complexes were observed by transmission electron microscopy after cross-linking. The purified pneumococcal GAPDH protein activated C1 in an in vitro assay unlike the human form. Deposition of C1q, C3b, and C4b from human serum at the surface of pneumococcal cells was dependent on the presence of surface-exposed GAPDH. This ability of C1q to sense both human and bacterial GAPDHs sheds new insights on the role of this important defense collagen molecule in modulating the immune response.

The soluble defense collagen C1q, as a major actor of the classical complement pathway, plays a crucial role in the response to microbial infection. However, its importance has been reinforced by the discoveries of its capacities to detect a wide variety of noxious altered-self substances such as β-amyloid fibrils, the pathological form of the prion protein, apoptotic and necrotic cells, or modified forms of the low density lipoprotein (1–8). Interestingly, unlike the other complement proteins, C1q is produced by macrophages and immature dendritic cells. Numerous studies have shown that C1q influences the phagocyte “status” through regulation of cytokine expression (7, 8), and it may be involved in the non-immunogenic presentation of self-antigens through its ability to modulate maturation of dendritic cells (9). C1q globular regions (GRs)3 mediate the binding to various molecules exposed at the surface of apoptotic cells such as phosphatidylserine, DNA, calreticulin, and annexins A2 and A5 (10–15). Opsonization with C1q facilitates the phagocytosis of pathogens and apoptotic cells, leading to different and appropriate adaptive responses (proinflammatory/immunogenic versus anti-inflammatory/tolerogenic). Consequently, pathogens might recruit C1q to subvert the host immune response by exploiting tolerance. To test this hypothesis, we looked for C1q ligands present at the surface of apoptotic cells that could be shared by pathogens.

The pathogen model used in this study is Streptococcus pneumoniae, a leading cause of meningitis, septicemia, and pneumonia responsible worldwide for the death of around 1 million children under 5 years of age every year (16). The nasopharynx of small children constitutes the normal habitat for pneumococci, and asymptomatic carriage usually occurs before and

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during the development of invasive diseases. The transition from the asymptomatic state to infectious behavior of the pneumococcus is poorly understood but is likely to be dependent on the host’s innate immune response. Within this context, the characterization of the immune response to *S. pneumoniae* is a high priority. One important component is the complement system among which the classical pathway is the dominant activation system, although the alternative and lectin pathways also play some roles (17–20). Data from experimental infections in mice have suggested that the classical pathway can be activated by various innate immune mediators. They include natural IgM, antibodies to capsular and noncapsular antigens, pentraxin components of the acute-phase response such as C-reactive protein and serum amyloid P component, and the macrophage lectin receptor SIGN-R1 (21). These data support the important role of the classical pathway for innate immunity to *S. pneumoniae* and suggest that different innate mediators act as bridging molecules by recognizing pneumococcus surface components and C1q. However, direct C1q ligands have yet to be identified at the surface of *S. pneumoniae*.

In the present study, we demonstrated that human and pneumococcal glyceraldehyde-3-phosphate dehydrogenases (named herein Hsa and Spn GAPDHs, respectively) exposed at the cell surface are C1q partners. Besides its well-characterized function as a glycolytic enzyme, GAPDH displays multiple functions in membrane fusion, transcriptional coactivation, DNA repair, apoptosis induction and regulation, transferrin binding, and bacterial virulence (22–26). GAPDH is present on the surfaces of both prokaryotic and eukaryotic cells, but the mechanism by which the protein is secreted and/or captured at the cell surface is not known (27–29). In pathogenic bacteria, including *Streptococcus*, surface-anchored GAPDH is associated with virulence because of its ability to bind different host proteins (30–32). Pneumococcal surface-exposed GAPDH binds plasminogen. Conversion of plasminogen to the proteolytically active plasmin form plays important roles in pathological processes such as the escape from blood clots and tissue invasion (33–35). To our knowledge, this is the first report of GAPDH-C1q interaction taking place in a comparable manner for eukaryotic and prokaryotic homologs but leading to different functional consequences in terms of complement activation.

**EXPERIMENTAL PROCEDURES**

*Proteins and Antibodies*—C1q and the proenzyme C1s-C1r-C1r-C1s tetramer were purified from human serum and C1qGRs were prepared and quantified as described previously (36). Mouse monoclonal anti-human GAPDH (Invitrogen, clone 258), rabbit polyclonal anti-human GAPDH (Sigma G9545), anti-human C4 (Abcam ab48612), and anti-human C3 (Abcam ab97462) antibodies were used. Rabbit polyclonal antibodies directed against human C1q and pneumococcal GAPDH were from the Immune response to pathogens and altered self and Pneumococcus groups (Institut de Biologie Structurale-Grenoble), respectively. Native purified human GAPDH was from Advanced Immunochemical Inc.

*Cell Culture*—HeLa cells were cultivated in GlutaMAX RPMI 1640 medium (Invitrogen). Medium was supplemented with 10% (v/v) FCS, 2.5 units/ml penicillin, and 2.5 mg/ml streptomycin. The cells were regularly tested for *Mycoplasma* contamination (Mycotest detection kit, Lonza). Apoptosis was induced and quantified as described previously (12). Briefly, cells were exposed to 1,000 mJ/cm² UV-B irradiation at 312 nm in fresh medium. Cells were then incubated for the indicated times at 37 °C under 5% CO₂.

*Pneumococcal Strains*—*S. pneumoniae* R6 is a nonencapsulated avirulent strain derived from strain R36A, which itself is derived from the capsular type 2 clinical isolate strain D39. R36A has multiple interruptions in the type 2 capsular locus inherited from D39. The R6 and D39 hydrophobic tail (Hbt) mutants were generated as addressed later. The wild-type (R6 and D39) and Hbt mutant (R6 Hbt and D39 Hbt) strains were cultivated under anaerobic conditions in either Todd Hewitt broth (BD Biosciences) for the R6 strains or Todd Hewitt broth supplemented with 0.5% yeast extract for the D39 strains. Whenever required, pneumococci were labeled with fluorescein isothiocyanate (FITC). A 10-ml bacterial culture in late exponential growth phase (A₅₀₀ = 0.6) was harvested by centrifugation (10 min at 3,000 × g). Cells were washed with PBS before incubation with FITC at 1 mg/ml in 500 μl of PBS for 30 min at 4 °C. Bacteria were washed three times with PBS before use.

*Isolation of HeLa Cell Surface Biotinylated Proteins*—HeLa cell surface proteins were biotinylated as follow. Cells were washed two times with ice-cold PBS and biotinylated using biotinamido caproate *N*-hydroxysuccinimide ester diluted in bicarbonate buffer (Amersham Biosciences). After incubation at 4 °C for 30 min with gentle shaking, cells were rinsed twice with ice-cold PBS to remove any remaining biotinylation reagent. The cells were then scraped and lysed in 1% Triton X-100, 1 mM CaCl₂, 1 mM MgCl₂, and protease inhibitors for 1 h at 4 °C. Soluble proteins in the supernatant were quantified with a bicinchoninic acid assay (complete EDTA-free, Roche Diagnostics) and left at 4 °C for 1 h. Insoluble material was removed by centrifugation at 20,800 × g for 30 min at 4 °C. Soluble proteins present in the supernatant were quantified with a bicinchoninic acid assay (Sigma) and incubated with streptavidin-conjugated magnetic beads (Dynabeads MyOne Streptavidin T1, Invitrogen Dynal AS, Oslo, Norway) with rotation for 90 min at 4 °C. The beads were collected with a magnet and washed eight times with PBS, 0.1% Tween 20. Biotinylated proteins were eluted by boiling the beads with 40 μl of 2 × Laemmli sample buffer for 5 min at 100 °C. The amount of biotinylated surface-exposed GAPDH was determined by Western blotting using an anti-human GAPDH antibody.

*Preparation of HeLa Cell Plasma Membrane Proteins*—Culture dishes (100 mm) of HeLa cells were harvested by scraping and incubated on ice for 20 min in hypotonic buffer (10 mM Tris-HCl (pH 7.6) containing protease inhibitors) and disrupted by Dounce homogenization. The homogenate was centrifuged at 3,000 × g for 10 min. The supernatant was collected and further centrifuged at 220,000 × g for 30 min. The cell membrane-containing pellet was then solubilized in PBS containing 1% Triton X-100, 1 mM CaCl₂, 1 mM MgCl₂, and protease inhibitors for 1 h at 4 °C. Solubilized membrane proteins
were collected from the supernatant by 30-min centrifugation at 220,000 \( \times g \).

**C1qGR Overlay Binding Assay**—Solubilized membrane proteins (10 \( \mu g \)) were subjected to electrophoresis using a 10% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore). The membrane was subsequently blocked with 3% BSA in PBS, 0.1% Tween 20 at room temperature for 1 h and washed three times with PBS, 0.1% Tween 20. Putative streptavidin binding sites were blocked using a streptavidin/biotin blocking kit (SP-2002, Vector Laboratories). The membrane was washed briefly before incubation with biotinylated C1qGRs (2.5 \( \mu g/ml \)) for 2 h (C1qGRs were biotinylated using the ECL protein biotin system (Amersham Biosciences) according to the manufacturer’s instructions). After extensive washes, the membrane was incubated with streptavidin conjugated to horseradish peroxidase for 1 h, which allowed detection of the bound biotinylated C1qGRs by chemiluminescence (SuperSignal West Pico Chemiluminescent substrate, Pierce).

**Pulldown Experiments**—Biotinylated C1qGRs were added to 80 \( \mu l \) of preequilibrated streptavidin-conjugated magnetic beads. The mixture was incubated for 30 min at room temperature with rotation. The beads were pelleted, and the supernatant was removed. After six washes with PBS, 1% BSA, the beads were incubated with plasma membrane proteins for 4 h at 4 °C with gentle rocking. The beads were then pelleted, and supernatants were collected. After seven washes with 0.1% Triton X-100, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\) in PBS, bound proteins were eluted by addition of 40 \( \mu l \) of 2× Laemmli sample buffer and incubated at 100 °C for 10 min. As a control, the same experiment was performed with 80 \( \mu l \) of streptavidin magnetic beads not coupled to biotinylated C1qGRs or coupled to free biotin. Detection of GAPDH was performed by Western blotting using an anti-human GAPDH antibody.

**Flow Cytometry**—HeLa cells were harvested using trypsin-EDTA solution (Invitrogen). Cells (0.5 \( \times 10^6/ml \)) were washed twice, resuspended in PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and resuspended in PBS, 1% BSA. Monoclonal anti-human GAPDH antibody was diluted 1:250 and incubated with cells for 45 min on ice. An isotype antibody was used as a control. After two washes, cells were resuspended in PBS and incubated on ice for 30 min with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1:200 and analyzed with a FACSscan flow cytometer using CellQuest software (BD Biosciences).

**Production and Purification of Recombinant Pneumococcal and Human GAPDHs**—Genomic DNA from the D39 strain of *S. pneumoniae* was used as a template to amplify the gapdh gene (Sp2012) by conventional PCR methodology. The resulting PCR product was cloned into the pLIM01 vector (PX’Therapeutics SA, Grenoble, France) that had been modified from pQE80 by insertion of patented sequences, allowing a ligase-independent cloning procedure. The resulting construct, pLIM01/gapdh, encodes the full-length GAPDH protein fused to a His\(_{6}\) tag at the N terminus. A tobacco etch virus protease cleavage site was inserted between the His\(_{6}\) tag and the N-terminal sequence of GAPDH. DNA sequencing confirmed that no mutation had been introduced during PCR. The human GAPDH gene was amplified from cDNA (GAPDH, NCBI Reference Sequence NM_002046, human cDNA clone, Origene), and a tobacco etch virus cleavage site was introduced at the 5’-extremity. The resulting PCR product was cloned into the pETDuet vector (Novagen) to fuse a His\(_{6}\) tag at the N terminus of the human GAPDH sequence. Overnight cultures of the *Escherichia coli* BL21(DE3)-CodonPlus-RIL (Stratagene) strain transformed with the Hsa or Spn GAPDH expression constructs were used for inoculation with 500 ml of Terrific Broth medium (Euromedex) supplemented with 100 \( \mu g/ml \) ampicillin and cultured at 37 °C for 3 h. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 16 h at 15 °C. After sonication and centrifugation of the lysate (20 min at 40,000 \( \times g \)), recombinant Hsa and Spn GAPDH proteins were recovered from the soluble fraction and loaded onto a 1-ml prepacked HisTrap HP column (GE Healthcare). Column equilibration buffer was 50 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole (pH 8.0). After extensive washing, recombinant proteins were eluted with 60, 100, 300, and 500 mM imidazole steps in 50 mM Tris-HCl, 200 mM NaCl (pH 8.0) buffer and subsequently dialyzed against 10 mM HEPES, 150 mM NaCl, 2 mM CaCl\(_2\) (pH 7.4) before use for biological assays. The degree of protein purity was checked by Coomassie Blue staining of SDS-polyacrylamide gels. Protein concentrations were determined by absorbance at 280 nm.

**Construction of the Hbt Mutant Pneumococcal Strains**—The R6 and D39 Hbt mutant strains were generated by insertion of a hydrophobic tail (IVLVGLVMLLLS) at the 3’-end of the gapdh gene as described previously (37). Complementary primers for insertion (QuickChange II XL Site-Directed Mutagenesis kit, Agilent Technologies) of this sequence in the pLIM01-rgAPDH vector were as follows: GAPDH-1, AATATCTCGAAAAGATTTGCTAAATTGTTCTGTGGCCTGTATTGCTTTTCTTTATCATAATTGATGTCCTGAGCC; GAPDH-2, GCTCGAGGTCACCTCAATGGAGTTAAGAAGAAAGCAGTAAACCGCCACCAAGAAACATTTTACATCTTGGAAGTATC. To construct the recombination product, three overlapping fragments were PCR-amplified: 1) a 604-nucleotide fragment comprising the 3’-end of the gapdh gene, the hydrophobic tail, and the 5’-region of the cat gene (GAPDH-3, GCTTGGCTCCAATGCTTAAAGCCTGT; GAPDH-4, TCAAAAAATTTCATCAAGCTTTTTAAGAAGAAAGCAGTAAACCGCCACCAAGAAACATTTTACATCTTGGAAGTATC); 2) a 1084-nucleotide fragment comprising the cat gene, the 3’-end of the hydrophobic tail, and the sequence downstream of the gapdh gene (GAPDH-5, GGCCCTGTTATGCTTTTCTTTTCTTTAAAGCTTGATGAAAATTGTGGTGA; GAPDH-6, GCTTCTTATCACTCAGAATTTCTGAAATGGATGATCCCCGG-3’); and 3) a 552-nucleotide fragment comprising the sequence downstream of the gapdh gene overlapping the cat gene (GAPDH-7, CCGGGGATCCATGGCTTTTACATGGCTTAAAGCCTGT; GAPDH-8, GCCAAAAATCTCTTATCTGC). These three fragments were PCR-assembled using primers GAPDH-3 and GAPDH-8, and R6 or D39 strain competent cells were transformed with the recombination product and plated onto chloramphenicol-containing Columbia blood agar. Mutant strains were selected twice, and insertion was verified by PCR amplifications using primers flanking the recombination product.
**GAPDH Is a Novel C1q Ligand**

Solid-phase Binding Assay—Solid-phase binding assays were performed to measure binding of Hsa and Spn GAPDH proteins to C1q and plasminogen. White 96-well microtiter plates (Greiner Bio One) were coated with 1 μg of C1q, plasminogen (Roche Applied Science) or BSA as a control in 100 μl of PBS at 4 °C overnight. Saturation was performed by adding 200 μl/well PBS, 2% BSA for 1 h at room temperature. Five washes were performed using 200 μl of PBS. The recombinant Hsa and Spn GAPDHs were added in each well (amounts ranging from 10 ng to 10 μg), and the mixture was incubated for 2 h at room temperature. Five washes were performed using 200 μl of PBS, 0.2% Tween. His-tagged bound Hsa and Spn GAPDH proteins were detected by adding 100 μl/well horseradish peroxidase-conjugated anti-His antibody (Sigma) (1:1,000 dilution) in PBS, 0.03% Tween 20, 0.2% BSA for 1 h at room temperature. Four washes with 200 μl of PBS, 0.2% Tween 20 were performed. ECL solution (Pierce) (100 μl) was added to each well, and chemiluminescence was measured using a multivwell luminescence reader (Fluostar Optima, BMG Labtech).

Surface Plasmon Resonance Measurements—Surface plasmon resonance measurements were performed using a BIAcore 3000 instrument (GE Healthcare). C1q and its GRs were covalently immobilized to the dextran matrix of a CM5 sensor chip via the primary amine groups (amine coupling kit, GE Healthcare). The carboxymethylated dextran surface was activated by the injection of a mixture of 0.2 M N-ethyl-N’-(diethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide. Ligands were injected in 10 mM sodium acetate buffer (pH 5.0). Ligand concentrations and contact time were adjusted according to the desired level of immobilization. The remaining N-hydroxysuccinimide esters were blocked by injection of 1 M ethanolamine hydrochloride (pH 8.5). All immobilization steps were performed at a flow rate of 10 μl/min in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% P20 (GE Healthcare) (pH 7.4). Immobilization levels for C1q and GR were 19,752 and 7,933 response units, respectively. No protein was immobilized on the control flow cell that underwent the activation and blocking steps. Binding experiments were performed at 25 °C at a flow rate of 30 μl/min in 10 mM HEPES, 150 mM NaCl, 2 mM CaCl₂, 0.005% P20 (pH 7.4). Spn GAPDH was injected at different concentrations ranging from 1 to 10 nM, and Hsa GAPDH was injected at concentrations ranging from 5 to 50 nM. Data were double referenced by subtraction of the control flow cell signal and of a blank run (buffer only). In all experiments, association phases ran for 180 s, and dissociation phases ran for 300 s. The surface was regenerated with pulses of guanidinium chloride ranging from 0.1 to 1 M. Data were analyzed by an alkaline elution strategy. A 50-ml culture in late exponential growth phase (A₆₀₀ ≈ 0.6) was centrifuged (15 min at 3,000 × g), and the pellet was resuspended in 1 ml of PBS containing 100 μg/ml lysozyme and 50 units/ml mutanolysin and incubated for 2 h at 37 °C, yielding the whole cell lysate extract. The remaining 90 ml were centrifuged (15 min at 3,000 × g), and the pellet was resuspended in 9 ml of PBS containing 100 μg/ml lysozyme, 50 units/ml mutanolysin, and 30% sucrose and incubated for 2 h at 37 °C. This lysate was centrifuged, and the supernatant containing the cell wall was collected. The pelleted protoplasts were resuspended in 9 ml of 10 mM HEPES, 10 mM KCl (pH 7.4); lysed by three cycles of freezing/thawing; and ultracentrifuged for 45 min at 190,000 × g. The soluble cytoplasm was collected, and the membrane pellet was resuspended in 9 ml of 10 mM HEPES, 10 mM KCl (pH 7.4). The different subcellular fractions were serially diluted, separated by SDS-PAGE, transferred on a nitrocellulose membrane, and analyzed by Western blot using rabbit anti-pneumococcal GAPDH antibody (1:5,000 dilution), horseradish peroxidase-conjugated anti-rabbit antibody (1:5,000 dilution; Sigma-Aldrich), and ECL solution (Pierce) as detection reagent. The intensity of the spots was quantified using ImageJ software. The amount of GAPDH in each sample was determined relative to the GAPDH released by the wild-type strain, used at the highest concentration, and this value was arbitrarily considered as 100%. If necessary, the intensity values were adjusted based on the CFU counting.

Pneumococcal Subcellular Fractionation—The amount of pneumococcal GAPDH in the different cell compartments of the wild-type and Hbt mutant strains was analyzed by cell fractionation. One-tenth of a 100-ml culture in late exponential growth phase (A₆₀₀ ≈ 0.6) was centrifuged (15 min at 3,000 × g), and the bacterial culture density was measured by CFU counting on blood agar plates. The cells were resuspended in PBS to adjust the bacterial suspension to 1.5 × 10⁹, 3 × 10⁹, 7.5 × 10⁹, 1.5 × 10¹⁰, 2.25 × 10¹⁰, and 3 × 10¹⁰ CFU in 100 μl of 100 mM carbonate (pH 10) buffer and incubated for 30 min at 37 °C. The suspensions were centrifuged, and the supernatants were collected, separated by SDS-PAGE, transferred on a nitrocellulose membrane, and analyzed by Western blot using rabbit anti-pneumococcal GAPDH antibody (1:5,000 dilution), horseradish peroxidase-conjugated anti-rabbit antibody (1:5,000 dilution; Sigma-Aldrich), and ECL solution (Pierce) as detection reagent. The intensity of the spots was quantified using ImageJ software.
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paraformaldehyde, incubated with 1% BSA, and then submitted to GAPDH and C1q detection by indirect immunofluorescence using a rabbit anti-C1q polyclonal antibody diluted 1:100 and a monoclonal anti-human GAPDH antibody diluted 1:250 in PBS. Bound antibodies were visualized with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and Alexa Fluor 488-conjugated anti-rabbit IgG diluted 1:250 in PBS. Cells were mounted on glass slides with Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Cells were photographed using an epifluorescence Olympus IX81 microscope equipped with differential interference contrast (Nomarski) and a reproducible optical section acquisition system (ΔZmin = 0.1 μm) for three-dimensional reconstruction. To assess spatial localizations within the cell and at the surface, serial optical sections were taken at 0.5-μm intervals throughout the thickness of all cells examined. Velocity 6 software was used for analysis and treatment (deconvolution/restoration, co-localization, and quantification). Pearson’s correlation coefficient was measured on each view to reinforce co-localization observations. Coefficients ≥0.75 were measured for all images shown.

Wild-type R6 pneumococcal strain was grown at an A600 of 0.3 and fixed with 4% paraformaldehyde for 20 min in ice. Cells were then deposited onto poly-L-lysine-coated Poly Prep slides (Sigma-Aldrich) and permeabilized in cold methanol for 5 min. Slides were blocked for 30 min at room temperature with 5% (w/v) nonfat dry milk in PBS (saturation buffer) and then incubated for 1 h with anti-pneumococcal GAPDH antibody in saturation buffer. The slides were then washed twice in PBS and incubated with a 1:300 dilution of Cy2-conjugated goat anti-rabbit antibody in saturation buffer. After successive washes with PBS and water, cells were incubated with 2 μg/ml DAPI (Tebu-Bio) for 15 min or with 0.5 μl of FM4-64 (1 mg/ml; Molecular Probes). Slides were mounted with Mowiol and stained with ammonium molybdate. Staining with ammonium molybdate took place after this step.

C1 Activation Assay—C1 activation was assayed as described previously (35). C1q (0.25 μm) and various amounts of GAPDH were mixed and incubated for 20 min at 37 °C before addition of equimolar amounts of the C1s-C1r-C1r-C1s tetramer (0.25 μM) to reconstitute the C1 complex. The mixture was then incubated for 20 min at 37 °C in 50 mM triethanolamine-HCl, 145 mM NaCl, 2 mM CaCl2 (pH 7.4). The reaction mixtures were submitted to SDS-PAGE analysis under reducing conditions using 10% acrylamide gels. The bands corresponding to C1s were revealed by Western blot analysis using a rabbit polyclonal antibody after electrotransfer to a nitrocellulose membrane. C1 activation was determined from the amounts of the A and B chains of activated C1s relative to that of the proenzyme. In a control experiment, it was verified that GAPDH does not activate the C1s-C1r-C1r-C1s tetramer in the absence of C1q.

Complement Deposition Assay—Complement deposition upon treatment with human serum was measured as follows. White 96-well microtiter plates (Greiner Bio One) were coated in triplicates with 1 μg of C1q, plasminogen, or BSA in PBS at 4 °C overnight. Saturation was performed by adding 200 μl/well PBS, 2% BSA for 1 h at room temperature. Five washes were performed using 200 μl of PBS. 100 μl of FITC-labeled bacterial suspensions (3 × 107, 7.5 × 107, 1.5 × 108, or 3 × 108 CFU/ml) were added to each well, and the mixture was incubated for 1 h at room temperature. Five washes were performed using 200 μl of PBS, 0.2% Tween 20. The bacterium-associated fluorescence was measured using a multwell fluorescence reader (Fluostar Optima, BMG Labtech). Bacterial suspensions of the wild-type and HBt mutant strains were plated for CFU counting to control the bacterial density. The data were corrected according to the wild-type/HBt ratio: the binding level was related to the value displayed by the wild-type strain, used at the highest concentration, and this value was arbitrarily considered as 100%.

Electron Microscopy Analysis—Isolated GAPDH and C1q samples at a concentration of approximately 0.05 mg/ml were applied to the clean side of carbon film on mica (carbon/mica interface) and negatively stained with 2% ammonium molybdate (pH 7.4). A grid was placed on top of the carbon film, which was subsequently air-dried. Images were taken under low dose conditions with a CM12 Phillips electron microscope at 120 kV and a calibrated nominal magnification of 45,000 using an ORIUS SC1000 charge-coupled device camera (Gatan, Inc.). Glutaraldehyde cross-linked complexes were also analyzed by EM. In addition, the recently described GraFix sample preparation technique was also used to obtain cross-linked C1q-GAPDH complexes (38). Briefly, the complexes were prepared by incubation of a 2:1 ratio mixture of GAPDH (0.1 mg/ml) and C1q (0.05 mg/ml) in 10 mM HEPES, 150 mM NaCl, 2 mM CaCl2 (pH 7.4) at room temperature for 30 min. 100 μl of the mixture was applied to a 0.5–20% (v/v) glycerol, 0.033–0.1% (v/v) glutaraldehyde gradient. After centrifugation at 20,000 rpm in an SW55 Ti rotor (Beckman Coulter) for 18 h at 4 °C, 50–μl fractions were collected from the top of the gradient, and excess glutaraldehyde was inactivated by adding 5 μl of 1 M Tris to each fraction. To remove the maximal amount of glycerol, the carbon/sample was rinsed by plunging it into buffer without glycerol. Staining with ammonium molybdate took place after this step.
necrosis was measured using a multiwell luminescence reader (Fluostar Optima, BMG Labtech). Two washes were performed with 100 μl of HBS-C between each step.

RESULTS

The Human GAPDH Exposed at the Cell Surface Is a Novel C1qGR-binding Protein—GAPDH was initially detected by blot overlay on plasma membrane extracts using the C1q globular region as the bait (Fig. 1A). This experiment was designed to identify unknown cell surface C1q ligand(s) potentially involved in the recognition and the uptake of apoptotic cells by macrophages. When incubated with plasma membrane proteins extracted from viable and early apoptotic HeLa cells (triggered by UV-B irradiation), biotinylated C1qGR strongly revealed a 36-kDa band that showed slightly increased intensity with the development of apoptosis (Fig. 1A, lanes 2, 3, and 4 compared with lane 1). N-terminal protein sequencing analysis demonstrated unambiguously that this band corresponds to GAPDH (data not shown). Because GAPDH is a major and widely expressed protein in all human cells, complementary analyses were conducted to validate this initial observation. The presence of GAPDH on the surface of HeLa cells was confirmed after cell surface biotinylation, purification of the biotinylated proteins using streptavidin magnetic beads, and Western blotting using an antibody directed against human GAPDH (Fig. 1B). As expected, GAPDH was detected in the pellet fraction containing biotinylated surface proteins. The supernatant fractions correspond to the intracellular compartments in which GAPDH was present independently of the biotinylation procedure. Surface GAPDH expression was also investigated by flow cytometry on HeLa and Jurkat cells. The human monocyte THP-1 cell line was used as a control because it was published which GAPDH was present independently of the biotinylation procedure. GAPDH detection by Western blotting among biotinylated HeLa cell surface proteins. GAPDH is detected in the pellet containing biotinylated surface proteins. The supernatants correspond to the intracellular non-biotinylated fractions. GAPDH was stained with anti-GAPDH or with an isotype control and analyzed by FACS. Viable and early apoptotic Jurkat cells are shown (Fig. 1C). The control (Ct) corresponds to purified GAPDH. The positions of molecular mass markers (expressed in kilodaltons) are shown (A, B, and E).

C1q and GAPDH Co-localize at the Surface of Apoptotic Cells—Based on the above observation, we questioned whether GAPDH could be a ligand of C1q involved in the recognition of apoptotic cells. To this end, co-localization experiments were conducted on viable and apoptotic HeLa cells using either the globular region of C1q or the complete molecule. As illustrated in Fig. 2, GAPDH was detected at the cell surface on both viable and apoptotic HeLa cells (Fig. 2, C, H, M, and R) in agreement
with the cytometry experiment (Fig. 1C). Binding of C1q was detected on apoptotic cells (Fig. 2A) and to a lesser extent on non-apoptotic cells (Fig. 2F), whereas C1qGR binding was mainly detected on apoptotic cells (Fig. 2K). The co-localization was supported by Pearson’s correlation coefficients measured on the merged images shown in Fig. 2, J, O, and T, as described under “Experimental Procedures.” In conclusion, C1q and C1qGR mostly co-localize with GAPDH detected on the surface of apoptotic cells characterized by plasma membrane blebbing. GAPDH-C1q co-localization is not restricted to HeLa cells because we obtained similar results on apoptotic Jurkat cells (not shown).

Expression and Purification of Human and Pneumococcal GAPDH Proteins—To further investigate the C1q binding to human and bacterial GAPDHs, comparable production strategies were conducted to produce Hsa and Spn recombinant GAPDH proteins. The expression vectors used allowed production of the recombinant proteins fused to a histidine affinity tag. SDS-PAGE analysis of the purified recombinant proteins indicated that Hsa and Spn GAPDHs were essentially pure and exhibited apparent molecular masses of about 38 kDa under reducing conditions (data not shown) in accordance with the calculated and measured masses. Indeed, the molecular masses determined by MALDI-TOF analysis were 38,198 ± 39 and 38,193 ± 9 Da for Hsa and Spn GAPDHs, respectively, consistent with the values predicted from the amino acid sequences after removal of the N-terminal methionine (38,198 and 38,180 Da, respectively). Transmission electron microscopy confirmed the homogeneity of the preparation and the tetrameric oligomerization state of both Hsa and Spn GAPDHs (see Fig. 5) (39–42).

Pneumococcal and Human GAPDHs Interact with C1q in Vitro—Spn and Hsa recombinant GAPDH proteins ranging from 0.01 to 10 μg were incubated with coated C1q. Significant dose-dependent binding responses of both GAPDH proteins to
C1q were observed when compared with BSA used as negative control (Fig. 3A). Similar results were obtained with coated plasminogen used as a positive control, showing that human GAPDH, like its bacterial homolog, binds to plasminogen (Fig. 3B).

**Human and Pneumococcal GAPDHs Bind to C1q Globular Region with High Affinity**—Surface plasmon resonance experiments confirmed the interactions between GAPDH and C1q (Fig. 4). Interaction was first tested using two different human GAPDH proteins: a commercially available native form and a His-tagged recombinant form. We compared the C1q and the C1qGR interaction with both immobilized human GAPDHs. In this configuration, the $K_D$ values determined using the simple 1:1 interaction model showed no significant differences between the native and recombinant human GAPDH forms ($K_D$ of $4 \times 10^{-7}$ and $3 \times 10^{-7} \text{ M}$, respectively). In the reverse configuration with C1q immobilized on the sensor chip surface, binding of human and pneumococcal His-tagged recombinant GAPDH proteins was concentration-dependent (Fig. 4, A and B). The data fit to the simple 1:1 interaction model proposed by the BIAevaluation software from which kinetic parameters were calculated. However, because of oligomeric organizations of GAPDH (tetramer) and C1q (hexamer), the interaction likely results from global avidity recognition. Consequently, the $K_D$ determinations correspond to apparent values (Table 1). The GAPDH binding to C1q is of high affinity because apparent $K_D$ values in the nanomolar range were determined for Spn and Hsa GAPDHs (0.34 and 2.17 nm, respectively). Comparison of the association and dissociation rate constant values indicates that the pneumococcal protein forms a slightly more stable complex with C1q than the human GAPDH (Table 1). This effect was also observed using the reverse experimental configuration where the GAPDH proteins were immobilized and C1q was used as the soluble analyte (data not shown). Both GAPDHs interacted with the C1qGR, although the apparent affinity was higher for the pneumococcal protein (0.73 nm) when compared with the value obtained with the human form (4.40 nm) (Table 1 and Fig. 4, C and D). As already observed with the whole molecule, the complex formed by Spn GAPDH and C1qGR was more stable than that involving the human protein (Table 1).

**Pneumococcal and Human GAPDHs Form Complexes with C1q in Solution**—Formation of the GAPDH-C1q complex in solution was analyzed by a cross-linking approach followed by Western blot detection (Fig. 5). Increasing quantities of GAPDH proteins were used (10, 50, and 100 μg). The proteins migrated as monomeric forms in the absence of glutaraldehyde with an apparent molecular mass around 37 kDa in accordance with the calculated masses (Fig. 5, A and B). The migration

![FIGURE 4. Surface plasmon resonance measurements of pneumococcal and human GAPDH protein interaction with C1q and its GR. Shown are binding of pneumococcal GAPDH (A) or human GAPDH (B) to C1q and binding of pneumococcal GAPDH (C) or human GAPDH (D) to C1qGR. C1q and GR proteins were immobilized on a BIAcore CMS sensor chip; GAPDH recombinant proteins were the analytes. Data represent overlays of sensorgrams resulting from the injection of different concentrations of GAPDH as indicated. The blank run was subtracted from each sensorgram. Fits are shown as dotted lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model. The kinetic parameters of the interactions determined by recording sensorgrams at varying GAPDH concentrations are listed in Table 1. Resp. Diff., response difference; RU, response units.](image-url)
pattern of the GAPDH proteins was not modified in the presence of C1q. Addition of glutaraldehyde induced the formation of dimers and multimers of human and pneumococcal GAPDHs (Fig. 5A and B). In these experimental conditions, pneumococcal GAPDH generated significantly more tetramers and oligomers (Fig. 5B). In the presence of C1q, cross-linking led to the appearance of species of high molecular weight range corresponding to the complex formed between GAPDH and C1q. Taking into account that both GAPDHs form oligomers and that the C1q molecule has a molecular mass of 460 kDa, it is likely that the covalent association of both partners is heterogeneous in size, impairing determination of the stoichiometry of the complex.

Electron microscopy was used to further investigate the association between GAPDH and C1q. Purified recombinant human and pneumococcal GAPDHs have a tetrameric structure (Fig. 5C). Isolated C1q displays the classical hexameric structure resembling a bouquet of tulips (Fig. 5C). The top view clearly shows the six globular regions of the molecules organized around the stem. The Hsa GAPDH-C1q complex was deposited on electron microscopy grids directly after the cross-linking experiment (Fig. 5D, left) or after the GraFix procedure (38), a sample preparation protocol combining the sedimentation of complexes through a glycerol gradient with mild chemical cross-linking (Fig. 5D, left). As shown in representative images corresponding to top views of C1q, complexes were clearly observed because GAPDH molecules characterized by their tetrameric-like structure are localized to each of the globular regions of C1q. Similar images were obtained from samples from GraFix and classical cross-linking experiments with human GAPDH. No difference was detected when comparing human and pneumococcal GAPDHs in complex with C1q.

**Construction of S. pneumoniae Strains with Reduced Level of Surface-exposed GAPDH**—The cellular localization of GAPDH was analyzed by immunofluorescence experiments. R6 S. pneumoniae was incubated with anti-GAPDH antibodies and with Cy2-conjugated secondary IgG. DNA and membranes were labeled with DAPI and FM4-64, respectively (Fig. 6A). Both merged images show that GAPDH is localized at the cell poles. Green GAPDH localization appears to be external to the red FM4-64 membrane labeling, indicating that GAPDH is associated with the cell surface (Fig. 6A).

Testing the physiological role of pneumococcal surface GAPDH requires the construction of a mutant strain with no or a limited amount of surface GAPDH. Because of its essential role in bacterial metabolism, the construction of a pneumococ-
**GAPDH Is a Novel C1q Ligand**

A hydrophobic tail is inserted at the 3'-end of the *gapdh* gene in the genome, which will anchor the protein to the membrane, prevent the export, and retain the enzymatic activity in the cytoplasm. The insertion of the hydrophobic tail was performed in the R6 and D39 strains. The mutant strains are designated HBt. The localization and expression level of GAPDH in the wild-type and mutants strains were compared in the different cellular compartments of R6 and D39 strains (supplemental Fig. S1). We could demonstrate that GAPDH expression is comparable in total extracts of both wild-type and mutant strains. We calculated that the proportion of GAPDH associated with the membrane fraction in the R6 HBt strain was increased by 50% when compared with the wild type. Conversely, 83% less GAPDH was associated with the cell wall fraction in the R6 HBt strain when compared with the wild type. Similar data were obtained when comparing the wild-type and mutant D39 strains. Taken together, these results show that the fusion of a hydrophobic tail indeed reduces the quantity of GAPDH at the pneumococcal surface to about 50%.

A complementary procedure was developed to quantify the level of surface-exposed GAPDH. Bacterial suspensions prepared at increasing cell densities were treated by alkaline pH to release GAPDH from the bacterial surface. The presence of GAPDH in the supernatant samples was detected by Western blot (Fig. 6, B and C). The quantity of eluted GAPDH was correlated to the increasing cell density used (from 1.5 × 10⁸ to 3 × 10¹⁰ CFU). Quantification of the chemiluminescence signals was performed. The values were adjusted based on the CFU counting for both strains and normalized to the value for the highest concentration of wild-type sample, which was considered as 100%. The mean difference between HBt mutant and wild-type strains was calculated: the R6 HBt strain displayed only 49% of the GAPDH normally expressed at the surface of the wild-type, and the D39 HBt strain displayed only 43%. These data are in accordance with the calculations based on the subcellular fractionation results (supplemental Fig. S1).

**Native GAPDH Exposed at the Surface of the Pneumococcus Binds to C1q**—The specific binding of FITC-labeled wild-type and mutant R6 and D39 strains to C1q was compared using a solid-phase assay (Fig. 7). Increasing the bacterial concentration of R6 strains led to a dose-dependent binding to C1q, which displayed lower intensity in the case of the HBt mutant strain (Fig. 7A). Although the mutant binding to C1q was decreased by 16 and 11% at 3 × 10⁸ and 7.5 × 10⁹ CFU/ml, respectively, when compared with the parental strain, the R6 HBt binding to C1q was decreased by 64 and 56% when used at 1.5 × 10⁹ and 3 × 10¹⁰ CFU/ml, respectively (Fig. 7A). Similar data were calculated from the analysis of the patterns of R6 wild-type and HBt strain binding to plasminogen (Fig. 7B). These results are in accordance with the half-decreased level of GAPDH exposed at the surface of the mutant strains. The less important decrease in binding of the R6 HBt strain to plasminogen compared with the binding to C1q might be explained by the presence of others plasminogen receptors like enolase and...
CbpE at the pneumococcal surface (43, 44). Similar data were obtained when comparing the wild-type and mutant D39 strains (Fig. 7, C and D). C1q binding to the D39 HBt mutant used at 3 × 10^7, 7.5 × 10^7, 1.5 × 10^8, and 3 × 10^8 CFU/ml ranged from 46 to 56% when compared with the binding level of the wild-type strain (Fig. 7C). As observed with the R6 strain, the binding of the D39 HBt strain to plasminogen was decreased from 53 to 72% when compared with the D39 parental strain (Fig. 7D).

**DISCUSSION**

We provide the first evidence that the eukaryotic and bacterial GAPDHs exposed at the cell surface are ligands of C1q, a major recognition molecule of the complement system. This conclusion is based on the following concordant observations. 1) Hsa GAPDH on HeLa cells bound C1q through its GRs. 2) C1qGR and GAPDH co-localized at the surface of apoptotic cells. 3) Cell surface GAPDH exposure increased rapidly at early steps of apoptosis. 4) The pneumococcal and the human GAPDH proteins interacted in vitro avidly with C1q through its GR. 5) Pneumococcal strains deficient for GAPDH surface exposure displayed significantly

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**FIGURE 7. Binding of pneumococcal R6 and D39 strains on C1q and plasminogen.** FITC-labeled bacteria at the indicated concentration in CFU/ml were incubated for 1 h at room temperature on 1 μg of proteins (C1q, plasminogen, and BSA) coated on a 96-well plate. After five washes, the fluorescence of FITC was measured. All experimental data points were triplicates in each single assay. The average of three independent experiments ± S.D. is shown. The ratios were calculated with respect to the parental strain maximal value. A, R6 wild-type and HBt mutant strain binding to C1q. B, R6 wild-type and HBt mutant strain binding to plasminogen. C, D39 wild-type and HBt mutant strain binding to C1q. D, D39 wild-type and HBt mutant strain binding to plasminogen. Background corresponding to the binding to BSA is subtracted for all points. Error bars correspond to S.D.
decreased C1q binding. 6) Pneumococcal GAPDH participated in complement activation unlike human GAPDH.

The complement system provides a first line of immune defense and plays multiple and central roles such as recognition and elimination of foreign invaders as well as damaged self-cells, clearance of immune complexes, connection between innate and adaptive immunity, and interaction with other host cascade systems like the coagulation system. The classical complement pathway is triggered by C1q recognition of IgG- and IgM-targeting microbes or cells to eliminate, microbial or eukaryotic surface moieties (LPS, phosphatidylserine, and polysaccharides), and the pentraxins C-reactive protein, PTX3, and serum amyloid P bound to targets (45). Interestingly, besides its role in activation of the complement cascade, C1q acts as a bridging molecule between apoptotic cell and antigen-presenting cell-like macrophages and dendritic cells, and furthermore, C1q influences the phagocyte immune status (8, 9). Although C1q is described as a pattern recognition molecule with the unique ability to sense an amazing variety of targets, very few proteins expressed at the surface of pathogens have been identified as C1q ligands. To our knowledge, the only reported bacterial protein ligand of the globular domains of C1q is the OmpK36 porin of Klebsiella pneumoniae (45).

In S. pneumoniae immunity, the complement classical pathway is an important component of the response (17, 18) and is activated by various innate immune mediators, including antibodies, C-reactive protein, serum amyloid P, and SIGN-R1. Recently, the lectin pathway has also been reported as a critical part of the innate immune response to pneumococcal infection (20). Many interactions between S. pneumoniae proteins and complement molecules have been identified but are likely involved in complement system evasion because mainly complement regulators are recruited by the bacteria. PsPC interacts with the complement inhibitor C4b-binding protein, which interferes with the assembly of the C4bC2a C3 convertase; acquisition of C4b-binding protein by the pneumococcus thereby inhibits classical pathway activation (46). CbpA recruits the host regulator Factor H, leading to immune protection and complement inhibition through the formation of a protective shield (47), and also binds to the C3 component of complement (48). The fact that human GAPDH might be involved in the apoptotic cell recognition by C1q raises the possibility of a novel bacterial subversion mechanism of the host immune system by exploiting tolerance.

Until the mid-1990s, it was thought that a protein translated from one gene displayed a unique function. Later on, a growing number of proteins were described to harbor several functions, leading to the generic term of moonlighting proteins (49, 50). This feature is encountered in eukaryotic and prokaryotic organisms and concerns highly conserved proteins involved in metabolic pathways or acting as molecular chaperones. In addition to multiple functions, multiple cellular localizations, including surface exposition (although the secretion mechanism remains unknown), are associated with the moonlighting proteins (51). Most of the bacterial enzymes involved in the glycolytic pathway exert various roles in pathological processes like adhesion and invasion of host cells (32, 51). GAPDH is among the most frequent glycolytic/moonlighting protein associated with the cell surface of pathogenic microorganisms (51) and has been extensively studied for its property to bind to plasminogen (33, 51–54).

Roles of GAPDH in bacterial immunity have also been reported. Group A Streptococcus-exposed GAPDH recruits complement C5a, which further inhibits the recruitment of neutrophils and H2O2 production, suggesting a role in immune system evasion (55). Streptococcus agalactiae GAPDH elicits B cell responses and induces production of the immunosuppressive cytokine IL-10, indicating that GAPDH modulates the immune response to promote host colonization by Group B Streptococcus (30). These data suggest that the role of GAPDH when exposed at the surface of bacterial pathogens might favor infection and/or persistence of the pathogen in the host organism by countering immune defense mechanisms.

The comprehensive role of externalized GAPDH on eukaryotic cells and in particular on apoptotic cell surfaces is still misunderstood. In this study, we showed an increased level of membrane-associated and surface-exposed GAPDH as soon as 2 h after apoptosis induction (Fig. 1, A and D). GAPDH was also recovered from cell culture medium of viable cells and to a
larger extent after the induction of apoptosis (data not shown). These data are supported by a study indicating that glycolytic enzymes such as GAPDH, enolase, and triose-phosphate isomerase are early biomarkers of apoptosis (56). Consequently, the GAPDH recognition by C1q at the early steps of apoptosis could be linked to the uptake of altered self-cells by phagocytes. An interesting aspect highlighted by this work concerns differential effects of human and pneumococcal GAPDHs on complement activation. Bacterial GAPDH activates the complement cascade through the classical pathway. However, the involvement of the lectin and/or the alternative pathways cannot be excluded at this stage. These data indicate that GAPDH is one of the bacterial ligands that lead to complement activation and to the clearance of bacteria. These observations are not consistent with previous reported roles of GAPDH in virulence processes and persistence of the bacteria in the host. This apparent paradox might be scrutinized in light of potential mimicry of apoptotic cells considering that the surface-exposed and/or soluble GAPDH recognition by C1q could be a strategy displayed by the pneumococcus to evade the immune system in some specific step of infection progression.

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