Human salivary agglutinin binds to lung surfactant protein-D and is identical with scavenger receptor protein gp-340

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Salivary agglutinin is a 300–400 kDa salivary glycoprotein that binds to antigen B polypeptides of oral streptococci, thereby playing a role in their colonization and the development of caries. A mass spectrum was recorded of a trypsin digest of agglutinin. A dominant peak of 1460 Da was sequenced by quadrupole time-of-flight (Q-TOF) tandem MS. The sequence showed 100% identity with part of the scavenger receptor cysteine-rich ('SRCR') domain found in gp-340/DMBT1 (deleted in malignant brain tumours-1). The mass spectrum revealed 11 peaks with an identical mass as a computer-simulated trypsin digest of gp-340. gp-340 is a 340 kDa glycoprotein isolated from bronchoalveolar lavage fluid that binds specifically to lung surfactant protein-D. *DMBT1* is a candidate tumour suppressor gene. A search in the human genome revealed only one copy of this gene. The molecular mass, as judged from SDS/PAGE and the amino acid composition of agglutinin, was found to be nearly identical with that of gp-340. It was shown by Western blotting that monoclonal antibodies against gp-340 reacted with salivary agglutinin, and monoclonals against agglutinin reacted with gp-340. It was demonstrated that gp-340 and agglutinin bound in a similar way to *Streptococcus mutans* and surfactant protein-D. Histochemically, the distribution of gp-340 in the submandibular salivary glands was identical with the agglutinin distribution, as shown in a previous paper [Takano, Bogert, Malamud, Lally and Hand (1991) Anat. Rec. **230**, 307–318]. We conclude that agglutinin is identical with gp-340, and that this molecule interacts with *S. mutans* and surfactant protein-D.

Key words: adhesion molecules, innate immunity, mucosa, *Streptococcus mutans*.

INTRODUCTION

Salivary agglutinin is a 300–400 kDa glycoprotein that was isolated originally by affinity adsorption of parotid saliva on to *Streptococcus mutans* [1]. It induces aggregation of *S. mutans* [1,2], and was shown to be present in the salivary pellicle on the tooth surface [3]. Immobilized on hydroxyapatite, it promotes adhesion of *S. mutans* [3–6]. In addition, agglutinin mediates binding between *S. mutans* and *S. sanguis*, which is one of the first colonizers of the dental surface [7]. It forms heterotypic complexes with IgA [8–10]. This complex formation is calcium-dependent [8,10], and is disrupted by reduction [9].

The primary structure of gp-340, a glycoprotein purified from lung washes with a molecular mass comparable with that of salivary agglutinin, has been described previously [11,12]. gp-340 was co-purified with lung surfactant protein-D (SP-D) from bronchoalveolar lavage (BAL) on carbohydrate-affinity columns, and purified SP-D bound gp-340 via protein–protein interaction in a calcium-dependent manner [11]. SP-D is a member of the collectin family. Collectins play an important role in innate immunity by binding to specific carbohydrate structures on the surfaces of pathogenic micro-organisms, including bacteria, viruses, yeast and parasitic protozoa. This binding enhances the phagocytosis and killing of micro-organisms by neutrophils and alveolar macrophages [13,14]. The gp-340 molecules exist both in a soluble form and in a form associated with the alveolar macrophages, and it has been suggested to be a potential opsonin receptor for SP-D.

gp-340 is an alternatively spliced form of DMBT1 (deleted in malignant brain tumours-1), a gene identified as a candidate tumour suppressor gene for tumours of the central nervous system [15]. It is a member of the scavenger receptor cysteine-rich (SRCR) superfamily, a group of molecules that are primarily involved in various types of ligand binding in the innate immune system. gp-340 is composed of 13 SRCR domains, two CUB (C1r/C1s Uegf Bmp1) domains separated by a fourteenth SRCR domain, a zona pellucida ('ZP') domain and a short sequence with no known homology. It was demonstrated by reverse transcriptase-PCR that gp-340 is also expressed in salivary gland tissue, and immunoreactive gp-340 was also found in the salivary ducts [12].

Until recently, nothing was known about the molecular structure of salivary agglutinin, making it difficult to perform functional studies. Recently, it has been demonstrated that salivary agglutinin is identical with gp-340/DMBT1 [16]. Here, we report our results corroborating this observation, and demonstrate that both proteins are not only identical on a genetic level, but also show similar binding characteristics for monoclonal antibodies, *S. mutans* and SP-D. Immunohistochemically, gp-340 expression is demonstrated in the salivary gland.

EXPERIMENTAL

Antibodies

Monoclonal antibody raised against salivary agglutinin (mAb143) with a specificity as described previously [17,18] was kindly provided by Dr D. Malamud (University of Pennsylvania, Philadelphia, PA, U.S.A.). Antibodies against gp-340 (Hyb213-1 and Hyb213-6) have been described previously [11,19].

Abbreviations used: BAL, bronchoalveolar lavage; DMBT1, deleted in malignant brain tumours 1; CUB, C1r/C1s Uegf Bmp1; Q-TOF, quadrupole time-of-flight; SP-D, lung surfactant protein-D; SRCR, scavenger receptor cysteine-rich; TBS, Tris-buffered saline.

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Alkaline-phosphatase-conjugated goat anti-(rabbit IgG) antibody (whole molecule) (A-8025) and alkaline-phosphatase-conjugated goat anti-(mouse IgG) antibody (whole molecule) (A-2527) were obtained from Sigma (St. Louis, MO, USA).

Purification of agglutinin

Parotid saliva was collected with a Lashley cup under stimulation with sugar-free candies and incubated on ice/water, resulting in the formation of precipitates containing agglutinin. After centrifugation (5000 ***g***, 15 min, 4 °C) the pellet was resuspended in a one-tenth volume of buffer A [10 mM Tris/HCl (pH 6.9)/10 mM EDTA/0.05 % (v/v) CHAPS]. Samples were applied on an Uno Q-6 column (BioRad Laboratories, Hercules, CA, U.S.A.). Retained proteins were eluted with a linear gradient from 0–0.5 M NaCl in buffer A. Agglutinin-containing fractions were pooled, and purity was checked by electrophoresis. This sample was used for binding studies with SP-D.

Isolation of gp-340 and SP-D

gp-340 was isolated from human bronchoalveolar lavage (BAL), as described previously [11]. SP-D was purified from amniotic fluid, as described by Strong et al. [20].

SDS/PAGE and Western blotting

SDS/PAGE was conducted on a Pharmacia Phast System (Pharmacia-LKB, Uppsala, Sweden) according to the manufacturer's instructions. Proteins were dissolved in sample buffer containing 15 mM Tris/HCl, pH 6.8, 0.5 % (w/v) SDS, 2.5 % (v/v) glycerol and 0.05 % (w/v) Bromophenol Blue. For reduction, samples were incubated with 25 mM dithiothreitol (ICN Biomedicals, Aurora, OH, U.S.A.). Samples were separated on 4–15 % (w/v) or 7.5 % polyacrylamide gels. For Western blotting, proteins were transferred on to nitrocellulose membranes by diffusion blotting. Nitrocellulose membranes were blocked with PBS containing 2 % (w/v) BSA and 0.1 % Tween 20. Incubation with antibodies was also performed in PBS containing 2 % (w/v) BSA and 0.1 % Tween 20. Bound antibodies were detected with alkaline-phosphatase-conjugated goat anti-mouse Igs (whole molecule) (A-2527, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics, Mannheim, Germany) was used as the substrate.

Determination of internal sequence

Agglutinin-enriched saliva was separated on 4–15 % Tris/acetate gels (Novex, San Diego, CA, U.S.A.). The gels were stained with Coomassie Brilliant Blue R250 (Sigma, St Louis, MO, U.S.A.) in 10 % (v/v) acetic acid and 25 % (v/v) methanol, and destained in the same solution without Coomassie Brilliant Blue. The agglutinin band was excised and incubated for 10 min with 100 μl of acetonitrile. After removing acetonitrile, gel fragments were dried for 30 min in a vacuum centrifuge. Trypsin digestion was conducted by overnight incubation in 20 μl of trypsin sequence grade (Roche Diagnostics) solution (20 ng/ml in 50 mM ammonium hydrogen carbonate). Peptide fragments were subsequently extracted, and wash fluid was pooled and concentrated into a volume of 30 μl. The sample was applied on a Poros 50 column. The column was washed with 5 % formic acid/5 % methanol. The sample was extracted in 10 μl of 5 % formic acid/60 % methanol, and applied on a quadrupole time-of-flight (Q-TOF; Micromass UK Ltd, Altrincham, Cheshire, U.K.) hybrid tandem mass spectrometer. In the MS mode, a mass spectrum of the total peptide mixture was recorded. A dominant peptide peak was selected and an MS–MS spectrum was recorded, from which the amino acid sequence was determined. Sequence similarity was searched for with the Protein Data Bank. Amino acid composition and location of determined sequences were determined with the Protean program of DNA Star (DNASTAR Inc., Madison, WI, U.S.A.). The *DMBT1* sequence [15] was used to do a database search on the human genome with the BLAST program at http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html [21].

Table 1 Comparison of a trypsin digest of salivary agglutinin with a computer-simulated digest of gp-340

Salivary agglutinin was digested with trypsin and a mass spectrum was run on a Q-TOF mass spectrometer. The peptide mass was compared with the mass of peptides of gp-340. The marked peptide fragment was sequenced and exhibited 100 % identity with a fragment of the SRCR domains of gp-340. *This fragment was present ten times in gp-340.

Mass spectrum peak of agglutinin	Computer-simulated digest of gp-340	Fragment in gp-340 sequence
535.245	535.31	2108–2111
795.428	795.36	2367–2373
1005.673	1005.57	1804–1812
1115.611	1115.55	1846–1854
1420.823	1420.65	2086–2098
1457.851	1457.65	2326–2338
1459.914*	1459.75*	418–431, 549–562, 657–670, 788–801, 917–930, 1048–1061, 1177–1190, 1306–1319, 1435–1448, 1695–1708
1501.787	1502.66	1033–1047
2254.195	2254.14	2034–2051
2477.365	2477.19	2086–2107
2813.615	2813.49	708–734

Bacterial binding assay

Binding experiments were conducted with *S. mutans* strain Ingbritt. The strain was stored frozen in Protect bacterial preservers (Technical Service Consultants Ltd., Bury, U.K.). Bacteria were cultured on blood-agar plates and grown for 48 h at 37 °C under micro-aerophilic conditions. One colony was used for inoculation in Todd–Hewitt broth (Difco Laboratories, Detroit, MI, U.S.A.), and cultures were grown overnight at 37 °C in completely filled 100 ml flasks under air without shaking. Cells were harvested by centrifugation (3000 ***g*** at 4 °C for 10 min), washed in PBS and resuspended in PBS to an attenuance at 700 nm of 1.0 (10^9 cells/ml). The bacterial suspension (0.5 ml) was pelleted by centrifugation for 2 min at 10000 ***g***. The pellet was resuspended in 40 μl of gp-340 solution (A_{280} 0.014), agglutinin or BAL fluid. After incubation for 1 h at 37 °C, bacteria were pelleted by centrifugation and washed with PBS. Hereafter, bacteria were resuspended in sample buffer and incubated for 1 h at room temperature. After centrifugation, the supernatant was prepared for SDS/PAGE by boiling for 5 min.

Analysis of SP-D binding to agglutinin and gp-340

Microtitre plates (Polysorp, Nunc, Kamstrup, Denmark) were coated with agglutinin or gp-340 (300 ng/ml) in coating buffer [60 mM Na_2CO_3/35 mM sodium hydrogen carbonate/0.02 % (w/v) NaN_3 (pH 9.6)] by overnight incubation. This incubation and all the following steps were performed in a volume of 100 μl per well at room temperature, and all washes and incubations were carried out with TBS-T [where the composition of Tris-buffered saline (TBS) is 140 mM NaCl, 10 mM Tris/HCl, 0.02 %

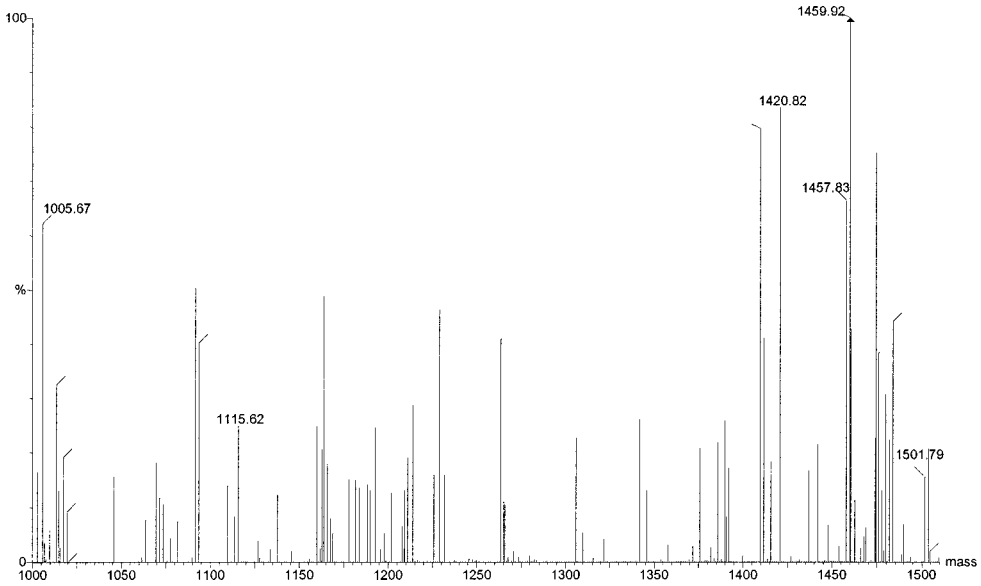

Figure 1 Mass spectrum of a trypsin digest of agglutinin as determined by Q-TOF mass spectrometry

Analysis of the spectrum revealed a particular strong peak at 1459.9 da. This peak was sequenced by tandem MS and exhibited 100% identity with a part of the SRCR domain of gp-340/DMBT1 (FGQGSGPIVLDDVR). The marked peaks are also found in a computer-simulated trypsin digest of gp-340. 100% on the *y*-axis represents the most intense peak currently on the display.

Table 2 Comparison of the amino acid composition of salivary agglutinin and gp-340

The amino acid composition as determined for agglutinin [9] was compared with the amino acid composition of gp-340, as determined by amino acid analysis [17] and from the polypeptide sequence [18]. ND, not determined. *Trp was not mentioned in [9].

		Amino acid composition (mol/mol protein)		
Amino acid	Glycoprotein ...	Agglutinin [9]	gp-340 [17]	gp-340 [18]
Ala		8.6	6.3	6.05
Cys		2.4	2.4	5.43
Asx		12.5	12.7	6.46
Glx		8.6	6.1	3.61(Glu)
Phe		2.3	3.2	2.11
Gly		11.4	11	10.73
His		2.5	2.4	3.11
Ile		4.4	3.2	3.03
Lys		1.2	4.3	0.41
Leu		8.0	8.5	7.05
Met		0.5	ND	5.14
Pro		4.3	6.2	5.30
Arg		6.2	6.1	3.27
Ser		11.6	10.7	12.06
Thr		6.0	6.0	5.72
Val		6.1	7.0	7.09
Trp*			ND	3.69
Tyr		3.4	3.4	3.07
Hyp		0.3	0.3	

(w/v) NaN_3 (pH 7.4); also containing 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate; Merck-Schuchardt, Hohenbrunn, Germany)] containing 5 mM $CaCl_2$, unless otherwise stated. The plates were washed and incubated with 200 μg of human serum albumin (Statens Serum Institut, Copenhagen, Denmark) in 200 μl of TBS for 2 h. After washing, the plates were incubated with dilutions of SP-D, or SP-D containing 100 mM maltose. As a control, the SP-D was also applied in buffer containing 10 mM EDTA instead of calcium. The plates were incubated overnight at 4 °C and washed. They were then incubated for 2 h with rabbit anti-(human SP-D) antiserum diluted 1:500 in TBS-T. After washing, the plates were incubated for 2 h with alkaline-phosphatase-coupled goat anti-(rabbit Ig) diluted 1:2000. After a final wash, the bound enzyme was estimated by adding *p*-nitrophenyl phosphate disodium salt (1 mg/ml) (Roche Diagnostics) in diethanolamine buffer. The absorbance of the 96 wells was read at 405 nm by means of a multi-channel spectrophotometer.

Immunohistochemistry

Four sections were cut from neutral-buffered formaldehyde-fixed paraffin-embedded tissue blocks. Sections were mounted on ChemMate Capillary Gap Slides (DAKO, Glostrup, Denmark) dried at 60 °C, deparaffinized and hydrated. Antigen retrieval was performed using microwave heating in Target Retrieval Solution (DAKO). Three Tissue-Tek containers (Miles Inc, Elkhart, IN, U.S.A.), each with 24 slides in 250 ml of buffer, were placed on the edge of a turntable inside the microwave oven. Slides were heated for 11 min at full power (900 W), and then for 15 min at 400 W. After heating, slides were allowed to remain in buffer for 15 min. Antigen retrieval was followed by blocking of endogenous biotin, using the Dako Biotin-Blocking System (DAKO). Incubation with Hyb 213-6 (17 μg/ml) was accomplished for 25 min at room temperature. Immunostaining was automated using the ChemMate HRP/DAB detection kit, K5001 (DAKO) on the TechMate 1000 instrument (DAKO). Immunostaining was followed by brief nuclear counterstaining in Mayer's haematoxylin. Finally, cover slips were mounted with AquaTex (Merck, Darmstadt, Germany). Controls were performed by replacing the primary monoclonal antibody with an unrelated

monoclonal antibody of the same subclass as the gp-340 antibodies.

RESULTS

Amino acid composition and sequence

Agglutinin was separated by SDS/PAGE under reducing conditions. A band was excised and digested with trypsin and analysed by Q-TOF MS. The amino acid sequence of a 1460 Da peak of the mass spectrum was determined by tandem MS (Figure 1). This sequence showed 100% identity with a part of an SRCR domain of gp-340 and DMBT1. Peaks of the mass spectrum were compared with a trypsin digest of gp-340 (Table 1), and 11 peaks with the same mass were revealed. The amino acid composition, as determined by Ericson and Rundegren [1], for agglutinin was compared with the amino acid composition as determined by Holmskov et al. [11], and the composition was developed from the amino acid sequence [12] (Table 2). A high degree of similarity was observed.

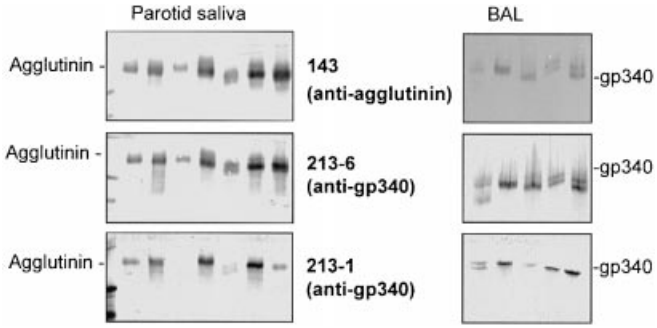

Figure 2 Specificities of monoclonal antibodies directed against agglutinin and gp-340 analysed by Western blotting

Parotid saliva from seven different human subjects (left boxes) and BAL samples from five different subjects (right boxes) were probed by Western blotting with monoclonal anti-agglutinin antibody (143) and with monoclonal anti-(gp-340) antibodies (213-6 and 213-1). The bound antibodies were visualized by means of alkaline-phosphatase-labelled goat anti-(mouse IgG) and substrate, as described in the Experimental section.

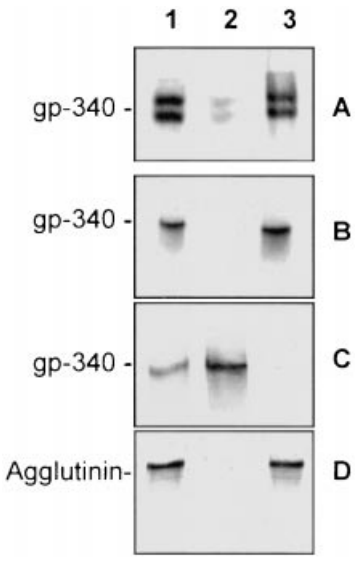

Figure 3 Binding of gp-340 and agglutinin to *S. mutans* as analysed by Western blotting

Bacterial pellets were suspended and incubated for 1 h in (**A**) crude BAL, (**B**) purified gp-340, (**C**) purified gp-340 in the presence of EDTA, and (**D**) partially purified agglutinin. After incubation, the bacteria were washed and the pellet was subjected to SDS/PAGE in the unreduced state, followed by Western blotting. Shown in the gels are starting material before (lanes 1) and after (lanes 2) incubation with *S. mutans*. Lanes 3, washed bacterial pellet after incubation with different solutions. The blots were incubated with monoclonal anti-(gp-340) antibody (Hyb213-1). The bound antibody was visualized by means of alkaline-phosphatase-labelled goat anti-(mouse IgG) and substrate as described in the Experimental section.

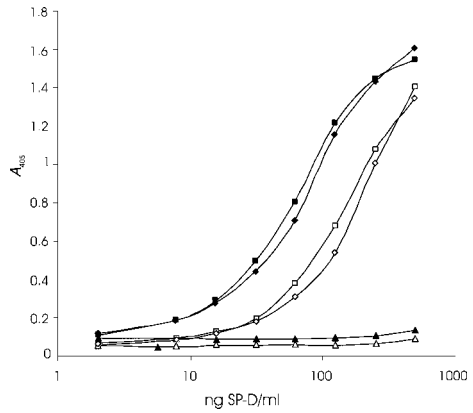

Figure 4 Binding of SP-D to gp-340 and agglutinin

Agglutinin (open symbols)- or gp-340 (filled symbols)-coated microtitre plates were incubated with serial dilutions of SP-D in the presence of calcium (◇, ◆), calcium and 100 mM maltose (□, ■) or with 10 mM EDTA (△, ▲). Bound SP-D was visualized by means of a polyclonal rabbit anti-(human SP-D) antiserum, alkaline-phosphatase-labelled goat anti-(rabbit IgG) and substrate, as described in the Experimental section.

Human genome search

The sequence of the *DMBT1* gene was used to search for homology in the human genome. No other candidate gene was found for a glycoprotein of 300–400 kDa containing this sequence, other than the *DMBT1* gene itself. This determines that the salivary agglutinin must be expressed from the *DMBT1* gene.

Western blotting with antibodies directed against agglutinin and gp-340

Agglutinin and gp-340 were compared on Western blots of parotid saliva and BAL fluid (Figure 2). The antibody directed against agglutinin (mAb143) and the antibodies against gp-340 (Hyb213-1) reacted with the same bands in saliva as in BAL. Heterogeneity of the band pattern was observed in both saliva and BAL, with some subjects showing two bands (band A and B) for both agglutinin and gp-340.

Binding of gp-340 and agglutinin to *S. mutans*

Binding of gp-340 and agglutinin to *S. mutans* was tested in a soluble-phase assay. After incubation, bacterial extracts were analysed by Western blotting (Figure 3). In the presence of calcium, both band A and band B of gp-340 from BAL fluid bind to *S. mutans* in solution (Figure 3A). Purified gp-340, containing only one band, and agglutinin both demonstrated binding to *S. mutans* (Figure 3C). Binding of gp-340 was inhibited by EDTA (Figure 3D), as described previously for agglutinin [22].

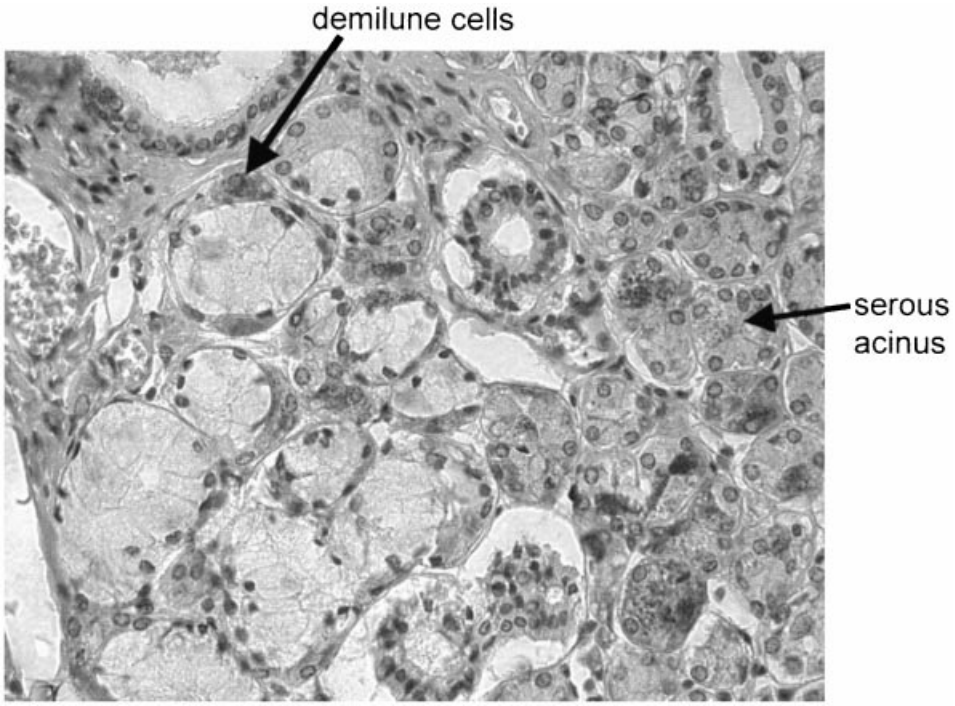

Figure 5 Immunohistochemical localization of agglutinin/gp-340 in the submandibular gland

The tissues were stained by an indirect immunoperoxidase technique and counterstained with Mayer's haematoxylin, as described in the Experimental section. Original magnification, × 400.

Binding of agglutinin and gp-340 to SP-D

In the presence of calcium, SP-D binds to microtitre plates coated with agglutinin (Figure 4). The binding was dependent on the concentration of SP-D and on the amount of agglutinin coated on the plate (results not shown). The binding was not influenced by the presence of maltose, but was totally inhibited by the chelation of calcium by EDTA. The same binding pattern was identified for SP-D binding to gp-340-coated microtitre plates.

Immunohistochemical analysis of gp-340

Immunohistochemical analysis was conducted with antibody 213-6 against gp-340. In the submandibular salivary gland, the serous acini and demilune cells were strongly stained. The ducts and mucous acini stained negative (Figure 5). Staining of controls was also negative.

DISCUSSION

The present paper demonstrates that human salivary agglutinin is identical with gp-340, and that both proteins show similar binding characteristics for antibodies, *S. mutans* and SP-D. Salivary agglutinin is a 300–400 kDa glycoprotein that was originally isolated from parotid saliva by affinity adsorption on to *S. mutans* [1]. It has been described as an aggregating factor for *S. mutans* and *S. sanguis* [23], the binding being mediated via the antigen B polypeptides on the surface of these bacteria [24]. It also exhibited complexation with S-IgA and bound to complement factor C1q, thereby activating C1 [25].

A mass spectrum of a trypsin digest of agglutinin revealed 11 peaks that were also present in a computer-simulated digest of gp-340. Three were fragments of SRCR domains, one was a fragment of a scavenger interspersed domain ('SID'), five were fragments of CUB domains and two were fragments of the C-terminal region of gp-340, which shows no similarity with other proteins. One fragment was sequenced, and the sequence showed 100% identity in a span of 14 residues with a fragment of an SRCR domain of gp-340. The amino acid composition of agglutinin and gp-340 were also found to be similar.

In the knowledge that high homologies are found among members of the SRCR superfamily, both within and among species, the binding characteristics of gp-340 and agglutinin were therefore examined. The specificity of three monoclonal antibodies raised against agglutinin and gp-340 were then compared, and the reaction pattern of these antibodies was shown to be nearly identical. Western blot analysis of crude parotid saliva and BAL fluid revealed interindividual heterogeneity in the band pattern, both in saliva and in BAL. This has been shown previously for both gp-340 and agglutinin. Two high-molecular-mass bands with an average mass of 340 kDa, and an additional band of 300 kDa, have been described for gp-340 [11,26], and three variant forms of a 300 kDa protein present in saliva that all bound to *S. mutans* have been described previously [27]. The heterogeneity of the molecule might be attributable to alternative splicing of the gene: at least three different splice forms have been cloned and sequenced for the *DMBT1* gene [12,15,28]. The variations in molecular mass might also be explained by differences in glycosylation, e.g. similar to the situation for blood-group-dependent differences. The presence of blood-group antigens has been demonstrated previously on agglutinin [22].

Not only the binding of antibodies, but also the binding of SP-D and *S. mutans* was identical. The calcium-dependent binding of gp-340 to *S. mutans* is similar that of agglutinin, and SP-D binding to agglutinin was similar to that of gp-340. This interaction was calcium-dependent, but was not inhibited by maltose.

It has been shown previously by reverse transcriptase-PCR that a mRNA for gp-340 was expressed in the salivary gland [12]. Immunohistochemical localization using specific monoclonal antibodies directed against gp-340 revealed strong and distinct granular staining of the serous acini and demilune cells in the submandibular gland. In a previous paper [18], staining for agglutinin was described both for the submandibular gland of the serous acinar cells and for demilune cells. Thus identical staining patterns are revealed with antibodies against gp-340 and agglutinin.

Structural data have indicated that ebnerin [29] and CRP-ductin [30], also called Vomeroglandin [31], are the rat and mouse proteins, respectively, that are homologous with gp-340. Recently, it was shown that purified CRP-ductin binds SP-D in a similar way as gp-340. Both ebnerin and CRP-ductin are found in soluble, as well as membrane-associated, forms, and they were cloned from the ebnerin and vomero glands respectively; the involvement of both molecules in pheromone/taste perception has been inferred [31]. It is therefore likely that ebnerin and CRP-ductin are the murine and rat proteins homologous with agglutinin.

On the basis of structural, as well as functional, data, we conclude that agglutinin is identical with gp-340/DMBT1. Up until now, salivary agglutinin has been described as a protein involved in the regulation of dental bacterial colonization, and gp-340 was known to be an SP-D-binding molecule. The results presented here show that salivary agglutinin can interact with micro-organisms and SP-D, emphasizing the putative role of this molecule in innate mucosal immunity.

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