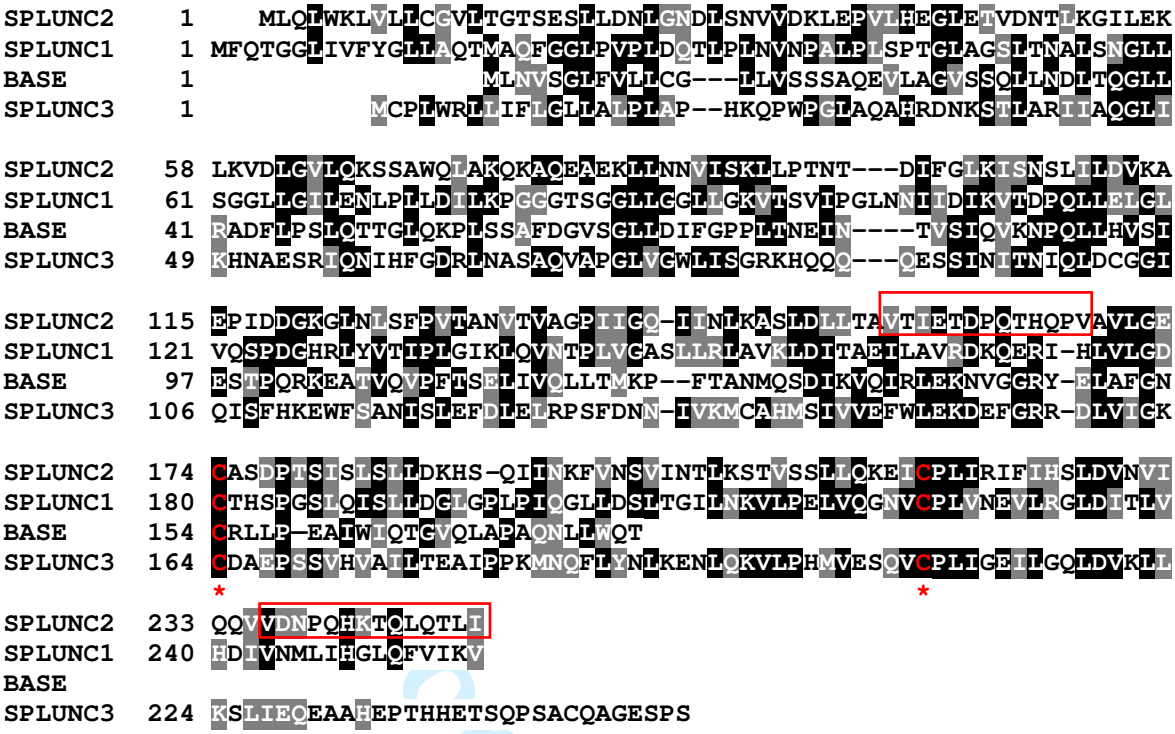

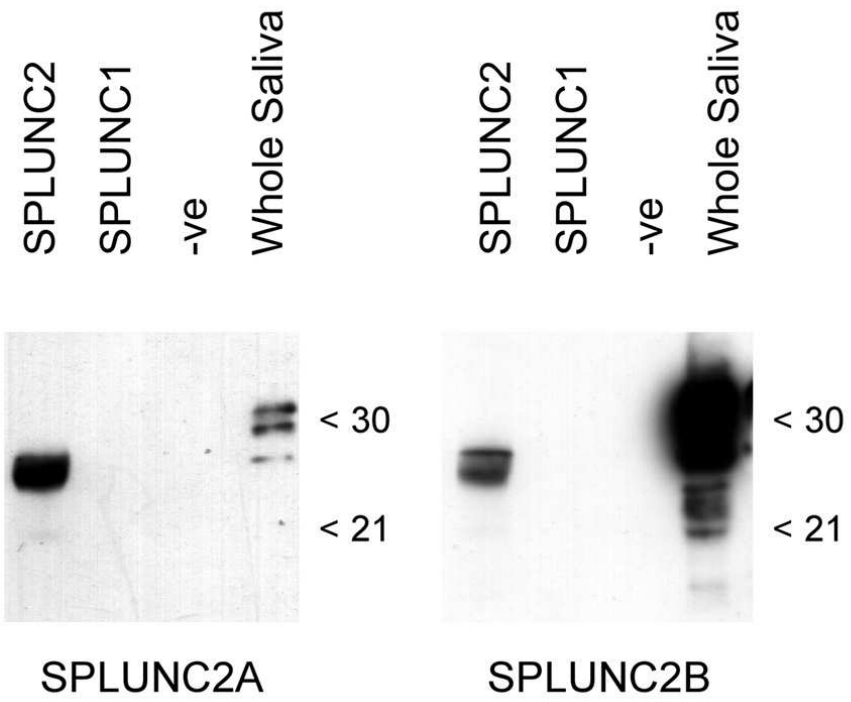

Fig 1 Characterisation of SPLUNC2 antibodies by western blotting (a) The position of the two-peptide epitopes used to generate the SPLUNC2 antibodies are illustrated (by red boxes) on a multiple sequence alignment of human SPLUNC proteins. The predicted amino acid sequences of human SPLUNC2, SPLUNC1, BASE and SPLUNC3 were aligned using CLUSTALX. In the alignment, identical residues are indicated by white on black background, whereas conserved residues have the grey background. Spaces introduced for maximum alignments are indicated by a -. The positions of the conserved cysteine residues are also indicated in red (*). (b) V5-epitope tagged SPLUNC proteins (SPLUNC1, SPLUNC2), were generated by coupled in vitro transcription and translation as outlined in the Materials and Methods section. 2µl of each reaction (and empty vector negative control reaction) was resolved on duplicate 12% SDS-PAGE gels alongside 5µl of whole human saliva. Gels were western blotted and probed with affinity purified polyclonal human SPLUNC2 antibodies (SPLUNC2A and SPLUNC2B). The positions of the molecular markers are indicated by the black arrows
83x63mm (300 x 300 DPI)

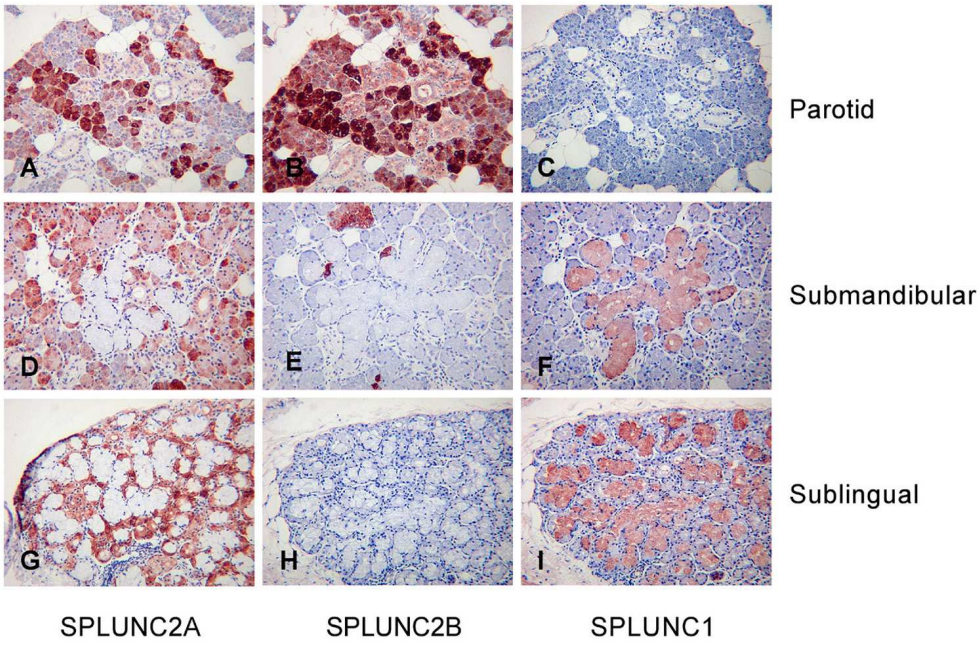

Fig 2 Distribution of SPLUNC2 in Major Salivary Glands Immunohistochemistry was performed on replicate sections as described in the Materials and Methods using the two SPLUNC2 antibodies (SPLUNC2A: A, D G; SPLUNC2B: B, E H) and a polyclonal antibody to SPLUNC1 (C, F, I). Tissues stained are Parotid gland (A, B, C), Submandibular gland (D, E, F) and Sublingual Gland (G, H, I) 129x84mm (300 x 300 DPI)

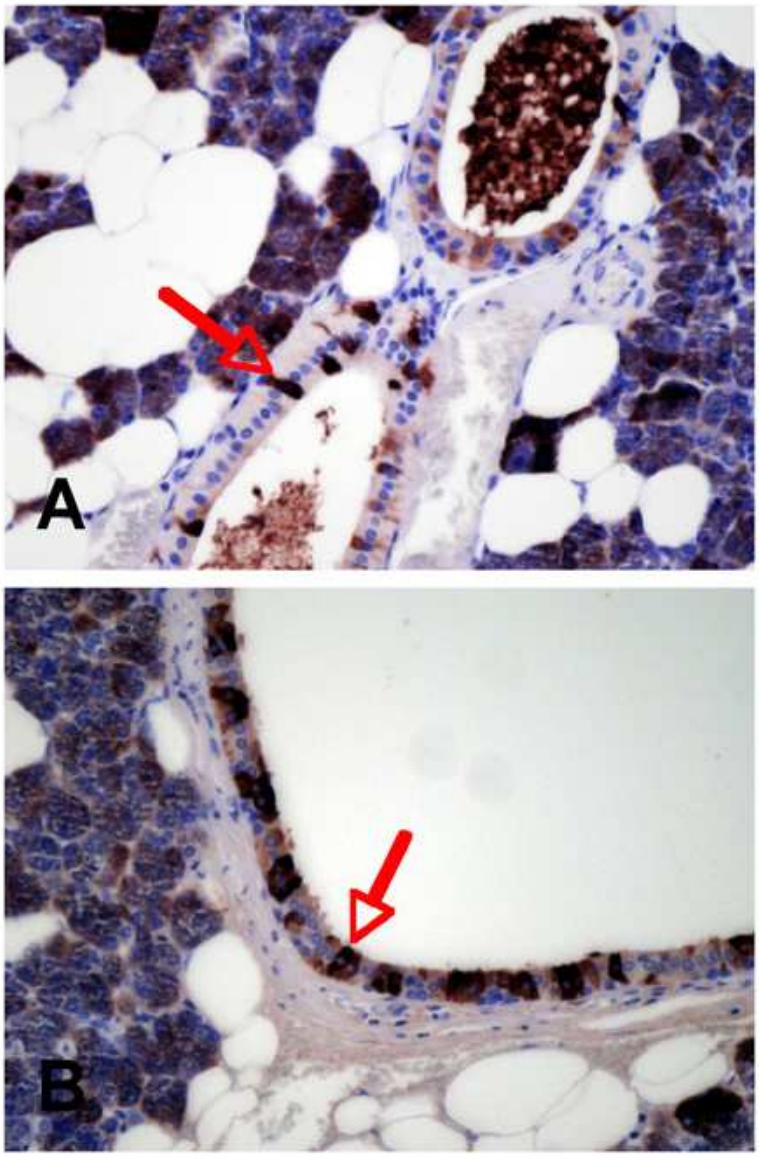

Fig 3 SPLUNC2 B immunoreactivity in parotid gland ducts Immunohistochemistry was performed on parotid gland with the antibody to SPLUNC2B as described in the Materials and Methods. High power images were taken to show staining in the larger interlobular ducts (A) and collecting ducts (B). Intensely staining duct cells are indicated by the arrows. Note that the contents of the interlobular ducts also stain intensely
39x58mm (300 x 300 DPI)

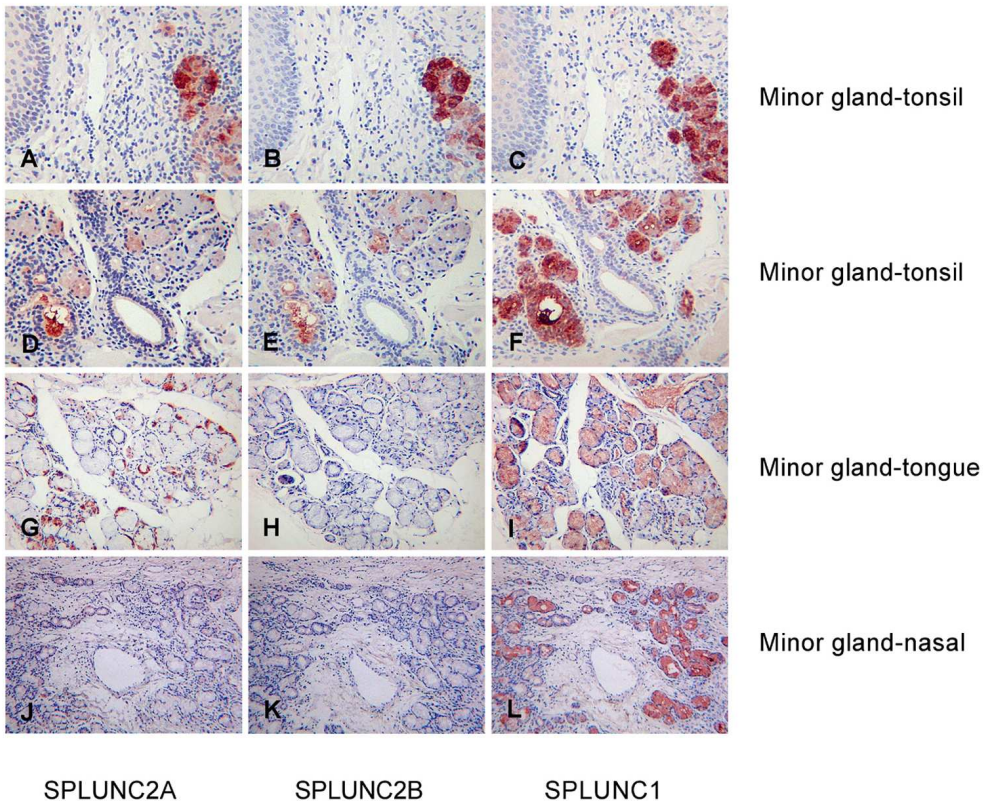

Fig 4 Distribution of SPLUNC2 in Minor Salivary Glands from the oro- and nasopharynx Immunohistochemistry was performed in replicate sections as described in the Materials and Methods using the two SPLUNC2 antibodies (SPLUNC2A: A, D, G, J; SPLUNC2B: B, E, H, K) and a polyclonal antibody to SPLUNC1 (C, F, I, L). Tissues stained are glands from the region of the palatine tonsil (A-F), glands from the posterior portion of the tongue (G-I) and glands from the nasal antral mucosa (J-L)
129x105mm (300 x 300 DPI)

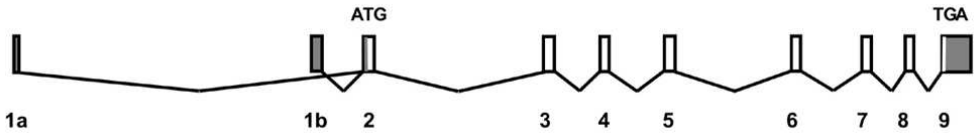

Fig 5 The human SPLUNC2 gene utilizes alternatively spliced 5′ non-coding exons
(a) Schematic representation of the SPLUNC2 gene with the exon numbers shown below the figure. Coding sequence is shown in white with non-coding sequence shown in grey. The position of the start (exon 2) and stop codons (exon 8) are illustrated above the figure. The two 5′ non-coding exons are shown as 1B and 1A. The scale bar represents 1Kb. (b) Expression of alternative splicing of SPLUNC2 was investigated by RT-PCR with three sets of exon-spanning primer pairs as described in the Materials and Methods section. The primers were designed to amplify an internal portion of SPLUNC2 as well as the two 5′ non-coding exons

83x17mm (300 x 300 DPI)

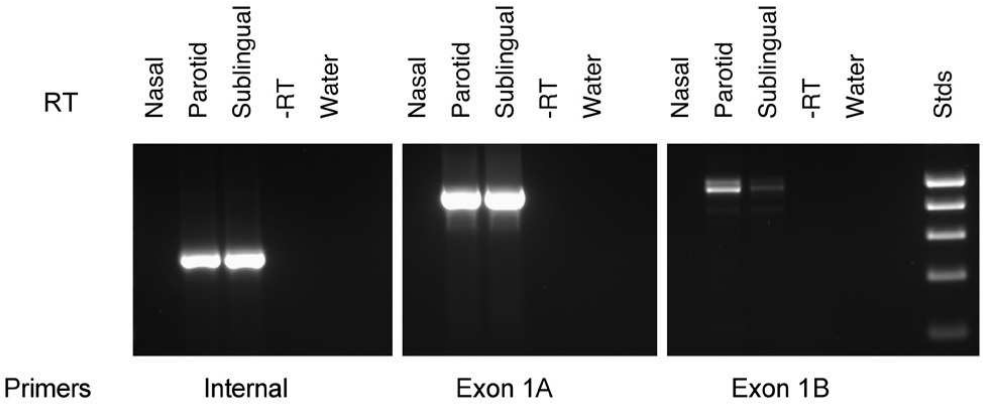

Fig 5 The human SPLUNC2 gene utilizes alternatively spliced 5′ non-coding exons
(a) Schematic representation of the SPLUNC2 gene with the exon numbers shown below the figure. Coding sequence is shown in white with non-coding sequence shown in grey. The position of the start (exon 2) and stop codons (exon 8) are illustrated above the figure. The two 5′ non-coding exons are shown as 1B and 1A. The scale bar represents 1Kb. (b) Expression of alternative splicing of SPLUNC2 was investigated by RT-PCR with three sets of exon-spanning primer pairs as described in the Materials and Methods section. The primers were designed to amplify an internal portion of SPLUNC2 as well as the two 5′ non-coding exons

83x36mm (300 x 300 DPI)

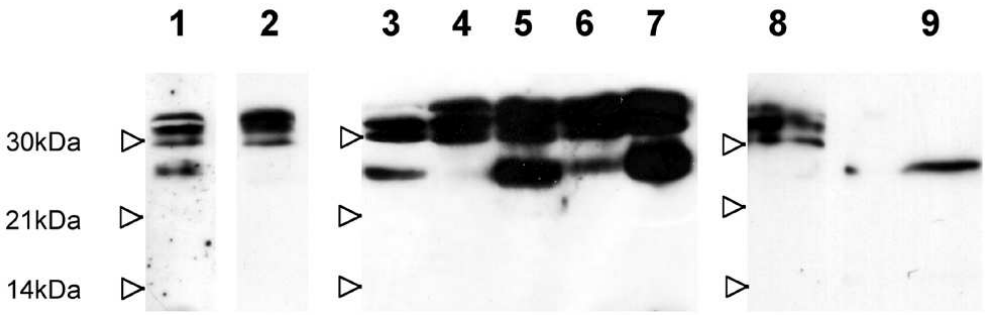

Fig 6 SPLUNC2 is present as multiple isoforms in saliva and is N-glycosylated Western blotting was performed on duplicate samples of unstimulated whole saliva using antibodies SPLUNC2A (lane 1) and SPLUNC2B (lane 2)). In an additional experiment a further four samples of unstimulated whole saliva (Lanes 3-6) and a sample sublingual/submandibular gland saliva collected at the same time as the whole saliva sample in lane 4 (lane 7) were western blotted with antibody SPLUNC2A. A sample of whole saliva was subjected to deglycosylation using N-Glycanase PNGase F and subjected to western blotting with antibody SPLUNC2B. The multiple bands present on the mock digested sample (lane 8) were reduced to a single band of lower molecular mass following PNGase treatment (lane 9). The positions of the molecular mass markers (30, 21 and 14kDa) are identified by the open arrowheads to the side of the images
83x27mm (300 x 300 DPI)

Characterisation and expression of SPLUNC2, the human orthologue of rodent Parotid Secretory Protein.

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ABSTRACT

We recently described the PLUNC family of proteins as an extended group of proteins expressed in the upper airways, nose and mouth. Little is known about these proteins but they are secreted into airway and nasal lining fluids and saliva where, due to their structural similarity with lipopolysaccharide binding protein (LBP) and Bactericidal/permeability-increasing protein (BPI), they may play a role in innate immune defence. We now describe the generation and characterisation of novel affinity-purified antibodies to SPLUNC2, and use them to determine expression of this, the major salivary gland PLUNC. Western blotting showed that the antibodies identified a number of distinct protein bands in saliva whilst immunohistochemical analysis demonstrated protein expression in serous cells of the major salivary glands and in the ductal lumens as well as in cells of minor mucosal glands. Antibodies directed against distinct epitopes of the protein yielded different staining patterns in both minor and major salivary glands. Using RT-PCR of tissues from the oral cavity, coupled with EST analysis, we have shown that the gene undergoes alternative splicing using two 5' non-coding exons, suggesting that the gene is regulated by alternative promoters. Comprehensive RACE analysis using salivary gland RNA as template, failed to identify any additional exons. Analysis of saliva showed that SPLUNC2 is subject to N-glycosylation. Thus our study shows that multiple SPLUNC2 isoforms are found in the oral cavity and suggest that these proteins may be differentially regulated in distinct tissues where they may function in the innate immune response.

KEY WORDS:

SPLUNC2, SPLUNC1, salivary glands, innate immunity, gene structure, alternative splicing.

INTRODUCTION

We have recently described a novel family of putative host defence proteins (known as PLUNCs) expressed in the upper respiratory tract and oral cavity, which may serve innate defence functions in these locations (Bingle & Craven 2002; Bingle et al 2004; Bingle & Craven 2004). The founding member of this extended family, **P**alate **Lu**ng **N**asal **C**lone (PLUNC), was first described in the nasal epithelium of the mouse embryo and the trachea and bronchi of adult mouse lung (Weston et al 1999). Our studies identified a family of human PLUNC proteins located on chromosome 20 (Bingle & Craven 2002) in close proximity to genes encoding the related proteins, lipopolysaccharide binding protein (LBP) and Bactericidal/permeability-increasing protein (BPI), key members of the innate immune response to Gram-negative bacteria. We have previously shown that the PLUNC family can be subdivided into short (SPLUNC) and long (LPLUNC) proteins containing domains predicted to be structurally similar to one or both domains of BPI (Bingle & Craven 2002; Bingle et al 2004; Bingle & Craven 2004). Due to the structural similarity to BPI and LBP we proposed that PLUNC proteins might function in host defence of the respiratory system, oro- and nasopharynx (Bingle & Craven 2002; Bingle et al 2004; Bingle & Craven 2004; Bingle & Gorr 2004).

Our comparative studies have revealed that PLUNC proteins are rapidly evolving and exhibit a high level of sequence diversity between orthologues as well as exhibiting lineage specific expansion in the number of paralogues (Bingle & Craven 2002; Bingle et al 2004; Wheeler et al 2007). The greatest diversity appears to exist in the SPLUNC portion of the family particularly with regard to proteins related to rodent parotid secretory protein (PSP). psp was identified as an abundant component of rat and mouse saliva (Madsen & Horth 1985; Shaw & Schibler 1986; Mirels & Ball 1992) and although the identification of psp far predates the identification of the wider PLUNC family (Madsen & Horth 1985; Shaw & Schibler 1986), a biological function for psp has not been shown. A second psp-related protein, submandibular gland protein B (smgb) was also identified in rats (Madsen & Horth 1985; Shaw & Schibler 1986) but is not present as a functional gene in mice (Ball et al 2003). We have recently shown that there are 4 psp-like proteins (called bsp30a-d) in the bovine genome that have arisen from a series of gene duplication events (Wheeler et al 2007). Our comparative molecular analysis has shown that SPLUNC2 is the orthologue of rodent psp (Bingle & Craven 2004). It has been noted by us and others that the *SPLUNC2/psp* gene pair represents one of the most divergent human/mouse gene pairs and that the level of amino acid sequence identity with the rodent psp is below 35% (Emes et al 2003; Bingle & Craven 2004). This low level of sequence identity coupled with the

diversity of the SPLUNC2/psp/smgb/bsp30 proteins in general suggest that extrapolation of studies from one species to another may be difficult. We have previously shown that like its rodent counterpart, *SPLUNC2* is predominantly expressed in salivary glands (Geetha et al 2005). The protein has also been identified in whole saliva by a number of independent proteomic studies (Vitorino et al 2004; Ramachandran et al 2006; Gou et al 2006; Walz et al 2006) as well as in minor gland saliva (Siqueira et al 2008) and as a component of acquired pellicle (Siqueira et al 2007). Consistent with our proposal that PLUNCs may function in a host defence capacity we have shown that peptides derived from SPLUNC2 are able to inhibit LPS mediated cytokine release and also inhibit LBP/LPS interactions (Geetha et al 2005).

As a prelude to the study of the biological activity of SPLUNC2 we have performed a systematic analysis of the human SPLUNC2 gene and studied the distribution of the protein in tissues of the oral cavity, nasopharynx and respiratory tract and in saliva.

METHODS

Generation and characterisation of SPLUNC2 antibodies

Anti-peptide antibodies were generated against human SPLUNC2 by Eurogentec using established methods as described (Vargas et al 2008). We generated SPLUNC2 antibodies by duel injection of two synthetic peptides corresponding to amino acids, 156-168: (VTIETDPQTHQPV) (designated SPLUNC2A) and amino acids 236-249-cooh: (VDNPQHKTQLQTLI) (designated SPLUNC2B). Pooled final serum was used for affinity purification against each of the individual peptides resulting in the generation of specific antibodies against each unique peptide. For validation of the antibodies we generated expression clones for human SPLUNC2 by direct cloning of PCR products into pcDNA5-frt-V5-His-Topo (Invitrogen) which generates fusion proteins with C-terminal His and V5 epitope tags. Positive clones, characterised by restriction mapping and sequencing, were then used for coupled *in vitro* transcription/translation reactions (Promega) as previously described (Bingle et al 2005). 2μl aliquots were run on SDS-PAGE gels and western blotted using the specific SPLUNC2 antibodies (1:500 dilution). Detection was performed using ECL (Amersham) as described (Vargas et al 2008). Fully characterised and sequenced clones were then used to generate stable CHO cell lines using the Flp-in Flp-mediated recombination system according to the manufacturers instructions (Invitrogen). Positive lines were generated by selection in hygromycin and successful integration was confirmed by identifying the fusion protein in the cell pellets by western blotting using the V5 antibody. Conditioned serum free media from each cell line was collected for use as

positive controls in western blots. The *in vitro* translated SPLUNC1 used for western blotting was generated as described (Bingle et al 2005).

Saliva collection and western blotting

Human parotid and submandibular–sublingual (SMSL) glandular salivas were stimulated with lemon juice and collected immediately before use from a single donor with Carlsonn Crittenden cups and a custom-made silicone impression device, respectively (Plummer et al 2006). Whole stimulated saliva was collected from several volunteers by chewing sugar-free gum and unstimulated saliva was collected over several minutes by allowing saliva to pool briefly in the mouth. Whole salivas were clarified by centrifugation at 13,000g for 10 minutes.

Saliva samples in SDS sample buffer were resolved on 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Whatman). The membranes were blocked overnight at 4°C with 5% skimmed milk in Tris-buffered saline. The SPLUNC2 antibodies were diluted in the same blocking solution + 0.05% Tween (1:250 SP2A and 1:500 SP2B) and incubated with the membrane for one hour at room temperature. Membranes were washed in TBS/0.05% Tween before incubating for one hour with an anti-rabbit HRP secondary antibody. Further washing with TBS/Tween was followed by visualisation with ECL.

Peptide: N-Glycosidase F (PNGase F) Treatment of Saliva

PNGase F (New England Biolabs) cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. In order to determine if the multiple bands, seen on western blots of saliva with the SPLUNC2 antibodies, were due to glycosylation of the protein PNGase F was incubated with whole saliva and then western blotted. Specifically, salivas were diluted in denaturing buffer and heated to 100°C for 10 minutes before adding the enzyme in the presence of NP-40. Following a one-hour incubation at 37°C the solution was mixed with 2X SDS sample buffer and loaded onto a 12% SDS polyacrylamide gel. The western blotting procedure was carried out as described above.

Immunohistochemistry

Ethical approval for use and processing of human tissues was granted by the Sheffield Research Ethics Committee. For oral, nasal, and lung tissue sections were cut from

formalin-fixed and paraffin-embedded tissue so as to be as representative of normal as possible, although all tissues were obtained from patients with an underlying clinical presentation. At least 3 individual samples were studied for each tissue. The slides were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase, incubated with 100% normal goat serum at room temperature for 30 minutes and then at 4℃ overnight with the affinity purified SPLUNC2 antibodies, 1:250 (SP2A) and 1:500 (SP2B), diluted in 100% goat serum. We also used a polyclonal SPLUNC1 antibody (1:300 dilution) as described (Campos et al 2004). A rabbit IgG (DAKO) was used as a negative control in duplicate slides of every case. A Vectastain Elite ABC kit (Vector Laboratories) containing an appropriate biotin-labelled secondary antibody was used according to the manufacturer's instructions. Peroxidase enzymatic development was performed using a Vector NovaRed substrate kit resulting in red staining in positive cells. Sections were counterstained with haematoxylin, dehydrated to xylene and mounted in DPX.

Molecular characterisation and bioinfomatic analysis of the SPLUNC2 gene

Total RNA was isolated from human salivary gland tissues, nasal epithelium and oral mucosa using TRI-reagent. Additional human RNA samples were purchased from Clontech and Ambion. All samples were provided with appropriate ethical approval. RT reactions were performed using oligo dT primed RNA reverse transcribed from 1μg of total RNA. Initial RT-PCR was performed with primers designed to amplify within the predicted coding region of SPLUNC2. (Forward: 5' AAC TGA TCC CCA GAC ACA CC 3'; Reverse: 5' CAG AAA TGG GCC TTT ATT GC 3'). 30 cycles of the following program: 94°C for 1', 60°C for 2' and 72°C for 3' generated the appropriately sized product, which was directly cloned in TOPO pCRII (Invitrogen) and sequenced. *SPLUNC2* ESTs were identified using Blast (NCBI) and the BLAT server from http://www.genome.ucsc.edu/. The genomic organisation confirmed by alignment of cDNAs with genomic DNA. Transcripts containing both 5' non-coding exons were identified by RT-PCR using the following forward primers. SP2 1AF1 5' TGA GCA TCC TCC TCT AAA CG 3'; SP2 1AF2 5' CAG TGG GGC AAG GAT TTC AT 3'; SP2 1BF1 5' GAG GAG GCT TTG GGA ATT GT 3' and SP2 1BF2 5' GGG AAT TGT CCA GCA GAA AC 3'. A common reverse primer (as shown above) was used in these reactions and the resultant products were cloned into pCR 2.1TOPO and sequenced. Rapid amplification of cDNA ends (RACE) was performed using the SMART RACE system (Clontech) according to the manufacturer's instructions. Parotid gland and submandibular gland total RNAs were used as templates with the following specific

primers; SPLUNC2RACER1 5' GAG TGG ATG AAG ATG CGG ATC AGT GG 3' and SPLUNC2RACER2 5' GGA GGA TAC AGT GCT TTT CAG CGT GTT G 3'. RACE products were cloned into pCR 2.1 TOPO and colonies screened by PCR using specific SPLUNC2 primers. Verified SPLUNC2 clones were then fully sequenced. We also searched for conservation of alternative splicing of primate SPLUNC2 by cross species alignments of the human cDNAs with genomic DNA as well as by PCR using primate specific primers and rhesus monkey salivary gland cDNA (Biochain, USA) as a template. Again products were cloned and sequenced for verification.

RESULTS

SPLUNC2 has a distinct distribution pattern within salivary glands

To study the localisation of human SPLUNC2 in tissues we generated two affinity purified anti-peptide antibodies. One of these, SPLUNC2-A, recognises an epitope that is found internally within the protein whilst the second antibody, SPLUNC2-B, recognises an epitope at the extreme C-terminus of the protein and because of the extremely low levels of sequence similarity between human PLUNC paralogues (Figure 1a) these antibodies should be highly specific. Following peptide affinity purification we characterised both antibodies by western blotting using *in vitro* translated recombinant SPLUNC2 and whole saliva (Figure 1b). These results clearly show that both antibodies are able to specifically recognise the recombinant SPLUNC2 but no reaction was seen with SPLUNC1 produced in the same manner. Similar results were seen with conditioned serum-free media collected from stable SPLUNC2 and SPLUNC1 expressing cell lines (results not shown). Both antibodies were also able to identify constituents of whole human saliva. The SPLUNC2A antibody recognised three clear bands, the smallest of which migrated with a similar apparent molecular mass to the recombinant protein generated by *in vitro* transcription/translation. The reactivity of the SPLUNC2B antibody with saliva was stronger than that seen with the SPLUNC2A antibody, however, it also recognised multiple bands in whole saliva as well as the recombinant SPLUNC2.

In view of our previous observations that SPLUNC2 mRNA is expressed in salivary glands (Geetha et al 2005) we initially performed immunohistochemistry on sections from major salivary glands using both SPLUNC2 antibodies as well as an antibody to the prototypic SPLUNC protein, SPLUNC1. In parotid gland, both SPLUNC2 antibodies reacted with serous acinar cells (Figure 2A and B). SPLUNC2B appeared to stain more strongly than SPLUNC2A. Cells within the interlobular ducts (both striated and intercalated) also stained

with both antibodies. Parotid tissue did not show SPLUNC1 immunostaining (Figure 2C). In submandibular gland the staining pattern of the two SPLUNC2 antibodies was somewhat different. SPLUNC2A stained the majority of the serous acinar tissues as well as the interlobular ducts. The cells of the mucous tubules were clearly negative for SPLUNC2A (Figure 2D). In contrast to the staining pattern described above, SPLUNC2B immunoreactivity was noted in only a few of the serous cells and the interlobular dusts did not stain (Figure 2E). Again the mucous tubule cells were clearly negative for staining. SPLUNC1 immunoreactivity was restricted to the mucous tubule cells (Figure 2F). A similar staining pattern was seen in sublingual glands (Figure 2G-H). SPLUNC2A strongly stained the serous demilunes whereas SPLUNC1 strongly stained the mucous secreting tubules. Interestingly SPLUNC2B staining was not seen in any sublingual gland samples.

In addition to the acinar staining we also noted intense staining in some cells within the larger striated and collecting ducts of parotid gland samples (Figure 3A and B). In these regions it was striking that within individual ducts there was significant heterogeneity of staining (as illustrated by arrowheads in Figure 3). In these regions either single or doublets/triplets of cells stained very intensely, whereas their neighbouring cells were negative. The lumenal content of the larger ducts also stained positively. Similar observations were made in sections stained with the SPLUNC2A antibody, although as before staining was less intense (results not shown). These findings show that SPLUNC2 appears to be a product of the serous acini and the interlobular ducts of the major salivary glands cells. They further suggest that distinct protein isoforms stain in these glands. The results also suggest that SPLUNC2 and SPLUNC1 staining is essentially mutually exclusive in these glands.

In the light of our previous observations that SPLUNC1 was a major protein product of the minor salivary glands of the oral cavity (Bingle et al 2005) we studied tissues from this region. Minor glands in the vallecular region of the tongue associated with the palatine tonsil also stained with both SPLUNC2 antibodies (Figure 4 A,B,D,E). In contrast to the situation seen in the major salivary glands where staining of SPLUNC2 and SPLUNC1 was mutually exclusive, minor salivary glands in this region stained for both proteins (Figure 1A-F). Staining was not seen in the tonsillar crypts or germinal centres. The staining patterns seen in the minor glands of the posterior portion of tongue was very similar to that seen in the sublingual gland. SPLUNC2A strongly stained the serous demilunes of the gland whereas SPLUNC2B staining was not seen in any samples (Figure 4G, H). Marked SPLUNC1 staining was seen in the mucous tubules (Figure 4I). The

surface epithelial layers of the tongue were not stained with either SPLUNC2 antibody (results not shown).

SPLUNC2 staining was not seen in minor glands associated with the upper respiratory tract. Sections of maxillary sinus did not show staining in the respiratory epithelium (results not shown) nor did either antibody detect staining in the minor glands underlying the respiratory mucosa (Figure 4 J, K) where strong SPLUNC1 staining is seen. Nasal polyps, tracheal and bronchial epithelium (with associated submucosal glands), were uniformly negative for staining with either SPLUNC2 antibody (results not shown). No staining was noted in peripheral lung tissues (results not shown).

These results clearly show that SPLUNC2 protein is produced by both the major and minor salivary glands. In major glands staining is found in the serous acini and populations of epithelial cells within both interlobular and collecting ducts. This staining contrasted directly with the staining of the related protein SPLUNC1, which was found in mucous cells. In some minor glands however, SPLUNC2 and SPLUNC1 staining overlapped. The most striking observation that we have made is that the two SPLUNC2 antibodies exhibit distinct staining patterns in glands (both major and minor) that have a significant mucous cell component. In these, SPLUNC2B does not detect protein within the serous demilunes of the glands. This is best illustrated in the sublingual gland where staining was never seen, and in the submandibular gland where only a few serous cells stained positively (Figure 2). It is possible that this staining pattern is caused by one of two factors. First, distinct SPLUNC2 protein isoforms could potentially be generated by alternative splicing such that the epitopes used to produce the antibodies would not be present in all protein isoforms. Second, differential post-translational modification, may render the different epitopes unavailable for antibody binding in a tissue specific manner. We set out to investigate these two possibilities

SPLUNC2 is alternatively spliced and utilises distinct 5' non-coding exons

To investigate the first of these two possibilities we studied the genomic organisation of the *SPLUNC2* gene in detail using sequences within public databases. This analysis allowed us to identify what appeared to be alternatively spliced sequences. One of these represented by CB985251 and AY359055 contains a novel 5' exon approximately 7kb upstream of exon 2. The second type of cDNAs, represented by BX484489, BX486580 and BX485187 represent sequences in which either exon 5 or exon 3 read through into the intron. No published ESTs with internally deleted exons appear to exist in the *SPLUNC2* gene. The organisation of the *SPLUNC2* gene is shown in Figure 5A.

To confirm the existence of the novel upstream non-coding exon, we generated specific forward primers in each of the exons and used them for PCR reactions with a common reverse primer and with parotid and submandibular gland cDNA as templates (Figure 5B). These reactions generated the expected sized products with both primer pairs and both were confirmed by sequencing of cloned products. In addition to the major transcripts, reactions performed with the primers to exon 1B generated an additional band. Cloning this band showed that it corresponded to a further transcript with a novel 3' end of exon 1B. To investigate the possibility that *SPLUNC2* generates other undescribed transcripts we performed 5' RACE using RNA from both parotid and submandibular gland as template. Using this technique we were, however, unable to amplify any additional transcripts containing either novel internal exons or any further upstream exons.

Due to the extreme diversity of the *SPLUNC2/psp* gene sub family that we have previously highlighted [6] we were keen to study the evolutionary basis of the alternative 5' exons. Alignment of over 1000 mouse *psp* ESTs with genomic DNA produced no evidence of a similar event in this species although some exist with a 3' extension to the first exon (results not shown). However, by mapping the human *SPLUNC2* cDNAs into the chimpanzee and rhesus monkey genomic assemblies using BLAT, we were able to predict that these species may contain both upstream exons. To test this observation we generated forward RT-PCR primers for both of the predicted exons in rhesus monkey *SPLUNC2* and used these with a reverse primer located in exon 9 of the rhesus monkey *SPLUNC2* gene. Both primer sets generated the expected sized product when used with rhesus monkey cDNA as a template (results not shown). Cloning and sequencing of these products produced sequences identical to those predicted and confirmed that the rhesus monkey *SPLUNC2* gene also utilizes two distinct 5' non-coding exons.

These studies clearly show that *SPLUNC2* can be alternatively spliced and is presumably under the control of two distinct regulatory regions. However, as translation of SPLUNC2 begins in exon 2, it is assumed that alternative splicing is not responsible for the distinct staining pattern seen in the immunohistochemical studies or the different bands seen in saliva. For this reason we next looked at salivary proteins in greater detail.

Multiple SPLUNC2 bands are detectable in human saliva

Western blotting of a single sample of saliva showed that both SPLUNC2 antibodies recognize multiple bands as shown in figure 1b. To study this in greater detail we performed western blotting analysis using whole saliva collected from 4 individuals. When such samples were blotted with the SPLUNC2A antibody it was clear that there was

variability in the banding pattern. Most striking was the difference in the smallest band, which was undetectable in one sample (Figure 6 lane 4 compared to lanes 3, 5 and 6). We also observed that submandibular/sublingual saliva collected from the same donor as the whole saliva in lane 6 also exhibited a marked difference in the amount of the fastest migrating (i.e. smallest) band suggesting that these two major salivary glands produced the majority of this isoform (compare Figure 6 lanes 6 and 7). The increase of the specific band identified with antibody SPLUNC2-A, in the submandibular/sublingual saliva is consistent with a recent proteomic study, which suggested that SPLUNC2 is found at greater levels in saliva from this source compared with either parotid or whole saliva [16].

To address the issue of possible differential glycosylation of SPLUNC2 we removed N-linked sugars on the protein in whole saliva by PNGase F treatment. This resulted in the reduction of the three distinct bands recognised by the SPLUNC2B antibody (Figure 6, lane 8) to a single band of approximately 25kDa (Figure 6, lane 9) suggesting that all three bands are differentially glycosylated isoforms of the same peptide. A similar removal of the higher mass bands from the sample blotted with SPLUNC2A was also observed (results not shown). This suggests that the smallest mass band in saliva represents a non-modified (non-glycosylated) isoform of the protein.

DISCUSSION

The PLUNC proteins make up the largest branch of the wider BPI/LBP/CETP/PLTP/PLUNC family of putative lipid transport proteins. The biological function of members of the PLUNC branch of the family is not clear, but on the basis of the sites at which they are expressed, there similarity to the host defence proteins BPI and LBP and their status as rapidly evolving proteins, we and others have suggested that they may function in host defence of mucosal surfaces. There is very little published data on the localisation of human PLUNC proteins with most information being available for the prototypic member, SPLUNC1. These have shown that the protein is predominantly localised to the minor mucosal glands or the oral cavity and respiratory tract as well as in a population of epithelial cells of the upper airways.

We have previously shown that *SPLUNC2* is expressed in a limited set of tissues with the most significant expression being seen in salivary glands (Geetha et al 2005). It is now firmly established that SPLUNC2 is the human orthologue of rodent psp despite the very low level of sequence similarity that exists between the two proteins (Emes et al 2003; Bingle et al 2004). psp was first identified as a significant component of rodent saliva and is known to appear during secretory granule formation in the major salivary glands in

postnatal development and increases to a high adult level (reviewed in Ball et al 2003). A second psp related protein, submandibular gland protein B (smgb) was also identified in rats (Mirels & Ball 1992). SPLUNC2 is not the orthologue of smgb, which appears to be a rodent specific member of the PLUNC gene family (Bingle et al 2004; Wheeler et al 2007). smgb and psp are two of the major products of the neonatal rat submandibular gland and both are also expressed in the sublingual and parotid glands. Expression of the two proteins in rats is discordant, since *smgb* is expressed at greater levels than *psp* in sublingual gland, whereas *psp* is a major parotid acinar cell product (Mirels & Ball 1992; Ball et al 2003). psp is produced by mouse salivary glands with similar temporal and cell specificity to rat psp, however, mouse *smgb* has been silenced through a viral integration event in the first intron (Ball et al 2003) and therefore mice are *smgb* deficient. Further psp-related proteins have been described in cow saliva and termed bovine salivary protein 30 kDa (bsp30) (Wheeler et al 2002). Cloning studies initially identified two distinct genes, *bsp30a* and *bsp30b,* that are highly expressed in the parotid gland and comprise approximately 30% of the protein in bovine saliva. The two forms of *bsp30* are highly expressed in the major salivary glands of cattle and appear to be expressed independently of one another (Wheeler et al 2002). bsp30a and bsp30b are highly related at primary sequence level (96% at DNA and 83% at amino acid level) and recent genomic analysis has identified the existence of two further bsp30 genes (*bsp30c* and *bsp30d*) and has shown that these four genes have arisen by a series of duplication events (Wheeler et al 2007). This finding reiterates our suggestion that the SPLUNC branch of the PLUNC gene has undergone major diversification during mammalian evolution (Bingle et al 2004; Wheeler et al 2007).

Despite multiple studies having been performed on rodent and bovine psp-related proteins a firm biological function for them has not been established. There is, however, some evidence that supports a host defence function for these proteins. For example, psp has been shown to bind to bacterial membranes (Robinson et al 1997) and appears to have anti-candidal activity (Khovidhunkit et al 2005). The cow proteins, bsp30a and b, exhibit growth-suppression activity against *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (Haigh et al 2008). In keeping with these studies, SPLUNC2 has also been shown to exhibit growth suppression effects on bacteria (Geetha et al 2003) and peptides derived from the protein also have anti-inflammatory effects (Geetha et al 2005) and can agglutinate bacteria (Gorr et al 2008). Furthermore, SPLUNC2 gene expression is elevated by pro-inflammatory cytokine treatment (Shiba et al 2005).

Consistent with the observations on expression of psp and bsp30 proteins we have shown that SPLUNC2 is found in both major and minor salivary glands. We have also shown that the protein is secreted into major gland saliva. Previously SPLUNC2 has not been identified in saliva by western analysis but has been identified in a number of proteomic studies of whole saliva, major gland saliva and, most recently, minor gland saliva (Vitorino et al 2004; Ramachandran et al 2006; Guo et al 2006; Walz et al 2006; Siqueira et al 2008). Our western analysis clearly shows that multiple protein isoforms of SPLUNC2 exist in saliva and that these are most likely due to differential glycosylation. This observation is supported by a proteomic study of glycosylated proteins in whole saliva (Ramachandran et al 2006). What function this differential glycosylation of SPLUNC2/psp serves, however, remains unclear. It is well known that many salivary proteins undergo complex post-translational modification, including glycosylation, and it is assumed that this has a major effect on their function (Helmerhorst & Oppenheim 2007). It is also note-worthy that rodent psp and smgb (Mirels et al 1998), as well as bovine bsp30a and b (Haigh et al 2008) have been shown to be N-glycosylated.

Our immunohistochemical data provides two observations of note. Firstly, the two SPLUNC2 antibodies exhibit discordant staining patterns in some of the salivary gland samples. This is particularly noticeable in both major and minor salivary glands with a significant mucous cell component. In some of these tissues, for example the sublingual gland, strong staining is seen with SPLUNC2A antibody whereas staining with SPLUNC2B is completely negative. We believe that this represents differences in the glycosylation status of the proteins within these tissues and suggest that tissue specific post-translational modifications of SPLUNC2 may on occasion mask the epitope to which the antibody binds. We do note, however, that the two N-glycosylation sites identified in a proteomic analysis of saliva are not within the peptide epitopes chosen for antibody generation (Ramachandran et al 2006).

The second observation that we have made is that in many (but not all) glandular tissues SPLUNC2 and SPLUNC1 staining is mutually exclusive. Although in a few of the minor glands of the oral cavity the two proteins do appear to be co-localised, for the most part cells positive for one protein appear to be negative for the other. It is interesting that these two related proteins, which presumably share common functional activities, should be expressed in this manner. This observation is not unique for members of the PLUNC family however, as we have previously shown a similar discordant localisation of two other putative innate immune molecules, secretory leukocyte proteinase inhibitor (SLPI) and HE4 (Bingle et al 2006). These are members of the WFDC-domain containing protein

family that contain two structural WFDC domains (Bingle & Vayarkarnam 2008). Perhaps these different cellular sites of protein production ensure that there is always a significant source of these molecules in the saliva.

In conclusion, we have systematically analysed the localisation of SPLUNC2 in the major and minor salivary glands. SPLUNC2 is predominantly expressed in the serous cells of major salivary glands as well as in the sero-mucus tubules of the minor glands in the oral mucosa, posterior tongue and tonsil. These are all sites of production of host defence proteins and support our suggestion that SPLUNC2 may function in the innate immune defence of the oral cavity. We have also shown that the protein is present in saliva where it appears to exist as several differentially glycosylated protein isoforms. It seems likely that these modified isoforms are also differentially distributed in the salivary glands. Analysis of the SPLUNC2 gene shows that it undergoes alternative splicing and utilizes two 5' upstream non-coding exons that are presumably under distinct regulatory control and are conserved in other primates.

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FIGURE LEGENDS

Fig 1 Characterisation of SPLUNC2 antibodies by western blotting (a) The position of the two-peptide epitopes used to generate the SPLUNC2 antibodies are illustrated (by red boxes) on a multiple sequence alignment of human SPLUNC proteins. The predicted amino acid sequences of human SPLUNC2, SPLUNC1, BASE and SPLUNC3 were aligned using CLUSTALX. In the alignment, identical residues are indicated by white on black background, whereas conserved residues have the grey background. Spaces

introduced for maximum alignments are indicated by a -. The positions of the conserved cysteine residues are also indicated in red (*). (**b)** V5-epitope tagged SPLUNC proteins (SPLUNC1, SPLUNC2), were generated by coupled *in vitro* transcription and translation as outlined in the Materials and Methods section. 2μl of each reaction (and empty vector negative control reaction) was resolved on duplicate 12% SDS-PAGE gels alongside 5μl of whole human saliva. Gels were western blotted and probed with affinity purified polyclonal human SPLUNC2 antibodies (SPLUNC2A and SPLUNC2B). The positions of the molecular markers are indicated by the black arrows

Fig 2 Distribution of SPLUNC2 in Major Salivary Glands Immunohistochemistry was performed on replicate sections as described in the Materials and Methods using the two SPLUNC2 antibodies (SPLUNC2A: A, D G; SPLUNC2B: B, E H) and a polyclonal antibody to SPLUNC1 (C, F, I). Tissues stained are Parotid gland (A, B, C), Submandibular gland (D, E, F) and Sublingual Gland (G, H, I)

Fig 3 SPLUNC2 B immunoreactivity in parotid gland ducts Immunohistochemistry was performed on parotid gland with the antibody to SPLUNC2B as described in the Materials and Methods. High power images were taken to show staining in the larger interlobular ducts (A) and collecting ducts (B). Intensely staining duct cells are indicated by the arrows. Note that the contents of the interlobular ducts also stain intensely

Fig 4 Distribution of SPLUNC2 in Minor Salivary Glands from the oro- and nasopharynx Immunohistochemistry was performed in replicate sections as described in the Materials and Methods using the two SPLUNC2 antibodies (SPLUNC2A: A, D, G, J; SPLUNC2B: B, E, H, K) and a polyclonal antibody to SPLUNC1 (C, F, I, L). Tissues stained are glands from the region of the palatine tonsil (A-F), glands from the posterior portion of the tongue (G-I) and glands from the nasal antral mucosa (J-L)

Fig 5 The human SPLUNC2 gene utilizes alternatively spliced 5' non-coding exons **(a)** Schematic representation of the *SPLUNC2* gene with the exon numbers shown below the figure. Coding sequence is shown in white with non-coding sequence shown in grey. The position of the start (exon 2) and stop codons (exon 8) are illustrated above the figure. The two 5' non-coding exons are shown as 1B and 1A. The scale bar represents 1Kb. **(b)** Expression of alternative splicing of *SPLUNC2* was investigated by RT-PCR with three sets of exon-spanning primer pairs as described in the Materials and Methods section. The

primers were designed to amplify an internal portion of SPLUNC2 as well as the two 5' non-coding exons

Fig 6 SPLUNC2 is present as multiple isoforms in saliva and is N-glycosylated Western blotting was performed on duplicate samples of unstimulated whole saliva using antibodies SPLUNC2A (lane 1) and SPLUNC2B (lane 2)). In an additional experiment a further four samples of unstimulated whole saliva (Lanes 3-6) and a sample sublingual/submandibular gland saliva collected at the same time as the whole saliva sample in lane 4 (lane 7) were western blotted with antibody SPLUNC2A. A sample of whole saliva was subjected to deglycosylation using N-Glycanase PNGase F and subjected to western blotting with antibody SPLUNC2B. The multiple bands present on the mock digested sample (lane 8) were reduced to a single band of lower molecular mass following PNGase treatment (lane 9). The positions of the molecular mass markers (30, 21 and 14kDa) are identified by the open arrowheads to the side of the images