Characterization of the rat salivary-gland B1-immunoreactive proteins

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The B1-immunoreactive proteins (B1-IPs) are major secretory products of rat submandibular gland acinar-cell progenitors, and are also produced by neonatal and adult rat sublingual and parotid glands. In order to characterize the B1-IPs, we have previously isolated cDNA clones encoding rat parotid secretory protein (PSP; the predominant parotid B1-IP) and the related clone ZZ3, which is developmentally regulated in the neonatal submandibular gland. The remainder of the B1-IPs were uncharacterized. This report demonstrates that all of the B1-IPs are derived from the PSP and ZZ3 transcripts. Molecular cloning and Western-blot analyses using PSP- and ZZ3-specific antisera show that, of the B1-IPs, only PSP and neonatal submandibular gland protein A (SMGA) are products of the *Psp* gene. This finding corrects our previous assertion that SMGA is derived from ZZ3. Neonatal submandibular gland proteins B1 and B2, as well as apparent M_r 26000–28000 and M_r 18000–20000 forms in submandibular, sublingual and parotid glands, are derived from the gene encoding ZZ3 by differential N-glycosylation and by proteolytic cleavage. The apparent M_r 18000–20000 proteolytic products are significant in secretion product collected *in vitro*, but rare in gland homogenate and submandibular/sublingual saliva. The gene encoding ZZ3 has been named *Smgb*. *Psp* and *Smgb* are regulated similarly in the developing submandibular gland, but differently in the sublingual and parotid glands. The expression pattern of *Psp* is conserved between rat and mouse. However, no evidence for proteins derived from an *Smgb*-like gene was observed in neonatal mouse submandibular or sublingual glands.

INTRODUCTION

The B1-immunoreactive proteins (B1-IPs) are a family of salivary proteins that are produced by rat submandibular, sublingual and parotid glands. These proteins were first identified as secretory products of the acinar-cell progenitors in the developing submandibular glands (SMGs) of neonatal rats [1]. In response to β-adrenergic stimulation, the acinar-cell progenitors secrete neonatal SMG proteins A (SMGA; apparent M_r 23500), B1 (SMGB1; apparent M_r 26000) and B2 (SMGB2, apparent M_r 27500) [2]. Rabbit antisera raised against either SMGB1 or SMGA cross-reacted with all three proteins, leading to their designation as the B1-IPs [3,4]. Western-blot analyses demonstrated immunologically related B1-IPs of apparent M_r 27000 and 18500 in sublingual glands, and of 27500 and 23500 in parotid glands. Several minor submandibular and parotid B1-IPs of apparent M_r 18000–20000 were also observed.

The acinar-cell progenitors of the neonatal SMG (type III cells) have been shown to mature through intermediate type IIIP cells to the seromucous acinar cells of the adult gland [5]. SMGA, SMGB1 and SMGB2 are abundant products of the type III cells, are present in reduced amounts in the type IIIP cells, and are not produced by adult seromucous acinar cells. By postnatal day 30, all acinar cells are seromucous, and B1-IP immunoreactivity is restricted to a subset of cells in the intercalated ducts [5,6]. Acinar-cell replacement in the adult submandibular gland is thought to occur at least partially by maturation of immature cells from the intercalated ducts (reviewed in [7]). The B1-IP-expressing intercalated duct cells may retain a neonatal phenotype, and differentiate to replace aging or damaged acinar cells in the adult gland [3].

To investigate the regulation of gene expression in salivary acinar-cell progenitors, we sought to characterize proteins SMGA, SMGB1 and SMGB2 of the neonatal SMG, and to determine their relationship to the immunologically cross-reactive proteins of the parotid and sublingual glands. Previously, we have shown that the apparent M_r 23500 parotid B1-IP is rat parotid secretory protein (PSP [8]), an abundant salivary protein originally described in mouse [9]. PSP may function as a bacterial binding protein in rodent saliva [10]. The transcripts of rat and mouse PSP are highly homologous throughout their 5′- and 3′-untranslated regions as well as within their protein coding sequences [8,11–13]. The mouse *Psp* gene has been mapped to chromosome 2 [9]. *Psp* expression is initiated early in mouse parotid development, and increases greatly with gland maturation [13,14]. In adult mice, *Psp* is expressed at high levels in the parotid gland, at low levels in the sublingual gland and is not detected in the SMGs of most strains [10,12,15]. The developmental onset and tissue specificity of rat *Psp* expression has not been described.

In the previous study, characterization of the B1-IPs in neonatal rat submandibular gland was initiated by screening a cDNA library from submandibular glands of 5-day-old rats under reduced stringency conditions using a PSP cDNA. cDNA clone ZZ3, encoding a related salivary protein with predicted M_r 21000, was isolated [8]. ZZ3 was shown to correspond to a 1 kb transcript that is developmentally regulated in the submandibular gland; transcript levels are high in neonates, but are greatly reduced in adults. The secreted form of the protein derived from ZZ3 has only 40 % amino acid similarity with PSP. However, sequence similarity between the signal-peptide-encoding, and 5′- and 3′-untranslated regions, of the PSP and

Abbreviations used: PSP, parotid secretory protein; B1-IPs, B1-immunoreactive proteins; SMG, submandibular gland; SMGA, neonatal SMG protein A; SMGB1, neonatal SMG protein B1; SMGB2, neonatal SMG protein B2; PNGase F, peptide N-glycosidase F.

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ZZ3 transcripts confirm that they are derived from two separate but evolutionarily related genes [8].

In the present study, secretion products and RNA from the submandibular, sublingual and parotid glands were examined to characterize the various B1-IPs. We demonstrate that all of these salivary proteins arise from *Psp* and the gene encoding ZZ3. Only the apparent M_r 23500 B1-IPs of the parotid gland and neonatal SMG, PSP and SMGA, are products of *Psp*. The remainder of the B1-IPs are products of the ZZ3 gene, which is now identified as *Smgb*.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other molecular biological reagents were purchased from New England Biolabs or Boehringer Mannheim unless otherwise specified. Synthetic oligonucleotides were obtained from Operon Technologies (Richmond, CA, U.S.A.) [α-^{32}P]dCTP (6000 Ci/mmol) and [α-^{35}S]dATP (1000 Ci/mmol) were purchased from New England Nuclear. L-[^{35}S]Methionine (1000 Ci/mmol) was obtained from Amersham.

Collection of salivary-gland materials

Sprague–Dawley rats and CB6F1 mice were obtained from Harlan. Animals were anesthetized with Metofane and killed by cervical dislocation or cardiac puncture. Salivary glands to be used for isolation of RNA or salivary-gland homogenate were frozen immediately in liquid nitrogen. Salivary-gland homogenates were prepared by grinding of frozen glands in Laemmli sample buffer using a polytron. Secretion product was collected *in vitro* as described previously [3]. Secretion was collected from minced salivary glands of adult rats by 20 μM DL-isoproterenol, during a 90 min incubation at 37 °C in Dulbecco's modified Eagle's medium under O_2/CO_2 (19:1). Secretion was elicited from minced glands of neonatal rats or mice by 1 mM dibutyryl cAMP under identical conditions. In the immature SMGs, direct stimulation of β-receptors with isoproterenol elicits secretion from only acinar-cell progenitors, whereas circumvention of β-receptors using the cAMP analogue activates secretion by both major secretory cell types of the neonatal gland [3]. Medium containing secretion products was cleared by centrifugation, and was immediately frozen. Submandibular/sublingual saliva, collected from the floor of the mouth of a lightly anaesthetized female rat, was elicited by chewing on a #7 wax spatula. Protein concentration was determined by the Bradford method using the Bio-Rad protein assay reagent.

Peptide N-glycosidase F (PNGase F) digestion

Salivary-gland secretions were denatured by boiling for 10 min in 0.5% (w/v) SDS/1% (v/v) β-mercaptoethanol. Secretion product was adjusted to a final concentration of 0.1 mg/ml in 50 mM sodium phosphate (pH 7.5)/1% (w/v) NP-40, 0.3% (w/v) SDS, 0.6% (v/v) β-mercaptoethanol, and digested overnight at 37 °C with 100 units of PNGase F (New England Biolabs) per μg of protein. Controls were treated identically to experimental samples, but without addition of PNGase F. Proteins were diluted directly into Laemmli sample buffer for analysis by SDS/PAGE.

Preparation of anti-ZZ3 antiserum

A mutated form of the ZZ3 gene product was prepared by removing amino acids number 137–149 of ZZ3 [8] by 'loop out' mutagenesis using the Bio-Rad Mutagene phagemid *in vitro* mutagenesis kit. The sequence of the mutagenic primer was GTCCTGAACATGCAGTTGAACAGAAAAAGT, corresponding to nt 418–432 and 472–486 of ZZ3. A glutathione S-transferase–ZZ3 fusion construct was created by cutting the pGEX-KG [16] polylinker with *Xba*I, digesting with mung-bean nuclease, and cutting with *Hin*dIII. The mutagenized ZZ3 cDNA, ZZ3Δ, was cleaved with *Eco*RV and *Hin*dIII. The *Eco*RV site in ZZ3 is at nt 91–96, four amino acids C-terminal to the signal-peptide-cleavage site, and the *Hin*dIII site is in the polylinker at the 3′ end of the clone [8]. The sequence of the transition region of the resulting glutathione S-transferase–ZZ3Δ fusion protein was GGT GGT GGA ATT ATC TTT CAA AAC, with Gly-Gly-Gly-Ile derived from pGEX-KG, and Ile-Phe-Gln-Asn from ZZ3. A rabbit antiserum against the glutathione S-transferase–ZZ3 fusion protein was prepared at Pocono Rabbit Farm. The fusion-protein preparation and immunization protocols were as described previously [17].

SDS/PAGE and Western-blot analyses

SDS/PAGE was performed by the method of Laemmli [18], using 12% acrylamide resolving gels and 3.5% acrylamide stacking gels. For direct detection of proteins, gels were silver stained by a modification of the method of Morrisey [19]. Proteins were electrophoretically transferred [20], and the membranes were blocked overnight at 4 °C with 5% (w/v) nonfat dried milk in 20 mM Tris/HCl/150 mM NaCl, pH 7.5 (TBS). For Figure 2, 5 and 7, proteins were transferred to nitrocellulose (Schleicher and Schuell). Primary antisera were diluted as follows: anti-ZZ3, 1:2000; anti-PSP [4], 1:3000; and anti-B1 antibody [3] was used at 1:10000. Blots were incubated in the presence of primary antisera in TBS plus 5% (w/v) nonfat dried milk for 2 h at 25 °C, and washed five times for 5 min in TBS containing 0.05% (v/v) Tween 20. Secondary antiserum (alkaline phosphatase-conjugated goat anti-rabbit IgG; Vector Labs, Burlingame, CA, U.S.A.) was diluted 1:1000 in TBS plus 5% (w/v) nonfat dry milk. Blots were incubated for 1 h at 25 °C, washed five times for 5 min in TBS containing 0.05% (v/v) Tween 20, and once in TBS. Reactive proteins were detected with 0.3 mg/ml Nitro Blue Tetrazolium/0.15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate in 100 mM Tris/HCl (pH 9.5) 100 mM NaCl/5 mM $MgCl_2$. For Figure 6, proteins were transferred to Immobilon P (Millipore), incubated with anti-ZZ3 at a 1:2500 dilution, and with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Jackson Immunoresearch, West Grove, PA, U.S.A.) at a 1:20000 dilution. Proteins were detected with the Renaissance chemiluminescence reagent from New England Nuclear as recommended by the manufacturer, with buffers, incubation times and washes as above.

RNA isolation, *in vitro* transcription and Northern-blot analysis

RNA was isolated by the method of Han et al. [21] or of Chomczynski and Sacchi [22]. Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo d(T) cellulose [23]. For use as a control in Northern-blot analysis, ZZ3 and PSP RNAs were transcribed *in vitro* according to the method of Melton et al. [24]. *In vitro* transcribed RNAs were examined by formaldehyde/1.5% agarose gel electrophoresis followed by ethidium bromide staining to ensure that they were intact and of the correct size.

RNA was separated by electrophoresis on 1.5% agarose/6.7%

formaldehyde gels, transferred to nitrocellulose, and hybridized under high-stringency conditions as described previously [8]. The PSP-specific cDNA probe was isolated from PSP cDNA RP3 [8] by digestion with *Xmn*I and *Avr*II. This 412 bp probe corresponded to nt 105–512 of the PSP cDNA. The 200 bp ZZ3-specific probe, obtained by digestion with *Eco*RV and *Nco*I, corresponded to nt 93–299 of the ZZ3 cDNA [8]. These probes were derived from the least similar regions of their respective clones. After digestion, probes were isolated by electrophoresis through low-melting-point agarose, excised, and labelled directly by random priming [25].

Reverse transcriptase-PCR, cDNA library screening and DNA sequence analysis

PSP and ZZ3 cDNAs were cloned from rat submandibular, sublingual and parotid glands using the 5′ rapid amplification of cDNA ends method as applied in the Clontech Marathon cDNA Amplification Kit. A pool of gland-specific cDNAs was synthesized using RNase H^- mouse mammary leukaemia virus reverse transcriptase followed by second-strand synthesis using *Escherichia coli* DNA polymerase I, RNase H and *E. coli* DNA ligase. A PSP-specific cDNA was amplified using the oligonucleotide ACGTCCCGGGATCCGAGAGGGACTGCTAGCTGTCC. The first 14 nucleotides encode *Xma*I and *Bam*HI sites for cloning; the remaining 21 nucleotides are complementary to nt 731–751 of clone RP3, which encode the C-terminal seven amino acids of PSP.

The ZZ3-specific primer, ACGTCCCGGGATCCGGTGGAGATTGCAGTCTGCGG, contains *Xma*I and *Bam*HI sites followed by the complement of nt 622–642 encoding the C-terminal seven amino acids of ZZ3. In each case, the second (5′) primer was the AP1 adaptor primer supplied by Clontech. PCR was performed using TaKaRa Ex Taq polymerase (Takara Shuzo product distributed by Pan Vera Corp., Madison, WI, U.S.A.) and TaqStart antibody (Clontech) with denaturation at 94 °C for 1 min and extension at 68 °C for 3 min over 30 cycles. PCR products were digested with *Bam*HI and *Not*I, isolated in low-melting-point agarose and ligated into pBluescript KS^-. PSP cDNA clones were isolated from 5-day-old-animal SMG and adult sublingual gland λZAP II cDNA libraries by hybridization under high-stringency conditions to the 412 bp *Xmn*I–*Avr*II fragment of PSP. The libraries and the high-stringency screening protocol were described previously [17]. DNA sequence analysis was performed by the dideoxy method [26] using Sequenase Version 2.0 enzyme and reagents (U.S. Biochemicals/Amersham). The ~ 670 bp ZZ3 cDNAs isolated by PCR were sequenced on one strand using T3 and T7 primers, as well as a synthetic 18-mer corresponding to nt 202–220 of ZZ3. The PSP cDNAs were sequenced on one strand using T3 and T7 primers, and synthetic primers corresponding to nt 156–172 of PSP, and 24-mer primers corresponding to nt 368–391 and 521–544 of PSP.

N-terminal sequence analysis of apparent M_r 27000 and 18500 proteins from 5-day-old animal sublingual gland

Secretion products from 5-day-old-animal sublingual gland were fractionated by SDS/PAGE with the addition of 0.1 mM sodium thioglycolate to the upper buffer reservoir [27]. Total secreted protein (20 μg) was applied to each of two gels. After electrophoresis, the gels were soaked for 10 min in 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid (Aldrich) (pH 11)/10 % (v/v) methanol, and then electroblotted to PVDF membrane (ProBlott; Applied Biosystems) in this buffer. Membranes were stained for 5 min in 1 mg/ml Coomassie Brilliant Blue in 50 % (v/v) methanol, and destained in 50 % (v/v) methanol until bands were visible. Protein bands corresponding to the apparent M_r 27000 and 18500 proteins were excised. Amino acid sequencing was performed by Matthew Williamson at the University of California at San Diego Biology Department protein-sequencing facility, using an Applied Biosystems model 470 gas-phase sequencer with a model 120 on-line HPLC for detection of PTH–amino acids.

RESULTS

The B1-IPs in salivary secretions

The major B1-IPs were revealed by SDS/PAGE of secretion products collected *in vitro* (Figure 1). In the sublingual gland, B1-IPs of apparent M_r 27000 and 18500 were present in both the neonate and the adult. SMGA (apparent M_r 23500), SMGB1 (apparent M_r 26000) and SMGB2 (apparent M_r 27500) were major secretory products of the neonatal gland, but were not detected in the adult secretion. SMGB1 and -B2 did not accumulate synchronously. Only SMGB2 was present from prenatal day 19 through postnatal day 1, and levels of SMGB2 declined more rapidly than SMGB1 during the second week of life (Figure 1 and [4]). In the parotid gland, the B1-IPs were at apparent M_r 27500 and 23500. The apparent M_r 23500 B1-IP, PSP, was also present in high proportion in the adult gland. Although the apparent M_r 27500 parotid B1-IP was abundant in the neonatal gland, levels diminished greatly during development.

The specificities of the previously prepared anti-SMGB1 and anti-PSP antisera are shown by Western blot in Figure 2. Anti-SMGB1 recognized all the major B1-IPs identified in Figure 1; in addition, it disclosed other reactive species at apparent M_r 18000–20000 (upper panel). The anti-PSP antiserum obtained

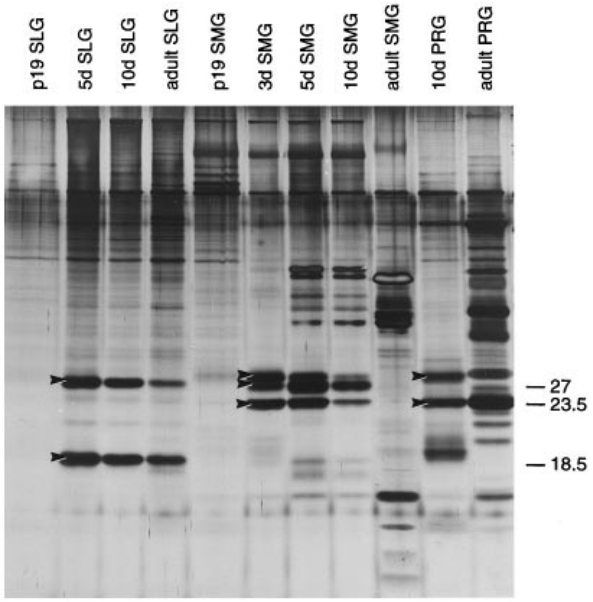

Figure 1 Expression of the B1-IPs in neonatal and adult rat salivary glands

Total secretion products of neonatal and adult rat sublingual (SLG), submandibular (SMG) and parotid (PRG) glands were revealed by SDS/PAGE followed by silver staining. The major B1-IPs described in [3] are identified by arrowheads. The ages [in days (d)] of the neonatal rats from which glands were obtained are listed above the Figure. Abbreviation: p19, prenatal day 19. The relative positions of the apparent M_r 27000, 23500 and 18500 B1-IPs are shown on the right-hand side.

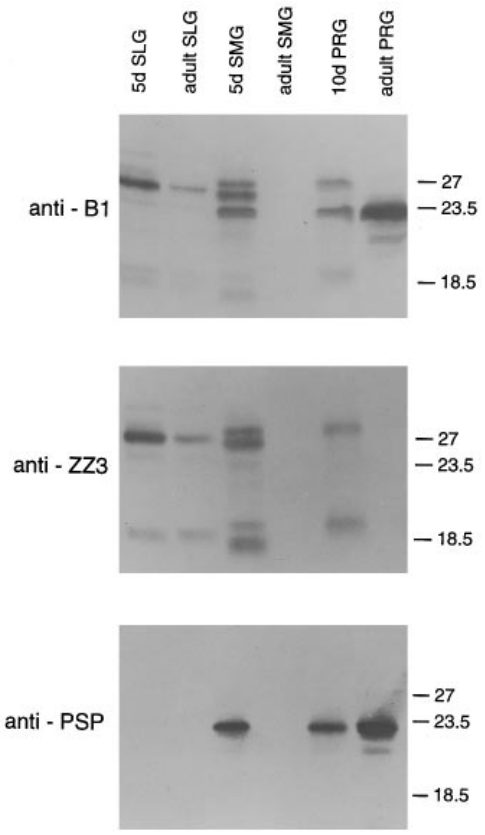

Figure 2 Reactivity of anti-B1, anti-ZZ3 and anti-PSP antisera with salivary-gland secretion products

B1-IPs in neonatal and adult submandibular (SMG), sublingual (SLG) and parotid (PRG) secretion were identified by Western-blot analysis. After SDS/PAGE, proteins were transferred to nitrocellulose and reacted with anti-B1 antiserum (which recognizes all the B1-IPs), with anti-ZZ3 or with anti-PSP. Total protein loaded for the anti-B1 and anti-ZZ3 blots was: 5-day-old-animal SLG, 01 μg; adult SLG, 0.5 μg; 5-day-old-animal SMG, 0.6 μg; adult SMG, 0.5 μg; 10-day-old-animal PRG, 0.3 μg; adult PRG, 2.4 μg. For the anti-PSP blot, the following amounts of protein were used: 5-day-old-animal SLG, 1 μg; adult SLG, 0.5 μg; 5-day-old-animal SMG, 0.15 μg; adult SMG, 0.5 μg; 10-day-old-animal PRG, 0.1 μg; adult PRG, 0.1 μg. The relative position of the apparent M_r 27000, 23500 and 18500 B1-IPs are shown on the right-hand side.

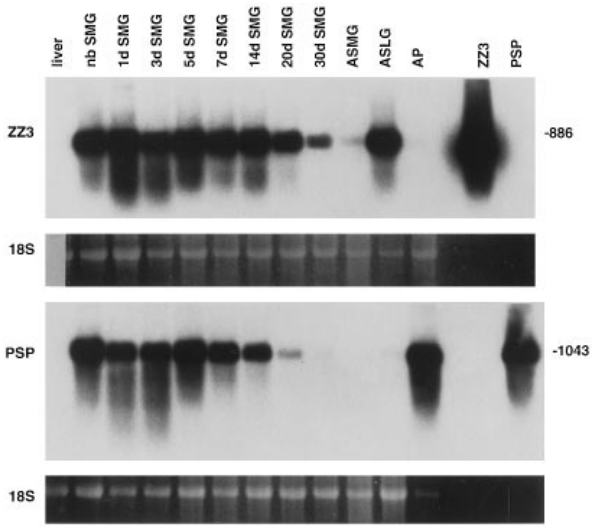

Figure 3 Both ZZ3 and PSP are developmentally regulated in the SMG

Northern-blot analysis of salivary-gland RNAs was performed using unique ZZ3 and PSP probes. The probes were hybridized to 8 μg of total RNA derived from adult liver, adult SMG (ASMG), adult sublingual gland (ASLG) and adult parotid gland (AP), and from neonatal rat SMG of ages 0 days (nb) through 30 days (d) as shown. The parotid RNA sample hybridized to PSP, and the sublingual sample hybridized to ZZ3 contained 2 μg of total RNA. Ribosomal RNA (18S) from these gels is shown as a loading control. The absence of reactivity of the ZZ3 probe with PSP and vice versa was demonstrated by inclusion of full-length *in vitro* transcribed ZZ3 (886 nt) and PSP (1043 nt) RNA on both blots.

by immunization with PSP isolated from parotid saliva [4] reacted with only the apparent M_r 23500 kDa proteins in neonatal SMG (SMGA) and in neonatal and adult parotid secretion (PSP) (lower panel).

A rabbit antiserum was raised against a glutathione S-transferase–ZZ3 fusion protein, in which ZZ3 had been mutagenized to eliminate amino acids 137–149. In the deleted sequence, 9/13 amino acids, including one Cys, are identical between ZZ3 and PSP. This region was considered a possible source of the cross-reactivity among B1-IPs seen with the anti-B1 antiserum [8]. The antiserum did not recognize PSP in neonatal or adult parotid, or SMGA in the neonatal SMG (Figure 2, middle panel). However, it did react strongly with all the other submandibular, sublingual and parotid products that are recognized by anti-SMGB1.

Expression patterns of PSP and ZZ3

Figure 3 shows a Northern blot of total RNA prepared from submandibular glands of rats aged 0–30 days, and from adult submandibular, sublingual and parotid glands. The blot was hybridized to radioactively labelled PSP and ZZ3 probes derived from the least similar protein-coding region of each clone. To ensure lack of cross-hybridization, full-length ZZ3 and PSP transcripts derived by *in vitro* transcription from each cDNA were included in the Northern blot.

Figure 3 demonstrates that both PSP and ZZ3 transcripts were present in neonatal rat submandibular gland. As was previously shown for ZZ3 [8], PSP transcript levels were highest in the first postnatal week, and diminish thereafter. It is apparent that by days 20 and 30, however, relative levels of ZZ3 RNA were higher than those of PSP. In the adult SMG, low levels of ZZ3 transcripts remained, whereas PSP transcripts were undetectable under these conditions. ZZ3 transcripts were present at high levels in the adult sublingual gland, but were barely detected in the adult parotid gland. Conversely, this blot confirmed the known abundance of PSP in adult parotid, but demonstrated little or no PSP mRNA in adult sublingual gland.

Figure 4 demonstrates the presence of transcripts homologous to ZZ3 and PSP in adult salivary glands. Highest levels of ZZ3 were present in adult sublingual glands; lower levels were present in adult submandibular and parotid glands. Similarly, highest relative levels of PSP were in an adult salivary gland (parotid). PSP RNA was present in adult sublingual glands, but was not detected in adult SMGs. No major difference in ZZ3 or PSP transcript levels between adult male and female salivary glands was observed. The RNA shown in Figure 4 was prepared from pooled glands of several rats. Additional salivary-gland RNAs prepared from at least three individual male and female rats contained similar levels of PSP and ZZ3 transcripts (results not shown). These Northern blots provided no evidence for multiple ZZ3 or PSP transcript sizes, or for expression of either gene in lacrimal glands.

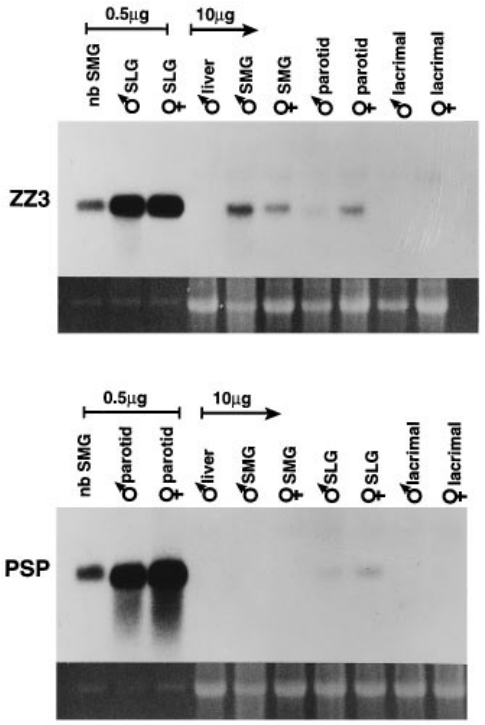

Figure 4 Relative levels of ZZ3 and PSP transcripts in the major salivary glands of adult male and female rats

Total RNA from SMGs, sublingual glands (SLG), parotid glands, lacrimal glands and liver (negative control) was examined by Northern-blot analysis using unique ZZ3 and PSP probes. In each blot, the first three lanes each contain 0.5 μg of total RNA, whereas the remaining lanes each contain 10 μg of total RNA. The lower panels in each half show the relative levels of ethidium bromide-stained 18S ribosomal RNA in the gels from which these blots were transferred. Abbreviation: nb, RNA obtained within 24 h of birth.

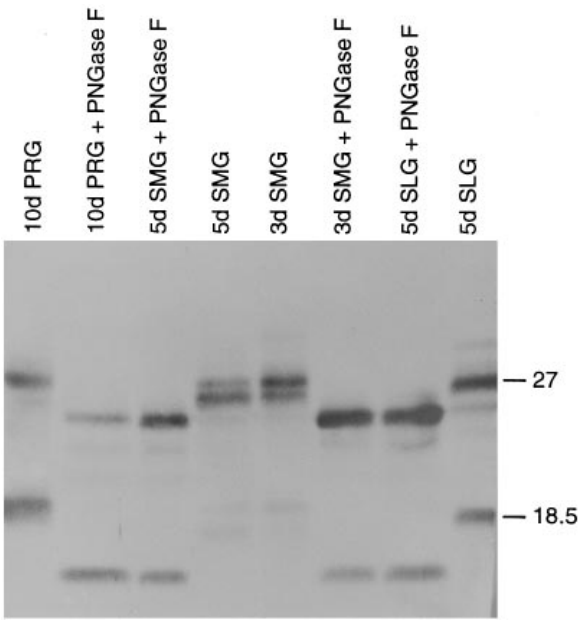

Figure 5 PNGase F digestion of neonatal salivary-gland secretion products

Secretion products from neonatal submandibular (SMG), sublingual (SLG) and parotid (PRG) glands were digested with PNGase F as described in the Materials and methods section. PNGase F-treated secretion product (1.5 μg) was fractionated by SDS/PAGE alongside an equal concentration of undigested secretory protein, and transferred to nitrocellulose. Proteins reactive with the anti-ZZ3 antiserum were detected colorimetrically. The relative positions of the apparent M_r 27000 and 18500 SLG proteins are shown on the right-hand side. Abbreviation: d, day.

The bands observed by Northern-blot analysis were shown to be bona fide PSP and ZZ3 transcripts by DNA sequencing. cDNA clones from each salivary gland were isolated and analysed. PSP clones were isolated from 5-day-old-animal SMG and adult sublingual gland cDNA libraries [17] by high-stringency hybridization to the 400 bp PSP-specific probe. Many PSP clones were isolated from the 5-day-old-animal SMG library, and one clone was identified from the adult sublingual-gland library. An additional PSP cDNA was isolated from adult sublingual-gland RNA using PCR methodology. Three 5-day-old-animal SMG PSP cDNAs and the two adult sublingual-gland PSP cDNAs were sequenced. The nucleotide sequences of all five cDNAs exactly matched that previously published from parotid gland [8], confirming that the *Psp* gene is also expressed in neonatal SMG and in adult sublingual gland.

ZZ3 cDNAs were isolated from adult submandibular, sublingual and parotid glands by PCR as described in the Materials and methods section. Three clones from adult sublingual gland, three from adult parotid gland and four from adult SMG were sequenced. Seven of ten cDNA clones were identical to the published sequence of ZZ3. In three of the ten clones (two from sublingual gland and one from SMG), nt 616 was C rather than T as in the published sequence. The single nucleotide change would result in a change from Ser to Pro at amino acid 198. It is possible that these changes represent errors by *Taq* polymerase, or may represent an allelic variation in these outbred rats.

Proteins derived from ZZ3 are N-glycosylated

Secretion products from 10-day-old-animal parotid gland, 3- and 5-day-old-animal SMG and 5-day-old-animal sublingual gland were digested with PNGase F to remove N-linked oligosaccharides. Digested and undigested secretion products were revealed by Western-blot analysis using the anti-ZZ3 antiserum (Figure 5). The apparent M_r 26000–27500 B1-IPs in each secretion were decreased to a single band of apparent M_r 24000. Additionally, the bands in the apparent M_r 18500 range were decreased to apparent M_r 15000. Despite the varied sizes of the fully glycosylated B1-IPs, the electrophoretic mobilities of the PNGase F-treated products were indistinguishable. Digestion of secretions from both 3-day-old-animal SMG, in which SMGB1 and SMGB2 are present in approximately equal proportion, and from 5-day-old-animal SMG, in which SMGB1 predominates, resulted in the appearance of only a single band. *In vitro* translation of 5-day-old-animal SMG RNA followed by immunoprecipitation with the anti-ZZ3 antiserum also suggested that SMGB1 and SMGB2 are differently N-glycosylated forms of a single polypeptide (results not shown).

The apparent $M_r \approx$ 18000–20000 B1-IPs are proteolytic products of the apparent $M_r \approx$ 26000–27500 B1-IPs

The apparent M_r 18500 sublingual gland B1-IP is a predominant component of both neonatal and adult sublingual-gland secretion product. To characterize this protein, the N-terminal six amino acids of the apparent M_r 18500 and 27000 sublingual-gland B1-IPs were determined after electrophoresis and transfer to PVDF

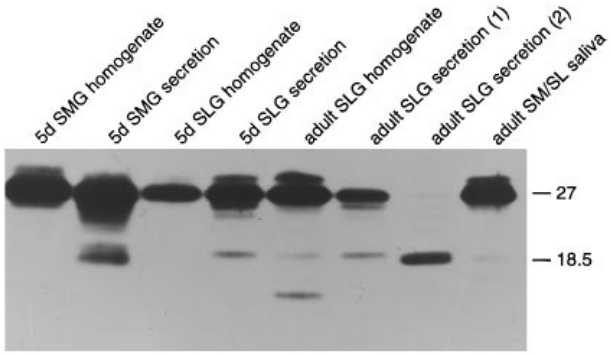

Figure 6 Relative abundance of apparent M_r 18000–20000 B1-IPs in salivary-gland homogenate and secretions

The presence of lower M_r B1-IPs in salivary-gland homogenate, secretion products and submandibular (SM)/sublingual (SL) saliva was examined by Western-blot analysis. Proteins from 5-day (d)-old-animal and adult SMG and sublingual glands (SLG) were transferred to PVDF membrane and reacted with anti-ZZ3 antiserum. Bound antibody was detected by chemiluminescence. This method did not allow resolution of SMGB1 and SMGB2 in neonatal SMG. Adult SLG secretion (1) was obtained under standard conditions; adult SLG secretion (2) was collected at a sevenfold-higher ratio of gland to medium.

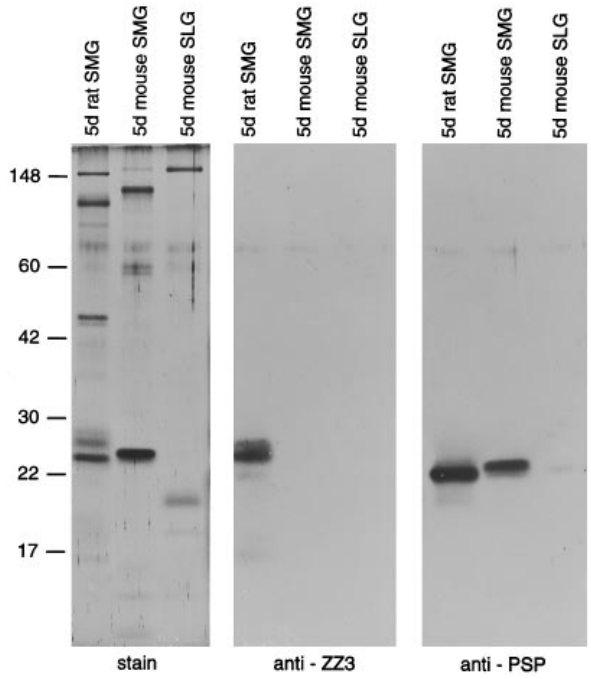

Figure 7 PSP but not ZZ3 protein is present in neonatal mouse SMG and sublingual glands (SLG)

Secretion products from 5-day (d)-old-rat SMG and 5-day-old-mouse SMG and SLG were separated by SDS/PAGE and revealed by silver staining (stain). The middle and right-hand panels show secretion products that were transferred to nitrocellulose after electrophoresis, and reacted with the anti-ZZ3 or anti-PSP antisera. Positions of M_r markers are shown on the left-hand side.

membrane. The apparent M_r 27500 sample contained a single major component, whereas the apparent M_r 18500 sample was a mixture of two salivary components. The amino acid sequences determined for both the apparent M_r 27000 protein and the predominant M_r 18500 component were identical: Ser-Leu-Phe-Asp-Ile-Phe. This corresponds to amino acids 20–25 of ZZ3, and defines the signal-peptide cleavage site for ZZ3 as C-terminal to amino acid 19 of the deduced amino acid sequence. The sequence of the lower-abundance component of the M_r 18500 sample was identical to amino acids 18–23 of CSP-1, an unrelated secretory product of sublingual glands [17]. Therefore, the apparent M_r 18500 'band' observed by silver staining of sublingual-gland secretion is actually a mixture of two components, CSP-1 and a protein derived from ZZ3 or a closely related transcript. These findings suggested that the apparent M_r 18500 protein may be derived from the apparent M_r 27000 species by proteolytic cleavage.

This possibility was further investigated by Western-blot analysis, by comparison of the proteins reactive with anti-ZZ3 antiserum in secretion product collected *in vitro* with those present in homogenates of frozen glands and in submandibular/sublingual saliva frozen immediately after collection (Figure 6). In each gland, the apparent M_r 18000–20000 bands were present in considerably greater proportion in secretion product than in homogenate or in adult submandibular/sublingual saliva. In neonatal homogenates the lower-M_r bands were not detected. Conversely, adult secretion product collected at a sevenfold-higher gland-to-medium ratio showed almost complete elimination of the apparent M_r 27000 band and increased intensity of the apparent M_r 18500 band. The identity of the additional smaller reactive protein observed in adult sublingual-gland homogenate is not known.

PSP is secreted by neonatal mouse submandibular and sublingual glands

Mouse SMG development follows a morphologically and temporally similar pattern to that of rat. To determine whether PSP and/or ZZ3 is present in neonatal mouse submandibular or sublingual glands, secretion products collected from 5-day-old-mouse glands were examined by Western blot using the PSP-specific and ZZ3-specific antisera. In the left panel of Figure 7, total secretion products from 5-day-old-rat SMG, and 5-day-old-mouse submandibular and sublingual glands are shown. Neonatal mouse SMG secretions contained a single major protein species in the apparent M_r 20000–30000 range with electrophoretic mobility slightly less than that of SMGA (PSP) in the neonatal rat secretion. On Western-blot analysis anti-PSP antiserum reacted with the major apparent $M_r \approx 24000$ protein in 5-day-old-mouse SMG, and detected low levels of a similar-sized protein in neonatal mouse sublingual gland. No evidence of reactivity of the anti-ZZ3 antiserum with either 5-day-old-mouse submandibular or sublingual secretion was observed.

DISCUSSION

The present study demonstrates that the rat salivary-gland B1-IPs are derived from two genes. SMGA and PSP are apparently identical, and are products of the *Psp* gene. SMGB1 and SMGB2 are differently glycosylated forms of a single protein, also expressed in sublingual and parotid glands. We suggest that the sublingual and parotid forms be called SMGB. The sequence of this protein was deduced from neonatal SMG cDNA clone ZZ3 [8]. Based on the findings presented here, the gene encoding cDNA ZZ3 has been named *Smgb*. The gene names *Psp* and *Smgb* have been approved by the International Rat Genetic Nomenclature Committee (Göran Levan, personal communication).

The salivary-gland specificity, relative expression levels and sizes of proteins derived from the *Psp* and *Smgb* genes are summarized in Table 1. In both submandibular and parotid

Table 1 Revised nomenclature for proteins derived from the *Psp* and *Smgb* (ZZ3) genes

Suggested nomenclature for the proteins formerly grouped as 'B1-IPs' is based on the findings given in the present report. -- indicates that PSP transcripts, but not protein, have been identified in rat sublingual gland to date; low (?) indicates that no PSP transcripts or protein were detected in adult SMG in the present study, although a band of apparent M_r similar to PSP was detected by Western blot previously [5]. SLG, sublingual gland; PRG, parotid gland.

Gene	Gland	Expression (pup/adult)	M_r	Former name	Proposed name
Psp	SMG	high/low(?)	23 500	SMG-A	PSP
	SLG	low/low	--	--	PSP
	PRG	high/high	23 500	PSP	PSP
Smgb	SMG	high/low	27 500	SMG-B2	SMGB2
			26 000	SMG-B1	SMGB1
	SLG	high/high	27 000	27k B1-IP	SMGB
			18 500	18.5 k B1-IP	SMGB
	PRG	high/low	27 500	27.5 k B1-IP	SMGB

glands, levels of *Psp* and *Smgb* expression vary between neonate and adult. No developmentally related changes in *Psp* or *Smgb* expression were noted in sublingual gland. This is consistent with the fact that the rat sublingual gland, unlike the submandibular and parotid glands, does not undergo a protracted period of postnatal development [29].

The relative absence of the apparent M_r 18 000–20 000 SMGB proteins in salivary-gland homogenate and in adult submandibular/sublingual saliva frozen immediately after collection suggests that these forms are not prominent in primary saliva. Proteolytic cleavage of SMGB protein was greatly enhanced under conditions in which secretion product was collected *in vitro*. A similar difference in the abundance of apparent M_r 27 500 and 19 000 proteins was previously present between homogenate and secretions of 5-day-old-animal parotid gland [3]. Proteolysis of human salivary proteins is known to occur in saliva and enamel pellicle [30,31]. For example, specific cleavage of human proline-rich proteins results in the production of low-molecular-mass proline-rich peptides that may have anti-microbial activity [32,33]. The extent or biological relevance of SMGB proteolysis *in vivo* in either neonatal or adult rats is not known.

Superimposed on the developmental regulation of *Smgb* gene expression in the neonatal rat SMG is a developmentally regulated pattern of N-glycosylation giving rise to SMGB1 and SMGB2. From prenatal day 19, when these proteins are first produced, through postnatal day 1, only or predominantly SMGB2 is synthesized. By postnatal day 3, approximately equal amounts of SMGB1 and SMGB2 are present, and the SMGB1 isoform is predominant from day 9 onward [4]. The apparent M_r 1500 kDa difference between SMGB1 and SMGB2 could result from occupancy of one N-glycosylation site in SMGB1 and both sites in SMGB2, or from utilization of an equal number of N-glycosylation sites by oligosaccharides of differing structure in SMGB1 and SMGB2.

The transition from the predominant SMGB2 to the SMGB1 isoform precedes but parallels an important morphologic transformation in the developing SMG. The transitional type IIIP secretory granules, which signify the initiation of mucin biosynthesis by the developing acinar cells, are commonly seen by day 3 [5,6]. By day 9, all secretory granules are mucous in appearance, indicating a widespread commitment to mucin biosynthesis. It is possible that developmentally regulated terminal glycosyl transferases required for mucin biosynthesis are able to glycosylate nascent N-linked oligosaccharides of SMGB. If so, these glycosyl transferases may give rise to the SMGB1 isoform from day 3 onward, whereas the SMGB2 isoform might be produced by transferases characteristic of the immature acinar cells.

The apparent M_r of glycosylated sublingual SMGB is consistently between that of SMGB1 and SMGB2 [3]. The parotid SMGB has apparent M_r 27 500, indistinguishable from SMGB2. The oligosaccharides on sublingual gland SMGB may be of different structure than those in submandibular gland, whereas glycosylation of this protein in the early neonatal submandibular and parotid glands may be identical. Previously it was shown that the salivary-gland protein CSP-1 is differently N-glycosylated in the adult sublingual and parotid glands [17].

Although the regulation of *Psp* gene expression is very similar between rat and mouse, some differences were observed. *Psp* expression in mouse sublingual gland is one-tenth of that in parotid gland [34], which is greater than the level of *Psp* transcripts found in rat sublingual gland. Similarly, *Psp* transcripts are detectable in mouse lacrimal gland by Northern-blot analysis [8,10], but were not detected in rat lacrimal gland under the conditions of Figure 4. No SMGB protein was observed in either neonatal mouse submandibular or sublingual secretion, the sites of highest levels in rat.

The expression patterns of *Smgb* and *Psp* in rat salivary glands suggest that regulatory elements directing high levels of gene expression in neonatal SMGs are conserved between these two genes. The salivary-specific enhancers regulating sublingual and parotid gland expression may be less similar. Future studies will be directed toward identification of salivary-gland-specific enhancers of the *Smgb* and *Psp* genes.

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