To elucidate the localization of post-translational modifications of different classes of human salivary proteins and peptides (acidic and basic proline-rich proteins (PRPs), Histatins, Statherin, P-B peptide, and “S type” Cystatins) a comparative reversed phase HPLC-ESI-MS analysis on intact proteins of enriched granule preparations from parotid and submandibular glands as well as parotid, submandibular/sublingual (Sm/Sl), and whole saliva was performed. The main results of this study indicate the following. (i) Phosphorylation of all salivary peptides, sulfation of Histatin 1, proteolytic cleavages of acidic and precursor basic PRPs occur before granule storage. (ii) In agreement with previous studies, basic PRPs are secreted by the parotid gland only, whereas all isoforms of acidic PRPs (aPRPs) are secreted by both parotid and Sm/Sl glands. (iii) Phosphorylation levels of aPRPs, Histatin 1, and Statherin are higher in the parotid gland, whereas the extent of cleavage of aPRP is higher in Sm/Sl glands. (iv) O-Sulfation of tyrosines of Histatin 1 is a post-translational modification specific for the submandibular gland. (v) The concentration of Histatin 3, Histatin 5, and Histatin 6, but not Histatin 1, is higher in parotid saliva. (vi) Histatin 3 is submitted to the first proteolytic cleavage (generating Histatins 6 and 5) during granule maturation, and it occurs to the same relative extent in both glands. (vii) The proteolytic cleavages of Histatin 5 and 6, generating a cascade of Histatin 3 fragments, take place after granule secretion and are more extensive in parotid secretion. (viii) Basic PRPs are cleaved in the oral cavity by unknown peptidases, generating various small proline-rich peptides. (ix) C-terminal removal from Statherin is more extensive in parotid saliva. (x) P-B peptide is secreted by both glands, and its relative quantity is higher in submandibular/sublingual secretion. (xi) In agreement with previous studies, S type Cystatins are mainly the product of Sm/Sl glands.

Saliva contains a complex mixture of proteins and peptides as well as fragments derived from these molecules. By RP1-HPLC-ESI-MS analysis of the acidic soluble fraction of human whole saliva we have identified in the chromatographic pattern more than 120 different proteins and naturally occurring peptides (1–6). Their characterization was performed by a variety of mass spectrometric techniques coupled with different enzymatic treatments and amino acid sequencing. The proteins and naturally occurring peptides belong to families of well characterized salivary proteins including Histatins, Statherin, acidic and basic proline-rich proteins (aPRP and bPRP), Cystatins, and Defensins (1–6). Two-dimensional gel electrophoresis has also been used by other researchers for analysis of salivary proteins and peptides, but this technique is not well suited for identification of small peptides as illustrated by the difficulty in identifying Histatins and the majority of bPRPs and bPRP fragments (7–9). However, knowledge of salivary proteins and peptides as well as their naturally occur-
rning fragments is important to understand the various stages of maturation occurring during their secretion pathway (10, 11).

Saliva originates from three pairs of major glands (parotid, submandibular, and sublingual) with a small fluid contribution from several minor glands (12). Most salivary proteins are synthesized in the acinar cells of the glands and follow a well-defined secretory pathway that for many proteins includes transit in the Golgi apparatus and storage in secretory granules preceding their release from the cell into the duct system and secretion into the mouth (13). During the transit in the cells the salivary proteins are subjected to a number of changes including removal of the signal peptide as well as various post-translational modifications (PTMs) such as proteolytic cleavage, glycosylation, phosphorylation, and sulfation. Further modifications of the proteins and peptides occur in ducts and in the oral cavity after secretion from the cells as a result of a number of proteolytic enzymes of different origin.

This study describes the comparative profiling of intact proteins and naturally occurring peptides of cellular fractions enriched in secretory granules (enriched granule preparations [EGPs]) as well as parotid (Pr), submandibular/sublingual (Sm/Sl), and whole saliva by the above cited RP-HPLC-ESI-MS methods (1–6). The results provide information useful for a better understanding of the spatial and temporal relationships of the modifications the proteins undergo from their synthesis on the ribosomes to their isolation from whole saliva. In fact, these analyses allow a comparison of the events taking place during synthesis and secretion from Pr and Sm/Sl glands and make it possible to distinguish between events occurring prior to storage in secretory granules, those taking place between granule release and secretion into the mouth, and those occurring after secretion into the mouth (Fig. 1).

MATERIALS AND METHODS

Reagents and Instrumentation—All general chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba (Milan, Italy), Merck, and Sigma-Aldrich.

For HPLC-ESI-MS a ThermoFinnigan (San Jose, CA) apparatus was used. The Surveyor HPLC system was equipped with a photodiode array detector and connected by a T splitter to the electrospray ionization/ion trap mass spectrometer, LCQ Deca XP Plus (ThermoFinnigan). The chromatographic column was a Vydac (Hesperia, CA) C8 with 5-μm particle diameter (column dimensions 150 × 2.1 mm).

Mass values of some peptides were confirmed by MALDI-TOF mass spectrometry performed on a Bruker Daltonics Autoflex apparatus (Bremen, Germany). Peptide sequencing was carried out with a Procise 610A Protein Sequencer (Applied Biosystems, Foster City, CA).

Sample Collection and Treatment—Resting whole saliva was collected from normal adult volunteers between 2 and 4 p.m. using a soft plastic aspirator. Parotid saliva was collected by a Lashley cup (14), and submandibular/sublingual saliva was obtained by means of an appliance described by Truelove et al. (15). Flow of glandular saliva was stimulated by sucking on sour lemon candy. An acidic solution (0.2% TFA) was added to the salivary samples exactly in 1:1 (v/v) ratio at 4 °C immediately after collection, and the solution was centrifuged at 8000 × g for 5 min (4 °C). The acidic supernatant was separated from the precipitate, and the supernatant was either immediately analyzed by HPLC-MS apparatus or stored at −80 °C.

Parotid and submandibular gland specimens were biopsies from patients undergoing removal of salivary gland neoplasia. The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine of the Catholic University. Pieces of surgically removed glands considered eligible on the basis of their normal histological appearance assessed by extemporaneous histopathological analysis were used to obtain enriched secretory granule preparations. The pieces obtained were immediately used to make enriched granule preparations.

Preparation of Salivary Granules and Their Extracts—Granules were isolated according to the procedure of Borges-Silva and Bento-Alves (16) with some modifications. The salivary gland was washed with 0.9% NaCl aqueous solution to eliminate blood, minced into small pieces by using a scalpel, and then homogenized in a glass Potter-Elvehjem homogenizer with a Teflon pestle as a 5% (w/v) suspension in 340 mM sucrose, 0.5 mM EDTA, 10 mM HEPES, pH 7.4, at room temperature. To remove fibrous connective tissue and insoluble particles, the homogenate was filtered through four layers of clean coarse gauze in the homogenizing medium and then centrifuged at 500 × g for 10 min at 4 °C. The supernatant was submitted to further centrifugation at 2500 × g for 15 min at 4 °C, and the pellet, corresponding to the crude fraction of secretory granules as verified by scanning electron microscopy (S4000 FE scanning electron microscope, Hitachi, Tokyo, Japan), was solubilized in 700 μl of 0.2% TFA. The granule fraction was centrifuged at 8000 × g for 10 min, and CHCl3 was added to the supernatant (1:1, v/v) to remove lipidic components. The aqueous phase was directly used for RP-HPLC-ESI ion trap MS analyses or stored at −80 °C.

HPLC-ESI-MS and MALDI-TOF-MS Experiments—Proteins (Cystatins, aPRPs, bPRPs, Histatins, Statherin, and P-B peptide) and naturally occurring peptides derived from aPRPs, bPRPs, Histatins, Statherin, and P-B peptide were already identified in previous studies (1–6). Identification was carried out by ESI ion trap MS and MALDI-TOF-MS coupled with different enzymatic treatments (trypsin, chymotrypsin, proteinase K, proteinase V8, Pronase, carboxy- and aminopeptidases, and phosphatase), chemical treatments (acidic hydrolysis for sulfoderivatives), and automated amino acid sequencing, the latter on some purified intact proteins and naturally occurring peptides as well as on some of their proteolytic fragments. The aqueous phase was directly used for RP-HPLC-ESI ion trap MS analyses or stored at −80 °C.
eluent B in 40 min at a flow rate of 0.30 ml/min. A T splitter addressed a flow rate of 0.20 ml/min toward the diode array detector and a flow rate of 0.10 ml/min toward the ESI source. Usually during the first 5 min of RP-HPLC separation the eluate was not addressed toward the ion trap MS apparatus to avoid instrument damage as a result of the high salt content. Mass spectra, in the positive ion mode, were collected every 3 ms. MS spray voltage was 4.50 kV, and the capillary temperature was 220 °C.

The structure of some bPRP fragments not previously characterized was confirmed by MS/MS analyses, generally carried out contemporaneously on different charged ions. MS/MS experiments were performed by detecting parent ions with a peak width of 2–4 m/z value and at 40% of the maximum activation amplitude with activation q = 0.25 and an activation time of 30 ms.

For MALDI-TOF-MS analysis samples were dissolved in 0.1% aqueous TFA, and the solution was treated with a C18 ZipTip micropipette to remove salts. The desalted solution was mixed 1:1 (v/v) with a solution of α-cyan-4-hydroxycinnamic acid prepared in acetonitrile/water (50:50, v/v) containing 0.1% TFA. Aliquots of 1 μl of the mixture were spotted onto the stainless steel target of the MALDI instrument. The calibration was performed using peptide calibration standards (angiotensins I and II, substance P, and bombesin; m/z range, 1000–3150 Da). Positive and negative MALDI spectra were acquired in either linear or reflectron mode with a pulsed nitrogen laser (337 nm). In linear mode an acceleration voltage of 20 kV, a detector gain voltage of 1300 V, a pulsed ion extraction time of 350 ns, and a laser frequency of 5 Hz were applied. In the reflectron mode an acceleration voltage of 19 kV, a detector gain voltage of 1400 V, a pulsed ion extraction time of 150 ns, and a laser frequency of 5 Hz were applied. Mass spectra were acquired over the mass range of 700–6000 Da with the low mass cutoff of 500 Da, and 400 scans were averaged for each spectrum.

Data Analysis and Determination of Relative Abundances—Deconvolution of averaged ESI-MS spectra was automatically performed either by using the Bioworks Browser software provided with the Deca XP instrument or by MagTran 1.0 software (17). As previously made for the identification of some small naturally occurring fragments derived from Histatins (4), bPRP (and aPRP) fragments not previously identified were characterized by manual comparison of their experimental MS/MS spectra with the theoretical spectra generated using the MS-Product program available with ProteinProspector. The sequences used to generate theoretical mass spectra were those originating from the cleavage of known acidic and basic PRPs as suggested by the ExPASy FindPept program with a mass tolerance of ±0.5 Da, with and without phosphorylation, and with no specific enzymatic cleavage. A peptide match was considered positive when all the experimental m/z values with a relative abundance higher than 10% were present in the theoretical fragmentation spectrum and when the differences between the experimental and theoretical values were less than ±0.2 m/z.

An extracted ion current (XIC) strategy was used to selectively reveal salivary peptides described in this study. The m/z values used in the multiple XIC strategy were carefully selected to exclude values common to other closely eluting proteins (±0.5 m/z). The area of the XIC peaks, measured when the signal to noise ratio was at least 5, was used for semiquantitative estimation of relative peptide/protein abundances. For Statherin, P-B peptide, and major Histatins (Histatins 1, 3, 5, and 6) UV peak area at 278 nm was also measured.

Samples Analyzed—Informed consent was obtained from all the subjects involved. To undertake a comparative analysis as outlined in Fig. 1, samples were obtained from 10 healthy adult volunteers (five females, five males, 26–55 years old) of Pr, Sm/Si, and whole saliva (WS). Because of the origin of the samples statistical analysis was performed utilizing paired tests. Flow rates of Pr and Sm/Si measured during the sampling varied between subjects (from 0.1 to 0.3 ml/min) but was similar in the same subject.

Ten samples of EGPs from parotid glands were analyzed (five females, five males, 27–76 years old). For three of these samples (one female, two males, 57–60 years old) it was possible to compare the results with WS obtained from the same subjects. Three samples of EGPs from submandibular gland were also examined (one female, two males, 40–65 years old). For one sample (female, 58 years old) it was possible to compare the results with WS from the same subject.

RESULTS

Analysis of the RP-HPLC-ESI-MS profiles of whole saliva, parotid, and Sm/Si secretions and parotid and submandibular EGPs (Fig. 2) allowed determination of the relative abundance of any peptide and protein belonging to the families of aPRPs, bPRPs, Histatins, Stattherin, P-B peptide, and salivary Cystatins. The results are reported in Tables I, II, and IV–VIII. From a comparison of the composition of parotid and Sm EGPs, parotid and Sm/Si secretions, and whole saliva it was possible to locate various postsynthetic modifications of parotid and Sm/Si proteins as outlined in Fig. 1. A comparative analysis of parotid and Sm/Si saliva provided information about differences in secretion from the glands.

aPRPs—The aPRP complex is expressed by two loci named PRH1 (three common alleles: PIF, Db, and Pa) and PRH2 (two common alleles: PRP1 and PRP2) localized close to each other on chromosome 12p13. The products of alleles commonly found in Caucasians together with their post-translational cleavage products are schematically illustrated in Fig. 3. More detailed information can be found in a previous study (3), in the literature therein reported, and in the Swiss-Prot database (accession number P02810). The masses of the different isoforms, their frequency of occurrence in the granule and saliva preparations, and their concentration as determined semiquantitatively are reported in Table I. It is worthwhile to observe that the masses may differ by as little as 1 Da (e.g., PRP1 and PRP2). All the aPRP isoforms have a pyroglutamatic C-terminal residue and are diphosphorylated on Ser<sup>8</sup> and Ser<sup>22</sup> as shown in earlier studies (18). However, minor amounts of triphosphorylated isoforms (Ser<sup>8</sup>, Ser<sup>17</sup>, and Ser<sup>22</sup>) as well as mono- and non-phosphorylated derivatives are present in whole saliva as observed previously (3). Four isoforms (PRP1, PRP2, PIF-s, and Db-s) are partially cleaved at the RRPR<sup>13</sup>-sequence (residue 106 in PRP1, PRP2, and PIF-s; residue 127 in Db-s) generating the PRP3, PRP4, PIF-f, and Db-f truncated isoforms, respectively, and the 44-residue P-C peptide common to all the isoforms. The Pa isoform is not prone to the conservative cleavage presumably due to an Arg→Cys substitution at residue 103 (19). It is almost completely present in whole human saliva as a Pa dimer, with a mass value of 30,922 ± 3 Da, generated by the formation of a disulfide bridge. Finally a carboxypeptidase activity generates small amounts of isoforms lacking the C-terminal residues (Fig. 3).

In agreement with previous studies the results of this study
indicated that all the aPRPs were secreted both from Pr and Sm/SI glands (Table I). In fact, analysis of subjects carrying Pa and Db phenotypes revealed their presence in Sm/SI.

The circles in Table I (as well as in Tables II and IV–VIII) represent the approximate relative abundance determined for the different isoforms, fragments and derivatives (four closed circles, 45–60%; three closed circles, 30–45%; two closed circles, 15–30%; one closed circle, 2–15%; one open circle, traces (<2%). The detection frequencies (on the total number of samples) are reported in parentheses.

Due to the sporadic detection of Pa and Db phenotypes, we carried out comparative analyses of aPRP phosphorylation level and of relative abundance of the truncated isoforms only on PRP1 type isoforms (PIF-s, PRP1, and PRP2) (Fig. 3). There were no significant differences in the relative abundances of differently phosphorylated isoforms and in the ratios of entire and truncated isoforms in EGP, parotid, Sm/SI, and whole saliva. These data indicated that both phosphorylation and proteolytic cleavage occur before sequestration into secretory granules in agreement with previous observations (20).

Small percentages of non-phosphorylated truncated isoforms and triphosphorylated entire isoforms are usually detectable. In this respect, the different groups under analysis did not show significant differences.

Furthermore we were able to detect in one sample of whole saliva a small relative abundance of the pentaphosphorylated Pa dimer (average $M_r$, 31,002 ± 3) that derived from the dimerization of diphosphorylated and triphosphorylated Pa monomers. Interestingly EGP from the parotid gland of three subjects carrying the Pa phenotype showed high amounts of Pa monomer (about 40% of total Pa), whereas the monomer was almost undetectable both in parotid and whole saliva samples suggesting that dimerization occurs during granule maturation.

aPRP derivatives missing the C-terminal residue (des-Gln150 and des-Arg106) (Table I) were detected in a few granule and saliva samples and always in very small relative abundance (<2%) (Table I). As a consequence, the localization of aPRP C-terminal removal by carboxypeptidase action cannot be conclusively established from these data.

Paired comparison of parotid and Sm/SI secretions showed that the phosphorylation level was significantly higher in parotid saliva for both PRP1 (PIF-s, PRP1, and PRP2) and PRP3 (PIF-f, PRP3, and PRP4) (Fig. 3) isoforms. Phosphorylation
levels were calculated from the XIC peak area of the different phosphorylated forms considering the number of phosphate groups by the following ratio: ratio = 100 × (monophos.)/(monophos. + 2 × (diphos.) + 3 × (triphos.)).

The statistical analysis (paired t test) gave the following results: PRP1 type isoforms (n = 10): parotid = 4.2 ± 1.7 (mean ± S.D.), Sm/Sl = 7.7 ± 2.4 (mean ± S.D.), p < 0.00002; PRP3 type isoforms (n = 10): parotid = 3.9 ± 1.5 (mean ± S.D.), Sm/Sl = 8.8 ± 3.0 (mean ± S.D.), p < 0.00002. These results indicated that the kinase involved in aPRP phosphorylation is more active in parotid gland.

Parotid and Sm/Sl secretions showed another interesting and very significant difference in the relative abundance of all the truncated PRP3 isoforms compared with the total sum of aPRPs (PRP1 + PRP3): ratio = 100 × (total PRP3)/(total PRP3 + total PRP1). The statistical analysis gave the following results (n = 10): parotid = 25.6 ± 8.7 (mean ± S.D.), Sm/Sl = 35.4 ± 8.6 (mean ± S.D.), p < 0.0002 (paired t test). The higher percentages of truncated PRP3 type isoforms indicated a higher activity of convertase cleavage of aPRP in Sm/Sl gland than parotid gland.

The validity of the use of the XIC peak areas for semiquantitative evaluations of the relative abundances of different peptides and fragments is supported by an examination of 42

| Isoform or derivative         | M, aver. (theor.) exper. | Sm | Pr | EGPGS | Sm/Sl | GS | WS | Pr | Sm/Sl | WS |
|-------------------------------|--------------------------|----|----|-------|-------|----|----|----|-------|----|
| PRP1 type non-phos. (P02810)  | (15,354–15,355)          |    |    |       |       |    |    |    |       |    |
| PRP1 type monophos.           | 15,355 ± 2               |    |    | (0/10)| (1/10)|    |    |    |       |    |
| PRP1 type diphos.             | (15,435–15,435)          |    |    | (10/10)| (10/10)|    |    |    |       |    |
| PRP1 type triphos.            | (15,514–15,515)          |    |    | (10/10)| (10/10)|    |    |    |       |    |
| PRP1 type non-phos. (P02810)  | (15,594–15,595)          |    |    | (8/10)| (10/10)|    |    |    |       |    |
| Db-s monophos.                | (17,553)                 |    |    | (1/1) | (2/2) |    |    |    |       |    |
| Db-s diphos.                  | (17,633)                 |    |    | (1/1) | (2/2) |    |    |    |       |    |
| Db-s triphos.                | (17,713)                 |    |    | (1/3) | (3/4) |    |    |    |       |    |
| Pa 1-mer diphos.             | (15,462)                 |    |    | (3/3) | (1/4) |    |    |    |       |    |
| Pa 2-mer diphos.             | (30,762)                 |    |    | (1/3) | (3/4) |    |    |    |       |    |
| Pa 2-mer triphos.            | (30,842)                 |    |    | (3/3) | (4/4) |    |    |    |       |    |
| Pa 2-mer tetraphos.           | (30,922)                 |    |    | (3/3) | (4/4) |    |    |    |       |    |
| Pa 2-mer tetraphos. des-Gln^150 | (30,793)             |    |    | (3/3) | (4/4) |    |    |    |       |    |
| PRP3 type non-phos.           | (11,001–11,002)          |    |    | (5/10)| (4/10)| (1/3) | (8/10)| (9/14)|       |    |
| PRP3 type monophos.           | (11,081–11,082)          |    |    | (10/10)| (10/10)| (3/3)| (10/10)| (14/14)|       |    |
| PRP3 type diphos.             | (11,161–11,162)          |    |    | (10/10)| (10/10)| (3/3)| (10/10)| (14/14)|       |    |
| PRP3 type triphos. des-Arg^106 | (11,005–11,006)      |    |    | (2/10)| (3/10)| (0/3)| (3/10)| (5/14)|       |    |
| PRP3 type non-phos.           | (13,200)                 |    |    | (1/1) | (0/2) | (0/1)| (1/1)| (2/2)|       |    |
| PRP3 type monophos.           | (13,280)                 |    |    | (1/1) | (2/2) | (1/1)| (1/1)| (2/2)|       |    |
| PRP3 type diphos.             | (4,371)                  |    |    | (10/10)| (10/10)| (3/3)| (10/10)| (14/14)|       |    |
| PRP3 type triphos. des-Gln^14  | (4,243)                  |    |    | (8/10)| (8/10)| (3/3)| (10/10)| (13/14)|       |    |
| PRP3 type triphos. des-Pro^43-Gln^14 | (4,141)    |    |    | (0/10)| (0/10)| (0/3)| (0/10)| (6/14)|       |    |
whole saliva samples obtained from PIF homozygotic subjects. There was a highly significant linear correlation \((r = 0.970)\) of the XIC peak areas of the P-C peptide and PRP3 type truncated isoforms as would be expected for products originating by cleavage of a common parent protein. As expected, paired analysis of whole saliva consistently showed relative abundances between those of parotid and Sm/Sl glands.

**bPRPs**—bPRPs constitute the most complex family of salivary proteins both in terms of genetic variability and PTMs. Four loci, named **PRB1**–**4**, clustered on chromosome 12p13.2 near the aPRP loci are responsible for their synthesis (21). Each locus comprises at least three alleles, named **S** (small), **M** (medium), and **L** (large). A fourth allele, **VL** (very large), was described for PRB-1 and PRB-3 loci (22–25). Fig. 4 summarizes the present knowledge of bPRP loci including coding regions for the various secreted proteins. The sequences of the majority of b-PRPs are similar and highly repetitive (26). After loss of the signal peptide, all proproteins are submitted to multiple cleavages, phosphorylation, glycosylation, and C-terminal removal (5, 27, 28). In contrast to aPRPs, uncleaved proproteins of bPRPs are not detectable in saliva. A preliminary proteomics study of bPRPs has been published (5).

Table II lists the results obtained for bPRPs. The masses of the different isoforms, their frequency of occurrence in the granule and saliva preparations, and their concentration as determined semiquantitatively are reported. In agreement with the results obtained for aPRPs, results were consistent between different samples.

| Basic PRP (Swiss-Prot accession no.) | \(M\), aver. (theor.) exper. | Pr | GS | WS |
|--------------------------------------|-----------------------------|----|----|----|
| IB-1 (P04281)                        | (9,593)                     | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| IB-1 non-phos. (P04281)              | 9,593 ± 2                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| IB-1 des-Arg96 (P04281)              | 9,513 ± 2                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| IB-1 des93–96 (P04281)               | 9,097 ± 2                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| II-2 (22)                            | (7,609)                     | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| II-2 non-phos.                       | 7,609 ± 2                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| II-2 des-Arg76                      | 7,453 ± 2                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| II-2 des72–75                      | 7,113 ± 2                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| P-D (or IB-5) (P02813)               | (6,950)                     | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| P-F (or IB-8c) (P02812)              | 5,843 ± 1                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| IB-6 (P04280)                        | (11,518 ± 2)                | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| P-H (or IB-4) (P04280)               | 5,590 ± 1                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| P-J (pending def. charact. (5))     | 5,590 ± 1                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| P-E (or IB-9) (P02811)               | (6,024)                     | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| P-E des-Arg61 (P02811)              | 5,868 ± 1                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| IB-7                                 | 5,769 ± 1                   | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| IB-8a (Con 1° variant (24))          | (11,898)                    | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| 10434 (5)                            | 11,898 ± 3                  | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| 23462 (5)                            | 10,434 ± 2                  | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
| 29415 (5)                            | 23,462 ± 2                  | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |
|                                          | 29,415 ± 3                  | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] | [●●●● ●●●● ●●●●] |

Table II: bPRPs and derivatives detected in the different samples under analysis. Symbols and abbreviations are the same as in Table I. The detection frequency in the number of samples is reported in parentheses. def. charact., definitive characterization.
with earlier investigations bPRPs were not detected in secretory granules and saliva from Sm/Sl (21).

In addition to the bPRPs Table II includes three unknown proteins with mass values of 10,434 Da, 23,462 Da, and 29,415 Da that are frequently detectable in the HPLC cluster of bPRPs (5). They were tentatively included in the bPRP family on the basis of their chromatographic properties, absence of absorbance in the near ultraviolet region, and resistance to tryptic digestion. Moreover these proteins are only found in parotid secretion. These are all characteristics of bPRPs.

Protein composition of granules and parotid saliva did not show significant differences except for derivatives of II-2, IB-1, and P-E missing the C-terminal Arg residue (II-2 des-Arg75, IB-1 des-Arg96, and P-E des-Arg61) that are present in parotid saliva but not detectable in granules (Table II). Therefore in agreement with previous investigations, phosphorylation, glycosylation, and convertase cleavage of bPRPs all occur prior to granule storage (28). Based on analysis of the nucleotide coding regions for P-F, IB-8a, and IB-7 it is likely that these proteins arise by removal of one or more C-terminal residues from precursor proteins (25). Because P-F and IB-8a are present in parotid granules it appears that this process occurs prior to granule sequestration. No conclusion could be reached with regard to IB-7 because it was only present in one saliva sample. In contrast, the absence of II-2 des-Arg75, IB-1 des-Arg96, and P-E des-Arg61 from parotid saliva but the presence in whole saliva indicates that in these instances C-terminal removal takes place after secretion into the mouth. As expected, because of their parotid origin, the relative abundances of bPRPs compared with other saliva proteins are lower in whole saliva, which in addition to parotid saliva contains proteins from other salivary glands.

A number of small peptides (7–21 residues) that potentially could have originated from bPRPs were found in WS but not in parotid secretory granules or parotid saliva (Table III). These proline-rich peptides were found during an extensive characterization of all the masses detectable in the first 5–12 min of elution during the chromatographic separation of whole saliva. The structures of these peptides were established by...
applying selected ion monitoring (SIM) MS/MS strategies and were considered potentially to originate by cleavage of all known acidic and basic PRPs. This possibility was examined using the ExPASy FindPept program. Matches of peptide sequences with PRP sequences were confirmed by manual comparison of the experimental and all theoretical MS/MS spectra using the MS-Product program with ProteinProspector. When possible, MS/MS fragmentation was simultaneously induced on mono- and bicharged ions (Fig. 5). The monocharged ion ([M + H]+) at 874.5 m/z, eluting at about 9.0 min and consistently observed in WS samples, is potentially attributable to more than 100 basic and acidic PRP fragments. A manual comparison of the theoretical MS/MS spectra of all the potential candidates with the experimental MS/MS spectra restricted the possibility to the two peptides GPPPPGKPQ or GPPPPQGPQ (the latter is a fragment of aPRP). To discriminate between them an MS3 experiment was necessary with a secondary SIM centered on internal fragments. Analysis of the MS3 fragmentation spectra allowed recognition of the internal fragments with m/z values of 477.3 and 380.3 as PPPPGK and PPQG, respectively, eliminating any ambiguity. For all the other peptides reported in Table III the first MS/MS fragmentation pattern allowed confirmation of their structures. Table III also shows the number of times the proline-rich fragment is present in the bPRP sequences. Due to the presence of multiple repeats it is impossible to establish from which protein the peptide originated. The presence of these fragments implied a common cleavage at Q→G sites sometimes followed by the removal of one or more C-terminal residues. Cleavage at this site has also been implied in the generation of some fragments of salivary peptides non-covalently bonded to enamel surface (29).

Histatins—Two Histatins, Histatin 1 and Histatin 3, are encoded by two genes located on chromosome 4q13 (30). Histatin 1 is a 38-residue peptide, whereas Histatin 3 has a sequence of 32 residues and is very similar to Histatin 1 (30, 31) (Fig. 6). Histatin 1 is phosphorylated on Ser2 (30), and the tyrosines are partly sulfated (1).

The masses of Histatins and fragments derived from them, their frequency of occurrence in the granule and saliva preparations, and their concentration as determined semiquantitatively are reported in Table IV. As expected, there were no differences in the relative concentration of Histatin 3 in secretion granules and saliva from the corresponding gland. Moreover the relative concentrations of some of the Histatin 3 fragments (1–25, 1–24, 26–32, 28–32, and 29–32) were the same in granule and saliva samples indicating that they are generated either before or during sequestration into the secretory granules. Although some Histatin 3 fragments were absent or present in very small amounts in secretory granules (Table V) they could be detected in increasing amounts in parotid, Sm/Sl, and whole saliva (Histatin3 1–13, 1–12, 1–11, 5–13, 5–12, 6–12, 6–11, 7–13, 7–11, 12–25, 12–24, 13–24, 14–25, 14–24, 15–25, and 15–24). Thus it appears that at least part of these fragments is generated after granule release into the salivary gland lumen. The observed peptide pattern supports a proposed sequential processing of Histatin 3 (4).

A comparison of parotid and Sm/Sl secretions based on the ratios of relative abundance of Histatin 3, Histatin 5, and Histatin 6 and total Histatin showed no significant differences, suggesting a comparable activity of the proteinases involved in Histatin 6 and Histatin 5 genesis in both glands (4). In contrast, the higher concentration of Histatin3 1–13, 1–12, 1–11, 12–25, 12–24, 13–24, 14–25, 14–24, and 15–24 in parotid than Sm/Sl saliva shows that parotid saliva is the main source of these fragments in whole saliva. Interestingly a comparison of parotid and Sm/Sl saliva
A statistical analysis was done based on the following (non-phosphorylated Histatin 1) in the various samples (Table III). Histatin 1 sulfation (Table VI). Histatin 1 derivatives with a theoretical; S, small; M, medium; L, large. showed that the ratio of relative abundance of Histatin 3 (or Histatin 5 or Histatin 6) to total Histatin 1 (Histatin 1 + Histatin 2 (non-phosphorylated Histatin 1)) is significantly higher in parotid than in Sm/Sl saliva, 0.51 (mean ± S.D.), Sm/Sl mean 0.55 (mean ± S.D.), p < 0.002; Histatin 6 (n = 10): parotid = 0.54 ± 0.34 (mean ± S.D.), Sm/Sl mean = 0.18 ± 0.07 (mean ± S.D.), p < 0.005. Because the abundance of Histatin 1 plus Histatin 2 was found to be similar in Pr and Sm/Sl saliva, the differences in the ratios were ascribed to an increased secretion of Histatins 3, 5, and 6 from the parotid gland. This is in agreement with previous investigations (2, 36). It is also apparent that phosphorylation of Statherin occurs prior to granule storage because the relative concentrations of variously secretory granules and the corresponding secretory granules. The detection of sulfated Histatin 1 in secretory granules in a ratio of relative abundances comparable with those observed in Sm/Sl and whole saliva indicated that Histatin 1 sulfation, similar to phosphorylation, is a PTM occurring prior to granule storage.

Statherin and P-B Peptide—Statherin is an unusual 43-residue phosphopeptide (phosphorylated on Ser2 and Ser3) found in human saliva (Fig. 7) (32) that is involved in oral cavity calcium ion homeostasis (33). Its gene (STATH) is localized on chromosome 4q13.3 (Ref. 34; Ensembl ENSG00000126549). Usually P-B peptide (Fig. 7) is included in the bPRP family. However, many characteristics of this peptide suggest a functional relationship with Statherin. Differently from bPRPs, P-B peptide is the product of PROL3 (Ensembl ENSG00000171201) localized on chromosome 4q13.3 very close to the Statherin gene (35). A similarity analysis performed by the Lalign program showed that the two peptides share more than 47% identity in the last 20 C-terminal residues.

The results of this study, summarized in Table VII, show that Statherin is synthesized in both the parotid and submandibular glands in agreement with previous investigations (2, 36). It is also apparent that phosphorylation of Statherin occurs before granule storage because the relative concentrations are the same in secretory granules and the corresponding glandular saliva. The vast majority of Statherin is diphosphorylated, and there is no evidence of dephosphorylation after secretion because the relative concentration of variously phosphorylated Statherin is the same in glandular and whole saliva.
paired to GPPPPGKPQ (see Table III). This derived was not detected in granules. Similarly small amounts of P-B des1–5 were found in all three types of saliva but not in secretory granules suggesting that also this N-terminal cleavage occurs after secretion.

In contrast to other bPRPs, P-B, being the product of a different gene (35), is present in both parotid and Sm/Sl saliva. Indeed the relative abundance of P-B is higher in Sm/Sl saliva: ratio = (P-B + derivatives)/Statherin + derivatives). The statistical analysis gave the following results (n = 10): parotid = 21.9 ± 26.6 (mean ± S.D.), Sm/Sl = 4.78 ± 4.44 (mean ± S.D.), p < 0.05 (paired t test).

The N-terminal removal generating Statherin des-Asp^1 is probably an event occurring after granule secretion because this derivative was not detected in granules. The statistical analysis gave the following results (n = 10): parotid = 0.52 ± 0.26 (mean ± S.D.), Sm/Sl = 0.98 ± 0.37 (mean ± S.D.), p < 0.002 (paired t test).

A similar calculation based on UV absorption at 278 nm showed that this difference is derived from an increased P-B secretion in Sm/Sl saliva. Cyclo-Statherin-Q37, recently identified as a Statherin derivative formed by transglutamination of Lys^6 and Gin^37 (37) was detected only and at very small relative abundance in WS samples, confirming that the site of action of transglutaminase 2 is in the oral cavity.

Cystatins—The major salivary Cystatins belong to family 2 of Cystatins (38) comprising Cystatins S, SN, and SA. Cystatin S may be phosphorylated on Ser^3 (Cystatin S1) or diphosphorylated on Ser^1 and Ser^3 (Cystatin S2). The loci producing all Cystatin S are closely clustered on chromosome 20p11.21 together with the loci of Cystatin C and D, named CST1–5. The masses of Cystatins, their frequency of occurrence in the granule and saliva preparations, and their concentration as determined semiquantitatively are reported in Table VIII.

All the Cystatins except Cystatins C and D are present in much lower concentrations in parotid than Sm/Sl saliva according to results of other researchers (39). In contrast to the other salivary proteins investigated, Cystatins were present in very low concentrations or absent from both parotid and Sm/Sl secretory granules. This low relative abundance compared with the high relative abundances observed in Sm/Sl and whole saliva suggests an "S type" Cystatin secretion that is not granule-mediated in agreement with studies that localized the transcript of Cystatin S as well as Cystatin S protein in cytoplasm of serous acinar cells of Sm glands (40).

Because of the sporadic presence and low abundance of Cystatins S, S1, and S2 in parotid saliva it was not possible to calculate the relative phosphorylation of Cystatin S type in parotid saliva, thereby precluding a comparison with Sm/Sl saliva. In Sm/Sl and whole saliva the relative abundance of Cystatin S1 (monophos.) is 60–70% of total Cystatin S forms, Cystatin S2 (diphos.) accounts for 20–30%, and Cystatin S accounts for about 5%.

Cystatin C was found sporadically in parotid, Sm/Sl, and whole saliva, but it was absent from secretory granules.
whereas Cystatin D was not found in any sample. Thus Cystatins C and D may have a different origin than the other salivary Cystatins.

**DISCUSSION**

The proteomics comparison performed in this study allowed us to establish that salivary peptides and proteins are submitted to a very complex and diversified maturation during and after secretion. The methodological strategy applied was centered on XIC searches of salivary proteins/peptides and their post-translational modification products characterized previously. This XIC approach made it possible to avoid interferences due to sample contamination. Moreover XIC peak area was utilized for semiquantitative measurements of the relative abundances of the peptides and proteins under investigation (41).

This study was mainly devoted to obtaining information about site of phosphorylation, sulfation, and endo- and exo-proteinase cleavages. The method utilized was not suitable for the study of bPRP glycosylation due to the great heterogeneity of the ESI spectra observed for these salivary proteins (5). However, like bPRPs, glycosylated PRPs were only detected in parotid samples.

Phosphorylation and sulfation, after removal of the signal peptides, are the first PTMs occurring probably in the cis-Golgi compartment. A unique kinase confined in the Golgi compartment (Golgi casein kinase) is probably responsible for the phosphorylation of all salivary peptides (20, 42). From the phosphorylation levels of Histatin 1, aPRPs, and Statherin, the present study shows that the kinase involved is more active in parotid than in Sm/Sl glands. This consistent observation is further support for a unique kinase acting on all salivary peptides.

**Fig. 6.** Sequences of Histatin 1 and Histatin 3 and schematic representation of the PTMs generating the derivatives and fragments reported in Table IV, peptid., peptidase.

**TABLE IV**

| Peptide or derivative | [M + H⁺]⁺ (theor.) exp. | Pr | Sm | Sm/Sl | WS |
|-----------------------|-------------------------|----|----|-------|----|
| Histatin 3 (P15516)   | (4062.4)                | ●●●● | ●●●● | ●●●● | ●●●● |
|                       | 4062.2 ± 0.4            | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 1–25   | (3192.5)                | ●●●● | ●●●● | ●●●● | ●●●● |
| (Histatin 6)          | 3192.4 ± 0.3            | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 1–24   | (3036.3)                | ●●●● | ●●●● | ●●●● | ●●●● |
| (Histatin 5)          | 3036.5 ± 0.3            | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 26–32  | (888.4)                 | ●●●● | ●●●● | ●●●● | ●●●● |
| Histatin 3 fr. 27–32  | (801.3)                 | ●●●● | ●●●● | ●●●● | ●●●● |
| Histatin 3 fr. 28–32  | (687.3)                 | (8/10) | (7/10) | (2/3) | (9/10) | (12/14) |
| Histatin 3 fr. 29–32  | (524.2)                 | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 30–32  | (524.2)                 | (10/10) | (10/10) | (1/3) | (10/10) | (14/14) |

*For Hist-3, -6, and -5, average mass; for the fragments, [M + H⁺]⁺ monoisotopic mass.

whereas Cystatin D was not found in any sample. Thus Cystatins C and D may have a different origin than the other salivary Cystatins.

**TABLE IV**

| Peptide or derivative | [M + H⁺]⁺ (theor.) exp. | Pr | Sm | Sm/Sl | WS |
|-----------------------|-------------------------|----|----|-------|----|
| Histatin 3 (P15516)   | (4062.4)                | ●●●● | ●●●● | ●●●● | ●●●● |
|                       | 4062.2 ± 0.4            | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 1–25   | (3192.5)                | ●●●● | ●●●● | ●●●● | ●●●● |
| (Histatin 6)          | 3192.4 ± 0.3            | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 1–24   | (3036.3)                | ●●●● | ●●●● | ●●●● | ●●●● |
| (Histatin 5)          | 3036.5 ± 0.3            | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 26–32  | (888.4)                 | ●●●● | ●●●● | ●●●● | ●●●● |
| Histatin 3 fr. 27–32  | (801.3)                 | ●●●● | ●●●● | ●●●● | ●●●● |
| Histatin 3 fr. 28–32  | (687.3)                 | (8/10) | (7/10) | (2/3) | (9/10) | (12/14) |
| Histatin 3 fr. 29–32  | (524.2)                 | (10/10) | (10/10) | (3/3) | (10/10) | (14/14) |
| Histatin 3 fr. 30–32  | (524.2)                 | (10/10) | (10/10) | (1/3) | (10/10) | (14/14) |

*For Hist-3, -6, and -5, average mass; for the fragments, [M + H⁺]⁺ monoisotopic mass.
Sulfation specifically involves the last four tyrosines (Tyr27, Tyr30, Tyr34, and Tyr36) of five present in the Histatin 1 sequence. The specificity of this PTM both in terms of peptide (only Histatin 1) and gland localization (submandibular) is puzzling and will require further studies (1). In any case, from our results, Histatin 1 sulfation is a PTM occurring at the Golgi level (see “Results,” Table VI, and Fig. 6). Tyrosine sulfation is a widespread PTM occurring by the transfer of sulfate from

### Table V

*Other Histatin 3 fragments detected in the different samples under analysis*

Symbols and abbreviations are the same as in Table I. The detection frequency in the number of samples is reported in parentheses.

| Peptide or derivative          | [M + H]+ (theor.) exper. | Pr          | Sm EGP | Sm/Sl EGP | WS
|-------------------------------|--------------------------|-------------|--------|----------|-----
| Histatin 3 fr. 1–13           | (1619.8)                 | (3/10)      | (0/3)  | (8/10)   | (14/14) |
| Histatin 3 fr. 1–12           | (1491.8)                 | (3/10)      | (0/3)  | (10/10)  | (14/14) |
| Histatin 3 fr. 1–11           | (1335.7 ± 0.2)           | (4/10)      | (0/3)  | (0/10)   | (14/14) |
| Histatin 3 fr. 5–13           | (1209.7 ± 0.2)           | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 5–12 (or fr. 6–13) | (1081.6 ± 0.2)     | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 5–11           | (925.5 ± 0.2)            | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 6–12           | (953.1 ± 0.2)            | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 6–11 (or fr. 7–12) | (796.9 ± 0.2)     | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 7–13           | (641.3 ± 0.2)            | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 7–11           | (641.3 ± 0.2)            | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 12–25          | (1875.0)                 | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 12–24 (or fr. 13–25) | (1718.9)              | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 13–24          | (1562.8 ± 0.2)           | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 14–25          | (1590.8 ± 0.2)           | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 14–24          | (1434.7 ± 0.2)           | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 15–25          | (1443.7 ± 0.2)           | 0           | 0      | 0        | 0    |
| Histatin 3 fr. 15–24          | (1287.6 ± 0.2)           | 0           | 0      | 0        | 0    |

* Monoisotopic.

### Table VI

*Histatin 1 and derivatives detected in the different samples under analysis*

Symbols and abbreviations are the same as in Table I. The detection frequency in the number of samples is reported in parentheses.

| Isoform or derivative          | M, aver. (theor.) exper. | Pr          | Sm EGP | Sm/Sl EGP | WS
|-------------------------------|--------------------------|-------------|--------|----------|-----
| Histatin 1 (P15515)           | (4928.2)                 | (10/10)     | (3/3)  | (10/10)  | (14/14) |
| Histatin 1 non-phos. (Histatin 2) | (4848.2)              | (10/10)     | (3/3)  | (10/10)  | (14/14) |
| Histatin 1 monosulfated       | (5008.2 ± 0.5)           | (0/10)      | (0/3)  | (2/3)    | (8/14) |
| Histatin 1 bisulfated         | (5088.2 ± 0.5)           | (0/10)      | (0/3)  | (2/3)    | (8/14) |
| Histatin 1 trisulfated        | (5168.2 ± 0.5)           | (0/10)      | (0/3)  | (2/3)    | (8/14) |
| Histatin 1 tetrasulfated      | (5248.2 ± 0.5)           | (0/10)      | (0/3)  | (2/3)    | (8/14) |
the universal sulfate donor adenosine 3' phosphate 5' phosphosulfate to the hydroxyl group of tyrosine residues (43). However, as we are aware, probably due to the analytical difficulties inherent to the detection of minor amounts of sulfoderivatives, only 37 human sulfated peptides are today reported in Swiss Prot/TrEMBL, and Histatin 1 represents the second example of a human phosphosulfopeptide detected in vivo. Two different tyrosylprotein sulfotransferases (TPST1 and TPST2) have been identified (44), and all mammalian cell types studied to date express both. In agreement with our results a TPST has been specifically detected at the level of the Golgi membranes of rat submandibular glands (45), and Statherin has been recently recognized as one potential substrate of salivary TPST in vitro (46). However, at the sensitivity

![Diagram of trafficking and PTMs of human salivary peptides](https://via.placeholder.com/150)

**TABLE VII**

| Peptide or derivative | Mr aver. (theor.) exper. | Pr | Sm | GEP | Sm/Sl | GS | WS |
|-----------------------|--------------------------|----|----|-----|-------|----|----|
| Statherin (P02808)    | (5379.7)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5380.0 ± 0.5          | (10/10)                  | (10/10) | (3/3) | (10/10) | (14/14) |
| Statherin monophos.   | (5299.7)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5299.9 ± 0.5          | (10/10)                  | (10/10) | (3/3) | (10/10) | (14/14) |
| Statherin non-phos.   | (5219.7)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5220.0 ± 0.5          | (8/10)                   | (8/10) | (3/3) | (10/10) | (12/14) |
| Statherin des-Phe43 (or SV-1) | (5232.5) | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5232.4 ± 0.5          | (10/10)                  | (10/10) | (3/3) | (10/10) | (14/14) |
| Statherin des-Thr42-Phe43 | (5131.4) | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5131.2 ± 0.5          | (8/10)                   | (6/10) | (1/3) | (9/10)  | (14/14) |
| Statherin des-Asp1     | (5264.6)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5264.7 ± 0.5          | (0/10)                   | (4/10) | (0/3) | (7/10)  | (12/14) |
| Statherin des6–15 (or SV-2) | (4148.2) | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 4148.0 ± 0.4          | (0/10)                   | (6/10) | (0/3) | (6/10)  | (6/14)  |
| Statherin des1–9       | (4127.6)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 4127.8 ± 0.4          | (6/10)                   | (8/10) | (1/3) | (10/10) | (14/14) |
| Statherin des1–10      | (3971.4)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 3971.3 ± 0.4          | (2/10)                   | (6/10) | (0/3) | (8/10)  | (14/14) |
| Statherin des1–13      | (3645.0)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 3645.0 ± 0.4          | (7/10)                   | (8/10) | (1/3) | (9/10)  | (14/14) |
| Cyclo-Statherin-Q37    | (5362.7)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5362.9 ± 0.5          | (0/10)                   | (0/10) | (0/3) | (0/10)  | (14/14) |
| P-B peptide (P02814)   | (5792.7)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5792.9 ± 0.5          | (10/10)                  | (10/10) | (3/3) | (10/10) | (14/14) |
| P-B peptide des1–5    | (5215.1)                 | ●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|●●●●●|
| 5215.0 ± 0.5          | (0/10)                   | (5/10) | (0/3) | (8/10)  | (14/14) |

**Fig. 7.** Sequences of Statherin (Stath.) and P-B peptide and schematic representation of the PTMs generating the derivatives and fragments reported in Table VII.
 Trafficking and PTMs of Human Salivary Peptides

**Table VIII**

| Protein (Swiss-Prot accession no.) | M, aver. (theor.) exper. | Pr | EGP | GS | Sm EGP | Sm/SI GS | WS |
|-----------------------------------|--------------------------|----|-----|----|--------|----------|----|
| Cystatin S (P01036)              | (14,185)                 | 14,186 ± 2 | (1/10) | (3/10) | (1/3) | (10/10) | (14/14) |
| Cystatin S1 (P01036)             | (14,265)                 | 14,266 ± 2 | (1/10) | (7/10) | (1/3) | (10/10) | (14/14) |
| Cystatin S2 (P01036)             | (14,345)                 | 14,346 ± 2 | (1/10) | (5/10) | (1/3) | (10/10) | (14/14) |
| Cystatin SN (P01037)             | (14,312)                 | 14,313 ± 2 | (2/10) | (10/10) | (2/3) | (10/10) | (14/14) |
| Cystatin SN oxidized (P01037)    | (14,328)                 | 14,328 ± 2 | (0/10) | (0/10) | (1/3) | (10/10) | (14/14) |
| Cystatin SA (P09228)             | (14,346)                 | 14,347 ± 2 | (0/10) | (7/10) | (2/3) | (10/10) | (14/14) |
| Cystatin C (P01034)              | (13,343)                 | 13,344 ± 2 | (0/10) | (3/10) | (0/3) | (4/10) | (6/14) |
| Cystatin D (P28325)              | (13,854)                 | (0/10) | (0/10) | (0/3) | (0/10) | (0/14) |    |

level of our apparatus, no sulfo-Statherin was detectable in the samples analyzed.

The majority of the consensus sequences observed in the cleavage sites are potentially recognized by enzymes identifiable in the convertase families (47). Differently from the unique enzymes potentially involved in phosphorylation and sulfation and despite similarities in the consensus sequences observed, the results of this study suggest that the different cleavages of salivary peptides are due to the action of a complex set of endo- and exopeptidases. The convertases responsible for acidic and basic PRPs cleavages act before granule storage. Moreover bPRP convertase is active only in parotid gland, and it is responsible for a complete digestion of the proprotein, whereas aPRP convertase is present in parotid and Sm/Sl glands where it is more active, and it is responsible for a partial cleavage of the proprotein. Two interesting previous studies of Bennick and co-workers (28, 48) addressed the characterization of these proteinases.

On the contrary, the proteinase(s) responsible for the first cleavage of Histatin 3 (Fig. 6) seems to work during granule maturation, and until now, nothing was known about its nature. Recently Helmerhorst and Oppenheim (49) proposed an interesting mechanism model explaining the constant ratio of Hst-3 to Hst-5. Also for this proteinase the presence of two Arg residues separated by two amino acid residues seems a mandatory requirement for the enzyme recognition, because Histatin 1, lacking the Arg26 residue present in Hst-3 (Fig. 6), is not cleaved.

The removal of a C-terminal residue by specific carboxypeptidases following a convertase cleavage is an event widespread in many secretory processes (50). This removal can be observed also during secretion of salivary peptides. Differently from the N-terminal removal of Statherin occurring after granule secretion and the C-terminal removal of some bPRPs occurring before granule storage, the majority of C-terminal removals should occur during granule maturation according to the abundant presence of the truncated products detected in the analysis performed on granules preparations. One of the most relevant removals, in terms of relative abundances, is that involving the C-terminal Arg-25 of Hst-3 fr. 1–25 (Hst-6), generating Hst-3 fr. 1–24 (Hst-5), which is probably linked to the specific antifungal activity of the latter peptide (30, 31).

After granule secretion Histatin 5 and Histatin 6, but not Histatin 3, are submitted to further cleavages at other minor sites (Fig. 6) by the action of unknown endo- and exopeptidases, generating a cascade of fragments. The results of this study indicate that this process occurs mainly during parotid secretion because these fragments were well detectable only in saliva of this gland (and in whole saliva).

Finally other unknown peptidases of endogenous origin are active in the oral cavity. bPRPs, which are their main substrate, are cleaved soon after secretion into the mouth. The main cleavage is at some Q↓G sites. This proteinase action generates a lot of small proline-rich peptides originating from repeats present in the parent proteins that were consistently found in almost all the WS samples analyzed. It is interesting to underline that some of these fragments have been detected in a recent study by Vitorino et al. (29) as recurrent non-covalently bound components of the enamel surface. This finding might provide a functional significance to this final bPRP maturation.

Except for phosphorylation, S type Cystatins seem not to undergo further PTMs unlike other salivary peptides. The results of this study show that it would be fruitful to investigate their secretion pathway, which cannot be granule-mediated.

Finally this study can serve as a reference for further clinical proteomics studies using salivary secretion for the diagnosis and prognosis of local and systemic pathologies. It not only
confirms that the secretion of salivary peptides follows a well-defined pathway where many and well-localized enzymatic activities contribute to a complex maturation but also demonstrates qualitative and quantitative differences in the secretion of the main salivary glands. Differences and similarities are summarized in Table IX. They suggest different molecular requirements of different areas of the oral cavity that are a stimulus for further investigations.

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