A Cascade of 24 Histatins (Histatin 3 Fragments) in Human Saliva

SUGGESTIONS FOR A PRE-SECRETORY SEQUENTIAL CLEAVAGE PATHWAY*

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The systematic search by tandem mass spectrometry of human saliva from four different subjects, of 136 possible fragments originated from histatin 3, allowed the detection of 24 different peptides. They include, with the exception of histatin 4, all the known histatin 3 fragments, namely histatins 5–12 and the peptides corresponding to 15–24, 26–32, 29–32 residues, and 13 new fragments corresponding to 1–11, 1–12, 1–13, 5–13, 6–11, 6–13, 7–11, 7–12, 7–13, 14–24, 14–25, 15–25, and 28–32 residues of histatin 3. On the contrary, none of 119 possible fragments of histatin 1, including histatin 2, was detected. The results suggest that the genesis of histatin 3-related peptides, being under the principal action of trypsin-like activities, is probably not a random process but rather follows a sequential fragmentation pathway. Lack of detection of C-terminal fragments, with the exception of 26–32, 28–32, and 29–32 fragments, suggested that arginine 25 should be the first cleavage site, generating histatin 6 and 26–32 fragments. The genesis of 28–32 and 29–32 fragments and histatin 5 should implicate a subsequent exo-protease action. Similarly, lack of detection of fragments having Lys-5 and Arg-6 at the N terminus and Arg-25 at the C terminus strongly suggested that sequences KRKF (11–14 residues) and AKR (4–6 residues) should be the second and the third cleavage sites, respectively. Lys-17 and Arg-22 are not cleaved at all.

Histatins are a class of salivary peptides probably present only in higher primates (1), deriving their name from the high histidine content (2, 3). The powerful antifungal action of this class of peptides stimulated intense investigations concerning their properties, activity, structure, and secretion (4). Until now, only two human genes, *HTN1* (*HIS1*) and *HTN2* (*HIS2*), localized on chromosome 4q13, have been recognized as responsible for their synthesis (1, 5). The products of these two genes are histatin 1 and histatin 3, respectively. The former is a peptide of 38 amino acids, phosphorylated at Ser-2, whereas the latter, 32 amino acid long with a sequence very similar to histatin 1, is not phosphorylated. Many other peptides of this family have been identified in human saliva, all sharing a sequence common to the two parent peptides. Although different classifications have been proposed, the current preferred nomenclature derives from the study of Troxler *et al.* (6), who identified in human saliva a peptide corresponding to the C-terminal 26 residues of histatin 1, named histatin 2, and nine peptides, all related to the sequence of histatin 3 and named histatins 4–12. Except for histatin 2, the other minor histatins likely originated by proteolytic cleavages from histatin 3. Among them, histatin 5, showing a sequence identical to the first 24 amino acids of histatin 3, represents the major fragment because it is present in human saliva at a higher concentration than the other fragments. Moreover, it appears to display the highest specific activity against *Candida albicans* species with respect to all the other histatins (7). By utilizing different separation protocols, other researchers identified several small peptides originating from histatin 3 (8). The sequences of histatins 1–12 and other fragments previously detected by other researchers are reported in Tables I and II. Studies by Xu *et al.* (9) demonstrated that histatins 2 and 4, not present in freshly collected parotid saliva, appeared only after autoproteolytic histatin degradation. Therefore, the main questions arising from these data concern the site and timing of the cleavage processes. Although some experimental evidence has suggested that cleavage events should be confined either before or during glandular secretions, the post-secretion involvement of endogenous and exogenous proteases present in the oral cavity cannot be completely excluded, due to the time-consuming experimental scheme utilized for purification. In this respect, relevant results were obtained by Xu *et al.* (10), who submitted several synthetic histatins to the action of salivary proteases *in vitro* and identified the different products of proteolysis. Fragments were detected only after a relatively long incubation time, and they were different from known histatins. Because the biosynthetic origin of histatin-derived peptides may be important to their function, interest in the synthesis of these peptides continues. In order to obtain further insight into the genesis of the fragments, we applied the high-throughput analytical potential inherent to electrospray multidimensional mass spectrometry-high performance liquid chromatography, which allows a fast and reliable analysis of complex protein mixtures without time-consuming pre-separation steps. Whole human salivary samples, a few seconds after collection, were treated with an acidic solution and, after centrifugation, immediately analyzed by RP-HPLC[1] ESI-

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[1] The abbreviations used are: RP-HPLC, reverse phase-high pressure liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; SIM, selected ion monitoring; MS/MS, tandem mass spectrometry; TIC, total ion current.
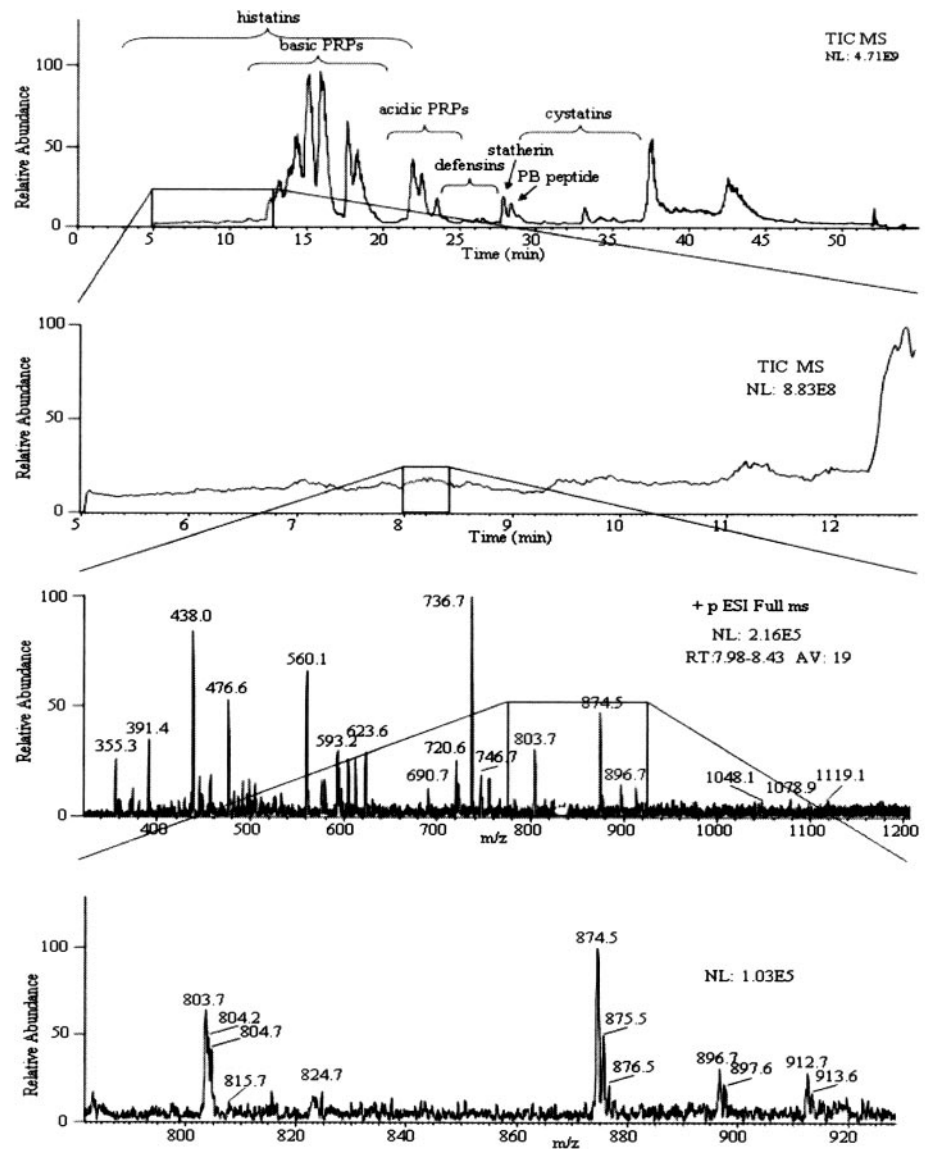
FIG. 1. *1st panel* (from the *top*), typical total ion current (*TIC MS*) profile obtained by RP-HPLC-ESI-MS analysis of human saliva. Elution ranges of the principal classes of salivary peptides/proteins soluble in acidic solution are indicated. *2nd panel,* enlargement of the 5–12-min elution range. *3rd panel,* averaged ESI mass spectrum (300–1200 *m/z* range) recorded in the elution range 7.98–8.43 min. *4th panel,* enlargement of the mass spectrum reported in the *third panel* (780–930 *m/z*) showing the presence of some mono- and di-charged ions.

MS^n. This procedure reduced the time during which artifacts can originate by salivary proteolysis, and thus it supported the assumption that the results obtained may represent a reliable picture of peptides present in the oral cavity immediately after secretion. This approach permitted detection and identification of several unknown peptides derived from histatin 3, and it provided the suggestion that a sequential cleavage pathway operates during the biosynthesis of these peptides.

MATERIALS AND METHODS

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

Apparatus—The HPLC-ESI-MS apparatus was a ThermoFinnigan (San Jose, CA) Surveyor HPLC connected by a T splitter to a PDA diode-array detector and to Xcalibur LCQ Deca XP Plus mass spectrometer. The mass spectrometer was equipped with an electrospray ion source (ESI). The chromatographic column was a Vydac (Hesperia, CA) C_8 column, with a 5-μm particle diameter (column dimensions 150 × 2.1 mm).

Sample Collection and Treatment—Whole human saliva was collected between 2 and 4 p.m. from four normal adult informed volunteers according to a standard protocol (11). Collection time was standardized in order to reduce concentration variability connected to circadian rhythms of secretion. An acidic solution (0.2% trifluoroacetic acid) was immediately added to salivary samples in 1:1 v/v ratio, and the solution was centrifuged at 8000 × g for 5 min. After centrifugation, the supernatant was separated from the precipitate and immediately analyzed by HPLC-mass spectrometry. The following solutions were utilized for reversed-phase chromatography: eluent A, 0.056% aqueous trifluoroacetic acid, and eluent B, 0.05% trifluoroacetic acid in acetonitrile/water 80:20 (v/v). The gradient applied was linear from 0 to 55% in 40 min, at a flow rate of 0.30 ml/min. The T splitter addressed a flow rate of about 0.20 ml/min toward the diode array detector and a flow rate of 0.10 ml/min toward the ESI source. The diode array detector was usually set at a wavelength of 214–276 nm. Mass spectra were collected every 3 ms in the positive ion mode. MS spray voltage was 4.50 kV, and the capillary temperature was 220 °C. Tandem MS experiments were performed by detection of parent ions with a peak width of 2–4 *m/z* values with 40–60% of the maximum activation amplitude.

Data Analysis—Deconvolution of averaged ESI mass spectra was automatically performed either by the software provided with the Deca-XP instrument (Bioworks Browser) or by MagTran 1.0 (13) software. Mass values obtained were compared with average theoretical values using PeptideMass and FindPept programs available at the Swiss-Prot Data Bank (us.expasy.org/tools), where human histatin 1 and histatin 3 are coded as P15515 and P15516, respectively. Theoretical tandem MS spectra were generated by utilizing the MS-Product program, available at the Protein Prospector site (prospector.ucsf.edu/).

RESULTS

Whole human saliva was collected between 2 and 4 p.m. in order to reduce variations connected to circadian rhythms (11,

FIG. 2. **TIC and SIM profiles registered during a tandem MS experiment were performed to search and confirm the sequence of fragments 1–11, 1–12, and 1–13 originated from histatin 3.** The SIM profiles correspond to the search of the di-charged ions at 668.7 *m/z* (1–11 fragment), 746.7 *m/z* (1–12 fragment), and 811.3 *m/z* (1–13 fragment) values, respectively.
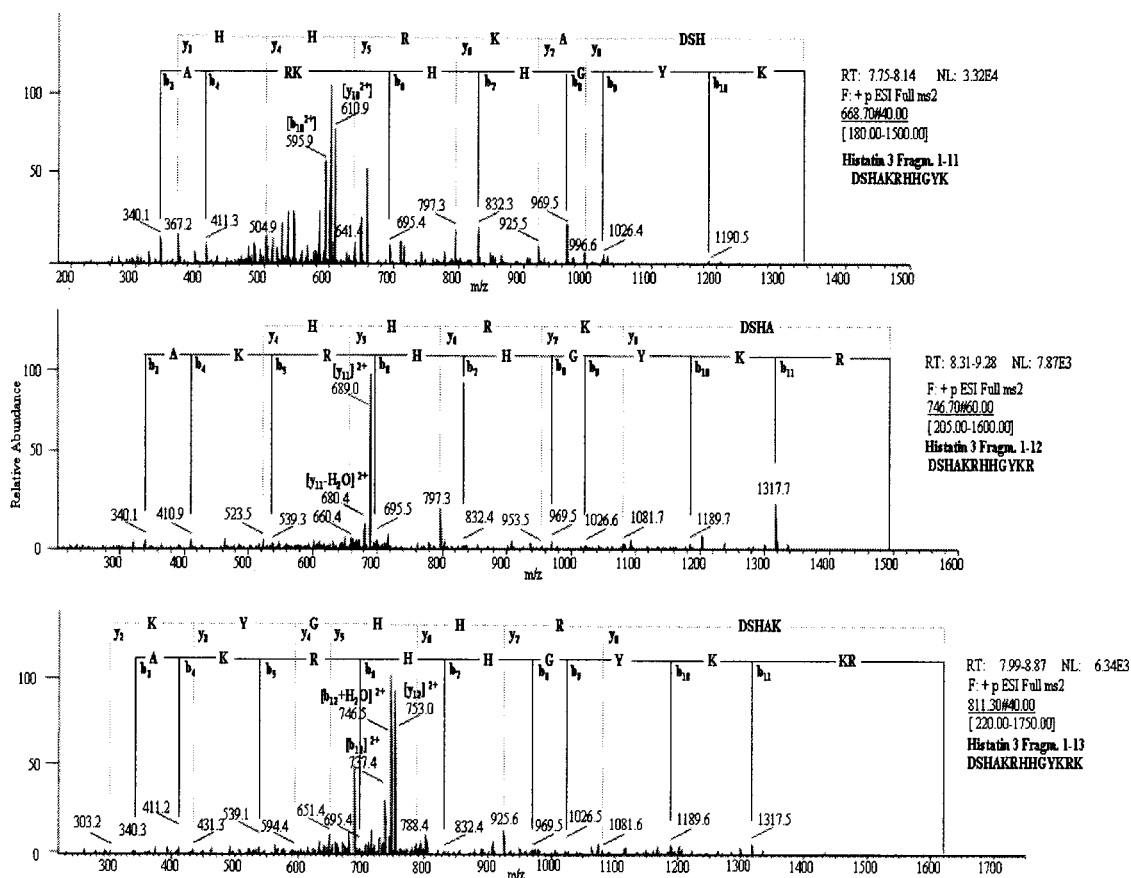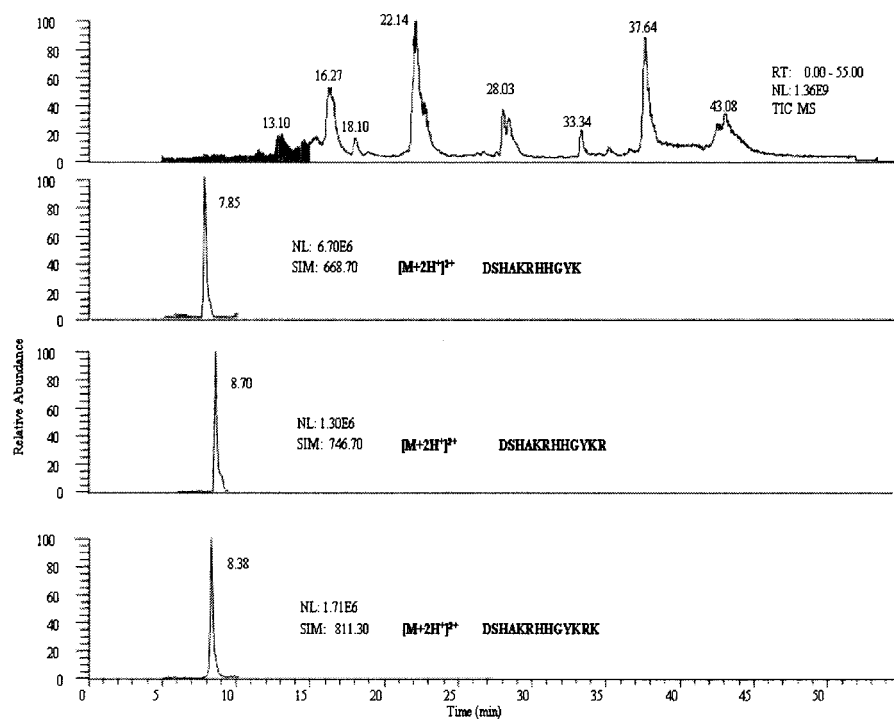
FIG. 3. **Fragmentation pattern resulting from the tandem MS experiment reported in Fig. 2.** The analysis allowed us to recognize the y and b fragmentation series, as well as many neutral losses and internal fragments, pertaining to peptides 1–11, 1–12, and 1–13 deriving from histatin 3.

12). Immediately after collection, samples were mixed with an equal volume of aqueous 0.2% trifluoroacetic acid and centrifuged, and the soluble fraction was injected into the RP-HPLC ESI-ion trap-mass spectrometer apparatus. Fig. 1 shows the typical RP-HPLC profile of human saliva, with the elution ranges of the principal classes of peptides and proteins detected by this method. They are histatins, basic and acidic proline-rich proteins, defensins, statherin, and cystatins. In Fig. 1, *upper*

FIG. 4. **MS/MS experiment performed on the doubled charged ion at 541.7 *m/z* value.** The analysis of the fragmentation pattern allowed to recognize the y and b fragmentation series, as well as many neutral losses and internal fragments, of both histatin 11 and the fragment 6–13 (sequence KRHHGYKR and RHHGYKRK, respectively).

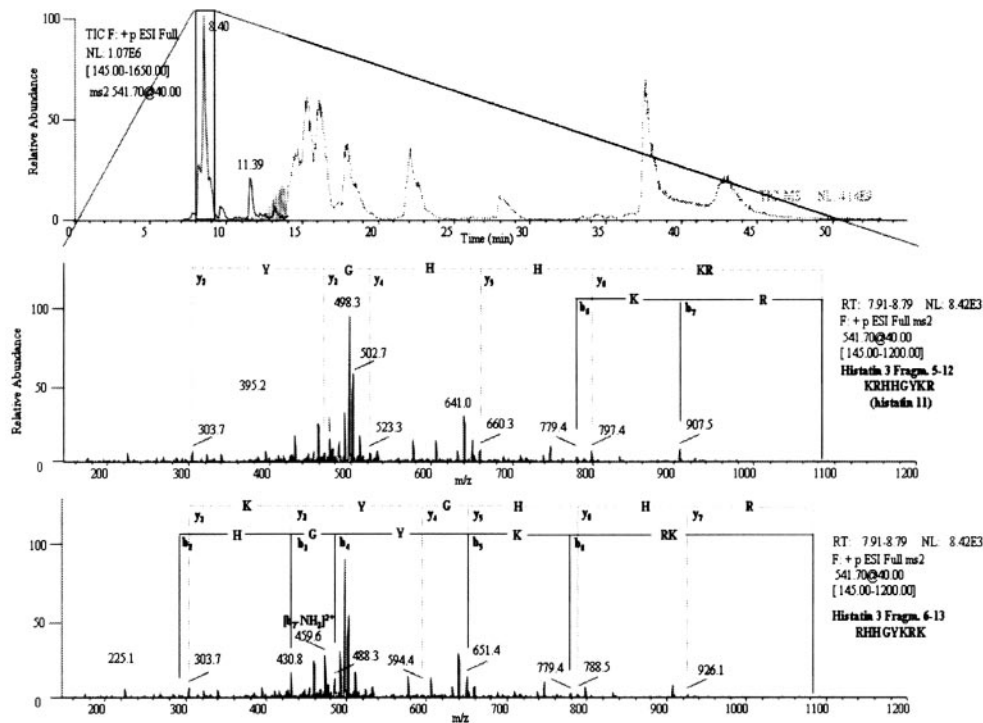*panel*, the total ion current (TIC) profile recorded by the mass spectrometer is reported. The HPLC separation was also revealed by UV detection at 214 and 276 nm (not reported). The eluent was not directed toward the electrospray source for the first 3–5 min of separation in order to avoid damage to the ion trap-mass spectrometer, due to the elevated concentration of electrolytes. Although TIC (and UV) profiles did not reveal significant signals between 3 and 12 min, the mass spectrometer detected numerous ions, with *m/z* values attributable to small peptides. As an example, the *3rd* and *4th panels* of Fig. 1 show the *m/z* values revealed between 7.98 and 8.43 min, which correspond to different mono-, di-, and multicharged ions. With the aim of identifying, among the different ions, those pertaining to histatin fragments, we adopted two strategies.

The first strategy was based on the following: (i) an extensive search for peptides with mass values lower than 4000 atomic mass units, performed along the entire chromatogram of the four salivary samples; (ii) a selection, among the masses detected, of the peptides with the masses expected for fragments originating either from histatin 1 or from its nonphosphorylated form or from histatin 3 by using FindPept program; (iii) an MS/MS experiment carried out on potential fragments, usually inducing the fragmentation of the double charged ion; and (iv) a comparison of the observed fragmentation patterns with those predictable for histatin fragments by the MS-Product program available on the ProteinProspector site. Figs. 2 and 3 exemplify the final steps of this strategy. Fig. 2 reports the selected ion monitoring (SIM) results obtained by searching the masses expected for the double charged fragments 1–11, 1–12, and 1–13 of histatin 3. Fig. 3 reports the MS/MS fragmentation spectra, which confirmed the sequence of these fragments.

The MS/MS experiments performed on the double charged ions allowed easy detection of the peaks corresponding to the y fragmentation pattern, but some fragments of the b pattern were undetectable. The first fragmentation of the double charged ions often corresponded to the neutral loss of one or two molecules of water or ammonia. In the presence of multiple arginine residues, a neutral loss of 44 atomic mass units, corresponding to the HN=CH–NH$_2$ group, was often observed.

Peptides having the same amino acid composition but different sequences usually show similar chromatographic behavior and therefore cannot be separated by HPLC. However, these peptides may generate different fragments by MS/MS experiments. Thus, an accurate analysis of fragmentation patterns may show the presence of two peptides in the same chromatographic peak. This is the case, for example, of the fragments 5–12 and 6–13 of histatin 3, which correspond to KRHHGYKR and RHHGYKRK sequences, respectively (Fig. 4).

On the basis of the results obtained with this strategy, we were able to identify many known and unknown fragments of histatin 3 in the different samples tested. It is worth mentioning that the fragmentation patterns observed for the majority of peptides submitted to the MS/MS experiment could not be ascribed to any possible histatin fragment.

Some of the fragments observed may also synthesized from histatin 1, which shares part of its sequence with histatin 3. However, no peptide fragments specific to histatin 1 were detected, suggesting that the fragments potentially resulting from both histatins originated solely from cleavage of histatin 3.

As the second strategy, we searched all the masses of peptides complementary to those found, as well as the masses of all possible fragments originating from histatin 1 and its nonphosphorylated form by cleavage pathways similar to those observed for histatin 3. To this end a SIM analysis was carried out, followed by the MS/MS analysis performed usually on the double charged ion. This second strategy allowed us to detect other fragments potentially originating from histatin 3, but none were specific to histatin 1. A search in the HPLC profile of histatins 1, 3, 5, and 6 was performed by SIM strategy. The simultaneous detection in the same HPLC position of four multicharged ions for each histatin was considered experimental evidence sufficient for its identification.

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TABLE I
List of histatin 3 fragments searched in human saliva
—, found only in this study; =, found in this study and in the references; – – –, found in the references but not in this study; ⋯, not found.

sequence

¹DS HA K⁰⁵ R HH GY¹⁰ KR KF H¹⁵ E KHH S²⁰ H RG Y R²⁵ SN Y LY³⁰ DN

av. Mass [MH⁺]	name	ref.
4063.4	histatin 3	[6]
429.4		[8]
557.6		[8]
713.8		[8]
988.1		
1336.5		
1492.6		
1620.8		
2162.4		
2523.8		
2817.1		
3037.3	histatin 5	[6]
3193.5	histatin 6	[6]
3280.6		
3394.7		
3557.9		[8]
598.7		
797.4	histatin 12	[6]
926.1	histatin 11	[6]
1082.3		
1210.4		
1494.7		
1752.0		
2113.4		
2406.7		
2627.0		
2783.1		
2870.2		
2984.3		
3147.5		
3653.0		
669.7		
797.9		
954.1		
1082.3		
1623.9		
2278.6		
2498.8		
2655.0		
2742.0		
2856.1		
3019.3		
3524.9		
531.5		
641.7		
797.9		
926.1		
1467.7		
2122.4		
2342.6		
2498.8		
2585.9		
2670.0		
2863.1		
3368.7		
799.0		
973.2		
1247.4		
1471.7		
1627.9		
1848.1		
2004.3		
2091.3		
2205.5		
2368.6		
2874.2		
845.0		
1499.7	histatin 7	[6]
1719.9	histatin 9	[6]
1876.1		
1963.2		
2077.3		
2240.5		
2746.0	histatin 4	[6]
688.8		
1343.5		
1400.5	histatin 8	[6]
1563.7	histatin 10	[6]
1719.9		
1807.0		
1921.1		
2084.3		
2589.8		
560.6		
1059.1		
1215.3		
1435.5		
1591.7		
1678.8		
1792.9		
1956.1		
2461.6		
912.0		
1068.1		
1288.4		[8]
1444.6		
1531.6		
1645.7		
1808.9		
2314.4		
673.7		
893.9		
1050.1		
1137.2		
1251.3		
1414.5		
1920.0		
536.6		
756.8		[8]
913.0		
1000.1		
1114.2		
1277.3		
1782.9		
688.8		
775.8		
889.9		
1503.1		
1558.7		
551.6		
638.7		
752.8		
916.0		
1421.5		
482.5		
596.6		
759.8		
1265.3		
539.6		
702.7		
1208.3		
539.6		
1045.1		[8]
888.9		[8]
801.8		[8]
687.7		
524.6		[8]

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

TABLE II
List of histatin 1 fragments searched in human saliva

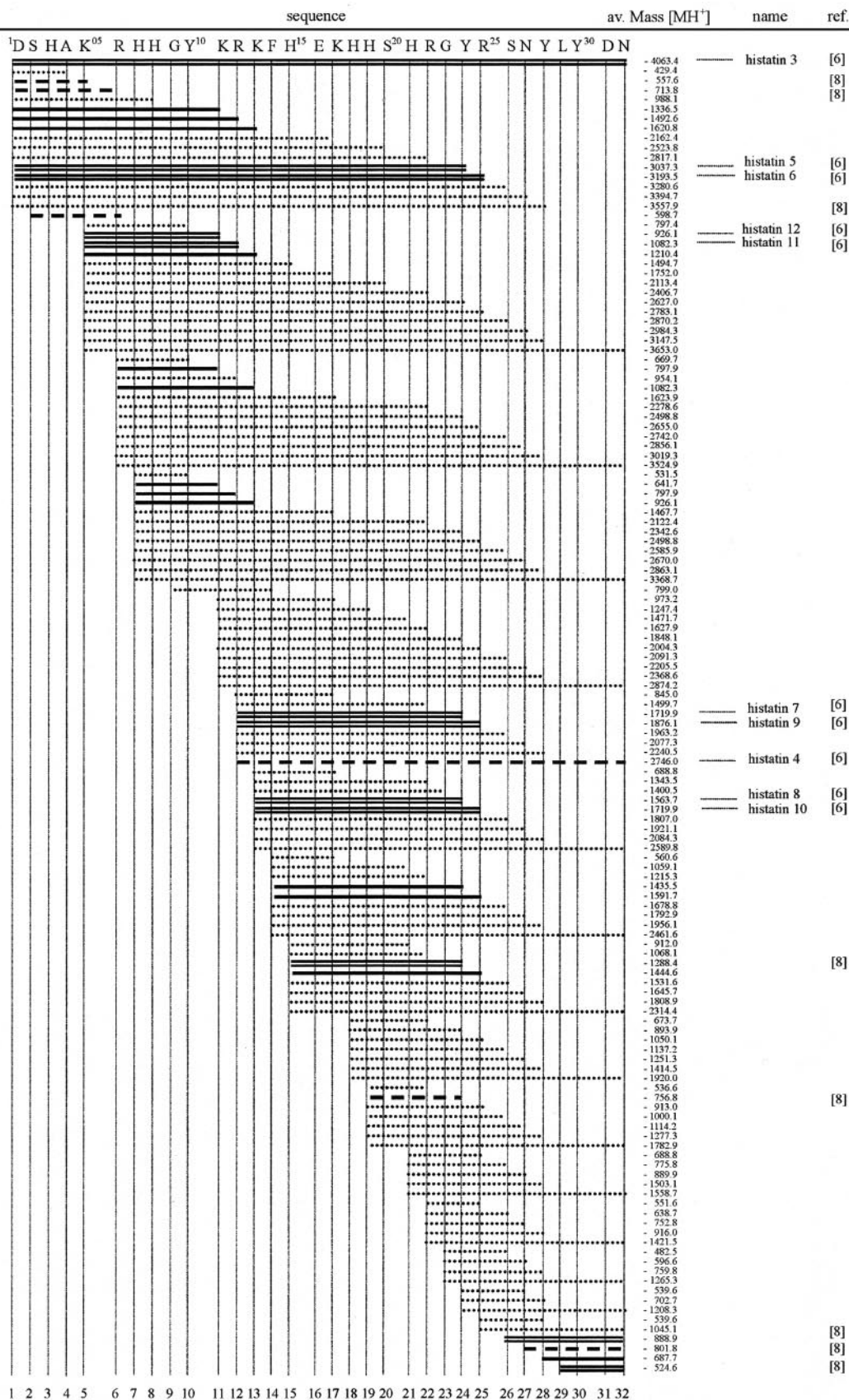—, found only in this study; =, found in this study and in the references; – – –, found in the references but not in this study; ···, not found; P corresponds to the phosphorylated peptide; asterisks refer to fragments of histatin 1 sharing a common sequence with fragments of histatin 3.

sequence	av. Mass [MH⁺]	name	ref.

¹D S H E K⁰⁵ R H H G Y¹⁰ R R K F H¹⁵ E K H H S²⁰ H R E F P²⁵ F Y G D Y³⁰ G S N Y L³⁵ Y D N

Mass values (top to bottom): 4929.2, 4849.2, 695.6, 615.6, 851.8, 771.8, 1502.5, 1422.5, 1658.7, 1578.7, 1786.9, 1706.9, 2328.5, 2248.5, 2983.2, 2903.2, 3666.9, 3586.9, 4059.3, 3979.3, 4146.4, 4066.4, 4423.6, 4343.6, 1591.0, 1956.1, 797.4, 954.1, 1110.3, 1238.4, 1780.1, 2434.7, 3188.5, 3510.9, 3597.9, 3875.2, 4380.8, 669.7, 825.9, 982.1, 1110.3, 1651.9, 2306.6, 2990.3, 3382.7, 3469.8, 3747.1, 4252.6, 531.5, 669.7, 825.9, 954.1, 1495.7, 2150.4, 2523.8, 2834.1, 3313.6, 3427.7, 3590.9, 4096.4, 1719.9, 1719.9, 1876.1, 1499.7, 2819.1, 845.0, 1499.7, 2183.4, 2575.8, 2662.9, 2940.2, 3445.7, 688.8, 1343.5, 1472.6, 2027.3, 2419.6, 2506.7, 2784.0, 3289.5, 560.6, 1059.1, 1215.3, 1344.4, 1899.1, 2291.4, 2378.5, 2655.8, 3161.3, 912.0, 1068.1, 673.7, 1357.5, 1749.8, 1836.9, 2114.2, 2619.7, 536.6, 702.8, 1095.2, 1182.2, 1459.5, 1965.0, 966.0, 1053.1, 1330.4, 1835.9, 721.7, 808.8, 1086.1, 1591.6, 574.6, 661.6, 938.9, 1444.5, 888.9, 801.8, 687.7, 524.6

histatin 1 [6]; histatin 2 [6]

Sequence position numbers: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

On the whole, among the 136 searched peptides potentially originating from histatin 3 by different cleavages (based on a total of 495 fragments resulting from any possible cleavage of histatin 3), we were able to identify the 24 fragments reported in Table I. As far as histatin 1 is concerned, the search of 119 peptides (based on a total of 775 possible fragments, comprising also the possible nonphosphorylated fragments) did not allow us to detect any fragment specifically originating from the parent peptide, comprising histatin 2 (see Table II). In Tables I and II, the double continuous line segments represent the fragments detected both in this work and in previous studies. Single continuous line segments represent the peptides de-

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tected in this study and, to our knowledge, not described until now. The boldface dashed line segments represent the peptides identified in other studies that we were not able to detect in our samples by using the strategies adopted. Finally, the dotted line segments represent peptides searched for and not found either in this study or in other studies reported in the literature. In Table II, the peptides originating from putative cleavage of histatin 1 sharing a common sequence with fragments derived from histatin 3 are labeled by an asterisk. Table II clearly shows that no fragment specific to histatin 1 was found.

Table III reports the detection (*asterisks*) of the fragments in Table I in the four subjects under study. Sixteen fragments were found in the saliva of all the subjects; four fragments were found in three subjects, and four fragments were found in the saliva of two subjects. As far as all the other peptides searched

TABLE III
Detection (asterisks) of the histatin 3 fragments in the four samples analyzed

Fragment	Synonim	Subject			
		1	2	3	4
Histatin$_3$ 1/11		*	*	*	*
Histatin$_3$ 1/12		*	*	*	*
Histatin$_3$ 1/13		*	*	*	*
Histatin$_3$ 1/24	Histatin 5	*	*	*	*
Histatin$_3$ 1/25	Histatin 6	*	*	*	*
Histatin$_3$ 5/11	Histatin 12	*	*	*	*
Histatin$_3$ 5/12	Histatin 11	*		*	
Histatin$_3$ 5/13		*	*	*	*
Histatin$_3$ 6/11		*	*	*	*
Histatin$_3$ 6/13		*		*	
Histatin$_3$ 7/11		*	*	*	*
Histatin$_3$ 7/12		*	*	*	*
Histatin$_3$ 7/13		*	*	*	*
Histatin$_3$ 12/24	Histatin 7	*	*	*	*
Histatin$_3$ 12/25	Histatin 9	*		*	
Histatin$_3$ 13/24	Histatin 8	*	*	*	*
Histatin$_3$ 13/25	Histatin 10	*	*	*	*
Histatin$_3$ 14/24		*	*	*	*
Histatin$_3$ 14/25		*		*	*
Histatin$_3$ 15/24		*	*	*	*
Histatin$_3$ 15/25			*	*	*
Histatin$_3$ 26/32		*		*	
Histatin$_3$ 28/32		*		*	
Histatin$_3$ 29/32		*	*	*	*

for are concerned, in one of the four volunteers we observed a mass corresponding to fragment 18–24 from histatin 3 but which showed an ambiguous tandem MS (not reported).

Finally, Fig. 5 reports the elution times of all the histatins and histatin fragments observed under the experimental conditions reported under "Material and Methods."

DISCUSSION

The different fragments of histatin 3, already detected in human saliva (4, 6, 8), are reported in Table I either as double continuous line segments (peptides found also in the present study) or as boldface dashed line segments (not found in the present study). These results were not obtained by a systematic search of histatin-derived peptides. The more systematic approach carried out in the present study by HPLC-tandem MS on whole human saliva allowed quite a comprehensive view of histatin 3 fragmentation patterns, because it was based on the search for the most probable fragments, and the information obtained was also derived from nondetected peptides (Tables I–III). For instance, lack of detection of histatin 2 and 4, the main and first products of salivary protease action, strongly supports the assumption that no artifacts are generated by salivary proteolysis under our experimental conditions (9). This assumption also agrees with lack of detection of almost all the fragments obtained by Xu *et al.*(10) through salivary proteolysis of synthetic histatins. The analysis was performed on whole saliva instead of selected glandular secretions with the aim of investigating any possible histatin source. In fact, preliminary data suggested that histatins are secreted not only by parotid glands but also by other minor glands of the oral cavity.

Most of the observed peptide cleavages are trypsin-like. The 26–32, 28–32, and 29–32 peptides were the only C-terminal fragments detected. This finding suggests that Arg-25 should be the first site of cleavage of histatin 3, and hence histatin 6 and the 26–32 peptide should be the first fragments generated. Then, an exo-protease should synthesize histatin 5 and the fragments 28–32 and 29–32. Similarly, the lack of fragments starting from Lys-5/Arg-6 and ending with Tyr-24/Arg-25 suggested that the KRKF residues (11–14 positions) represent the second cleavage site and the AKR residues (4–6 positions) the final third cleavage site. Arg-22 and Lys-17 do not represent cleavage sites at all. The observed cleavages suggest the in-
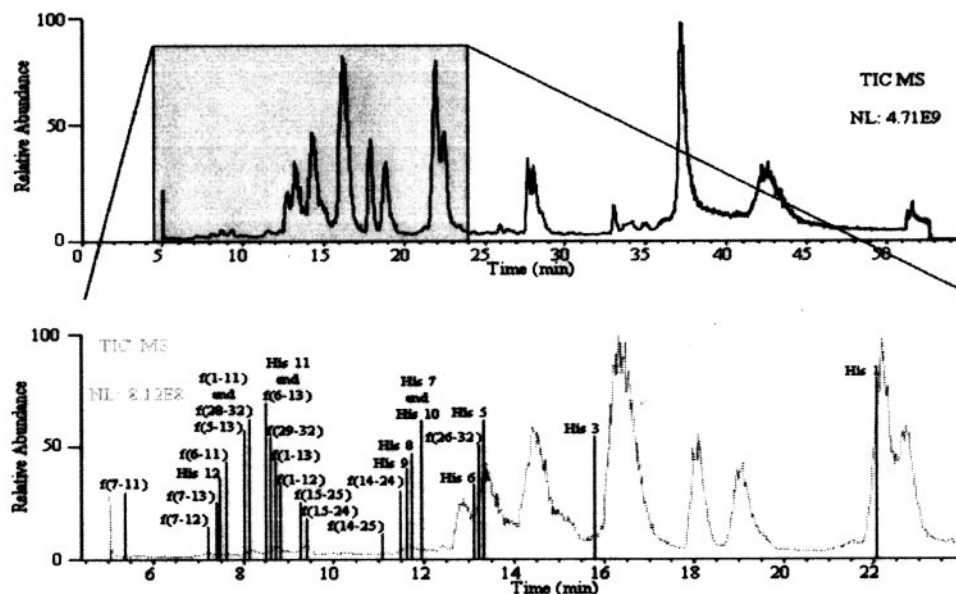

FIG. 5. *Top panel,* typical TIC profile obtained by RP-HPLC-ESI-MS analysis of human saliva. In the enlargement (4.5–24 min, *bottom panel*) the approximate elution position of all the known histatins and the histatin 3 fragments detected in this study is reported. A shift of the position of all the histatins (maximum shift about 0.5 min) was sometimes observed in chromatograms of different salivary samples.

volvement of a proprotein convertase belonging to the kexin/subtilisin family, enzymes expressed in many tissues, where their active forms are localized in the *trans*-Golgi network and small secretory vesicles (14). Indeed, the human proprotein convertase 1 (hPC1), an endocrine and neural convertase, was able to cleave *in vitro* synthetic histatin 3 primarily at the Arg-25 residue with minor cleavage at Arg-6 (15). Chan and Bennick (16) showed that intrinsic furin, the first proprotein convertase to be identified, may be responsible for *in vivo* cleavage of a basic proline-rich protein precursors in human submandibular cell lines. Moreover, the expression of the trypsin-like serine protease gene, *PRSS20*, a putative new member of the human kallikrein family, has recently been recognized in human salivary glands (17). However, in the absence of any information regarding its specificity, the action of this enzyme on histatin 3 processing cannot be confirmed.

Lack of detection of specific fragments related to histatin 1 agrees well with other studies (9). One significant structural difference between histatin 1 and histatin 3 is represented by the phosphorylation at Ser-2 of the former peptide. Even though the phosphate group does not represent a hindrance to exploiting protease action (indeed histatin 1 can be cleaved by salivary proteases (10)), phosphorylation can potentially represent a signal for differential processing. The results of the present study suggest another possible rationalization of the different sensitivity of the two major histatins to proteolysis. In fact, histatin 1 lacks an amino acid equivalent to the Arg-25 residue of histatin 3, which seems to represent the first crucial cleavage site for this peptide.

In conclusion, two main questions arise from the results of the study. (*a*) Can the present fragmentation pattern of histatins be considered definitive? The answer should be negative for the following reasons. (i) Different fragmentation pathways linked to individual variations could be observed, and it must be outlined that our analysis was performed on a limited number of subjects. (ii) Some fragments could be present at a concentration lower than the detection limit of our analytical method. (iii) Some fragments could not be detected by the mass spectrometer. For example, fragment 6–12 of histatin 3 was not found, even though it should be present in salivary secretion, because of similarity with other identified fragments (see Table I). Moreover, fragments 1–4, 1–5, and 1–6, potentially present in human saliva on the basis of the presence of other complementary peptides (*i.e.* 7–11), two of them effectively observed by other researchers, (Table II) (8) were not detected by us. These very polar peptides are expected to elute in the first minutes of the chromatographic separation, together with high salt amounts, when the eluent cannot be directed toward the MS apparatus. In order to overcome this problem we reduced salt concentration by applying a desalting C18 ZipTip procedure to the sample before the HPLC-MS analysis (data not reported). Unfortunately, after this desalting step not only were we unable to detect the 1–4, 1–5, and 1–6 fragments but we also verified the loss of almost all the small fragments detected in the undesalted sample. On the other hand, the direct injection in the MS apparatus of whole saliva and the attempt to begin the MS detection at 1.50 min caused a strong increase in noise and a significant sensitivity reduction because of ion suppression.

(*b*) What does this histatin proteolytic cascade mean from a physiological point of view? Antifungal properties of histatin 5 against *C. albicans* species were largely demonstrated (4, 6, 10), implying an action on energized mitochondria (18). Moreover, various studies performed with peptide-histatin analogues (19–21) pointed out that the central sequence starting from Lys-11 and ending with Tyr-24 seems to be crucial for the antifungal activity. For instance, Arg-22 seems essential for the biological activity (22). The fragmentation pathway shown in this study, directed toward the genesis of different central fragments, agrees well with the results of structure-activity investigations. However, little is known about the role of the small terminal fragments, which may also play a significant role in the oral cavity and should be further investigated.

Finally, we wish to observe that even though the name "histatins" is a suitable choice, the large number of new histatin fragments detected in this study suggests adoption of a more rational nomenclature, capable of including present and future identifications. To this purpose we suggest the nomenclature "histatin$_x$ y/z", where x refers to the parent histatin (1 or 3) and y/z represents the position in the parent sequence of the first and last amino acid residues. Histatin 3 fragments reported in Table III have been named following the proposed nomenclature.

REFERENCES

1. Sabatini, L. M., Ota, T., and Azen, E. A. (1993) *Mol. Biol. Evol.* **10,** 497–511
2. Mac Kay, B. J., Pollock, J. J., Iacono, V. J., and Baum, B. J. (1984) *Infect. Immun.* **44,** 688–694
3. Oppenheim, F. G., Xu, T., Mc Millian, F. M., Levitz, S. M., Diamond, R. D., Offner, G. D., and Troxler, R. F. (1988) *J. Biol. Chem.* **263,** 7472–7477
4. Tsai, H., and Bobek, L. A. (1998) *Crit. Rev. Oral Biol. Med.* **9,** 480–497
5. Vanderspek, J. C., Wyandt, H. E., Skare, J. C., Milunsky, A., Oppenheim, F. G., and Troxler, R. F. (1989) *Am. J. Hum. Genet.* **45,** 381–387
6. Troxler, R. F., Offner, G. D., Xu, T., Vanderspeck, J. C., and Oppenheim, F. G. (1989) *J. Dent. Res.* **69,** 2–6
7. Xu, T., Levitz, S. M., Diamond, R. D., and Oppenheim, F. G. (1991) *Infect. Immun.* **59,** 2549–2554
8. Perinpanayagam, H. E. R., Van Wuyckhuyse, B. C., Ji, Z. S., and Tabak, L. A. (1995) *J. Dent. Res.* **74,** 345–350
9. Xu, L., Lal, K., and Pollock, J. J. (1992) *Oral Microbiol. Immunol.* **7,** 127–128
10. Xu, L., Lal, K., Santarpia, R. P., III, and Pollock, J. J. (1993) *Arch. Oral Biol.* **38,** 277–283
11. Castagnola, M., Congiu, D., Denotti, G., Di Nunzio, A., Fadda, M. B., Melis, S., Messana, I., Misiti, F., Murtas, R., Olianas, A., Piras, V., Pittau, A., and Puddu, G. (2001) *J. Chromatogr. B.* **751,** 153–160
12. Guzman, H., Leone, C., Helmerorst, E. J., Nunn, M., Flora, B., Troxler, R. F., and Oppenheim, F. G. (2004) *Arch. Oral Biol.* **49,** 11–22
13. Zhang, Z., and Marshall, A. G. (1998) *J. Am. Soc. Mass Spectrom.* **9,** 225–233
14. Zhou, A., Webb, G., Zhu, X., and Steiner, D. F. (1999) *J. Biol. Chem.* **274,** 20745–20748
15. Basak, A., Ernst, B., Brewer, D., Seidah, N. G., Munzer, J. S., Lazure, C., and Lajoie, G. A. (1997) *J. Pept. Res.* **49,** 596–603
16. Chan, M., and Bennick, A. (2001) *Eur. J. Biochem.* **268,** 3423–3431
17. Yousef, G. M., Scorilas, A., and Diamandis, E. P. (2000) *Genomics* **63,** 88–96
18. Helmerorst, E. J., Breeuwer, P., van't Hof, W., Walgreen-Weterings, E., Oomen, L. C. J. M., Veerman, E. C. I., Nieuw Amerongen, A. V., and Abee, T. (1999) *J. Biol. Chem.* **274,** 7286–7291
19. Helmerorst, E. J., van't Hof, W., Veerman, E. C. I., Simoons-Smit, I., and Nieuw Amerongen, A. V. (1997) *Biochem. J.* **326,** 39–45
20. Rothstein, D. M., Spacciapoli, P., Tran, L. T., Xu, T., Roberts, F. D., Dalla Serra, M., Buxton, D. K., Oppheneim, F. G., and Friden, P. (2001) *Antimicrob. Agents Chemother.* **45,** 1367–1373
21. Brewer, D., and Lajoie, G. (2002) *Biochemistry* **41,** 5526–5536
22. Tsai, H., and Bobek, L. A. (1997) *Antimicrob. Agents Chemother.* **41,** 2224–2228