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Structure of non-(1-84) PTH fragments secreted by parathyroid glands in primary and secondary hyperparathyroidism

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Structure of non-(1-84) PTH fragments secreted by parathyroid glands in primary and secondary hyperparathyroidism.

Background. Non-(1-84) parathyroid hormone (PTH) fragments are large circulating carboxyl-terminal (C) fragments with a partially preserved amino-terminal (N) structure. hPTH (7-84), a synthetic surrogate, has been demonstrated to exert biologic effects in vivo and in vitro which are opposite to those of hPTH (1-34) on the PTH/PTHrP type I receptor through a C-PTH receptor. We wanted to determine the N structure of non-(1-84) PTH fragments.

Methods. Parathyroid cells isolated from glands obtained at surgery from three patients with primary hyperparathyroidism and three patients with secondary hyperparathyroidism were incubated with ^{35}S-methionine to internally label their secretion products. Incubations were performed for 8 hours at the patient-ionized calcium concentration and in the presence of various protease inhibitors. The supernatant was fractionated by high-performance liquid chromatography (HPLC) and fractions were analyzed with PTH assays having (1 to 4) and (12 to 23) epitopes, respectively. The serum of each patient was similarly analyzed. Peaks of immunoreactivity identified were submitted to sequence analysis to recover the ^{35}S-methionine residues in positions 8 and 18.

Results. Three regions of interest were identified with PTH assays. They corresponded to non-(1-84) PTH fragments (further divided in regions 3 and 4), a peak of N-PTH migrating in front of hPTH (1-84) (region 2) and a peak of immunoreactivity corresponding to the elution position of hPTH (1-84) (region 1). The last corresponded to a single sequence starting at position 1. Region 2 gave similar results in all cases (a major signal starting at position 1) but also sometimes minor sequences starting at position 4 or 7. Regions 3 and 4 always identified a major sequence starting at positions 7 and minor sequences starting at positions 8, 10, and 15. Surprisingly, a major signal starting at position 1 was also present in region 3. The HPLC profile obtained from a given patient's parathyroid cells was qualitatively similar to the one obtained with his/her serum in each case.

Conclusion. These results indicate that non-(1-84) PTH fragments are composed of a family of fragments which may be generated by specific or progressive cleavage at the N region. The longest fragment starts at position 4 and the shortest at position 15. A peptide starting at position 7 appears as the major component of non-(1-84) PTH fragments. The generation process is similar to the one described for smaller C-PTH fragments a number of years ago, suggesting a similar production mechanism and source for all C-PTH fragments.

Key words: parathyroid hormone, primary hyperparathyroidism, non-(1-84) parathyroid hormone, secondary hyperparathyroidism, calcium.

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Non-(1-84) parathyroid hormone (PTH) fragments or amino-terminal (N) truncated PTH fragments are large circulating carboxyl-terminal (C) fragments of PTH with a partially preserved N structure. They differ from other circulating C-PTH fragments (which make up the majority of PTH in circulation) by their capacity to react in intact (i) or (13-34) PTH assays [1–3]. Non-(1-84) C-PTH fragments were initially described during a study of parathyroid function in normal individuals [1]. When circulating PTH molecular forms were fractionated by high-performance liquid chromatography (HPLC) at various calcium concentrations, a slightly less hydrophobic peak of immunoreactivity was identified in front of hPTH (1-84) and named non-(1-84) PTH. This peak represented about 20% of iPTH in a normal individual under normocalcemic condition but up to 50% in patients with terminal renal failure [1–3]. Like other C-PTH fragments [4], non-(1-84) PTH fragments are cleared by the kidney and thus accumulate in renal failure patients proportionally to the degree of renal failure [5]. They are also secreted by the parathyroid glands [6] and generated during the peripheral metabolism of hPTH (1-84) [6]. The exact N-terminal structure of non-(1-84) PTH fragments is unknown but a recent immunologic study based on the use of antibodies with proximal or distal epitopes in the region (13-34) of the PTH structure suggests that all

non-(1-84) PTH fragments start their N structure prior to position 19 [7].

Our interest in non-(1-84) PTH fragments lies in the fact that they could exert biologic effects different from those of hPTH (1-84). hPTH (7-84), a commercially available surrogate for all non-(1-84) PTH fragments, was initially used to demonstrate that such fragments reacted in iPTH assays [2, 3]. It has been also used to demonstrate that this category of C-PTH fragments exerts biologic effects different from those of hPTH (1-84) via a C-PTH receptor [8–11]. hPTH (7-84) causes hypocalcemia, hypophosphatemia, and antagonizes the calcemic response to hPTH (1-34) and hPTH (1-84) in parathyroidectomized rats [8, 9]. It is also an inhibitor of bone resorption in vitro [11] and of bone turnover in parathyroidectomized 5/6 nephrectomized rats [10]. It is thus important to determine the N-terminal structure of non-(1-84) PTH fragments to see if they could duplicate the biologic effects observed with hPTH (7-84).

METHODS

Experimental subjects

Three patients with primary hyperparathyroidism and three others with secondary hyperparathyroidism, who, upon investigation, had a large amount of parathyroid tissue, were recruited for this project. The study was approved by the Research Ethics Committee of our center, and all participants signed an informed consent.

Experimental protocols

Blood was obtained from each subject prior to surgery to measure indices of calcium metabolism and to perform HPLC separation of circulating PTH molecular forms. Parathyroid tissue taken at surgery was brought to pathology where a portion was kept for pathologic analysis and another was used for experimentation. Five hundred milligrams of tissue proved necessary for successful experimentation with parathyroid cells.

Biochemical measurements

Ionized calcium (Ca^{++}) was measured by a specific electrode (Rapid Lab 348) (Bayer, Toronto, Ontario, Canada), while total calcium (Ca_t), phosphorus (PO_4), creatinine, and alkaline phosphatase (AP) were quantified by automated colorimetric methods. PTH was measured in serum and in HPLC fractions by two enzyme-linked immunosorbent assays (ELISAs) provided by Immutopics Inc. (San Clemente, CA, USA), the Human Bioactive PTH ELISA and the Human PTH ELISA. Both assays use a capture antibody purified by affinity chromatography against hPTH (39-84). The Human Bioactive PTH ELISA has a (1-4) epitope [12]. The revealing antibody of the Human PTH ELISA was purified by affinity chromatography against hPTH (13-34). We also developed a carboxyl-terminal radioimmunoassay (RIA) with a (79-84) immunoaffinity purified antibody provided by Immutopics Inc. and ^{125}I [tyr^{53}] hPTH(53-84) as tracer. The antibody was first purified by affinity chromatography against hPTH (39-84) and further purified by the same method against hPTH (79-84). The behavior of each assay was studied using appropriate hPTH standards. These included hPTH (1-84), hPTH (7-84), [tyr^{34}] hPTH (19-84), [tyr^{34}] hPTH (24-84), hPTH (39-84), hPTH (53-84), hPTH (64-84), and hPTH (53-83). Saturation analysis of the revealing antibody of the two PTH ELISAs was also performed with hPTH (1-34), hPTH (13-34), and hPTH (18-48). All peptides were purchased from Bachem (Torrance, CA, USA) except for hPTH (53-83), [tyr^{34}] hPTH (19-84), and [tyr^{34}] hPTH (24-84) which were generously provided by Dr. H. Jüppner of the Massachusetts General Hospital in Boston.

Parathyroid cell incubation

Parathyroid tissue obtained at surgery was separated from fat and connective tissue, and minced into small pieces. These pieces were digested with collagenase type I (Worthington, 239 U/mg) and DNase type I (Worthington, 3.08 U/mg) [13]. The first was used at a concentration of 20 mg/200 mg of tissue while the second was used at 500 μg/200 mg. Both were dissolved in Dulbecco's modified Eagle's medium (DMEM)/F-12, 10% fetal calf serum (FCS), 0.2% bovine serum albumin (BSA) at 10 mL/200 mg of tissue. Digestion proceeded for 60 minutes at 37°C or overnight at 4°C. The medium was then filtered through a nylon filter (250 μmol/L) and centrifuged at 3500 rpm at 4°C over 10 minutes. After the supernatant was discarded, the pellet was resuspended in digestion medium lacking methionine and washed three times. The cells were then incubated in 10 mL of the same medium in a 25 cm^3 flask at 37°C for 4 hours. Ca^{++} concentration was adjusted to the original Ca^{++} concentration of the patient with $CaCl_2$. Again, after centrifugation, the supernatant was discarded, and the cells were incubated for 8 hours in the same medium to which ^{35}S-methionine 50 μCi/mL had been added with a cocktail of protease inhibitors (Complete, EDTA-free) (Roche P8340). At the end, the medium was centrifuged and the supernatant containing the secreted peptides was kept for further processing. Up to three 8-hour incubations could be achieved with the same cells. When this was done, the HPLC profiles were qualitatively comparable.

HPLC analysis

Circulating PTH molecular forms from all sera and from parathyroid cell incubations were extracted with Waters Sep-Pak Plus C-18 cartridges, as described by

Bennett et al [14]. One C-18 cartridge was used for each 3 mL of serum or medium. Samples were eluted from the cartridge with 3 mL of 800 mL/L acetonitrile in 1 g/L trifluoroacetic acid. Acetonitrile was evaporated from the eluate with nitrogen, and the residual volume was freeze-dried, then reconstituted in 2 mL of 1 g/L trifluroacetic acid for HPLC analysis. Each 2 mL sample was loaded on a Waters C_{18} μBondapak analytic column [300 × 3.9 mm (inner diameter)] and eluted with a noncontinuous linear gradient of acetonitrile in 1 g/L trifluoroacetic acid. The gradient ranged from 15% to 23% in 25 minutes, 23% to 30% in 5 minutes, and 30% to 33% in 30 minutes. The gradient was delivered at 1.5 mL/min with a Hitachi Model L-6200 solvent delivery system. The 1.5 mL fractions were evaporated, freeze-dried, and reconstituted to 1 mL with 7 g/L BSA in water; adequate volumes were then measured in the various PTH assays. Controls experiments were performed with hPTH (1-84) added to hypoparathyroid serum and with internally labeled hPTH (1-84) added to a parathyroid cells incubation experiment where ^{35}S-methionine was omitted to insure that PTH degradation did not occur during the various procedures. After HPLC separation, a single peak of radioactivity coeluting with hPTH (1-84) was seen and single peak of immunoreactivity coeluting with hPTH (1-84) was detected by the two PTH assays. Immunoreactive PTH recovery with the two PTH ELISAs through all of these procedures was calculated by comparing original serum or medium PTH value with the sum of PTH immunoreactivity across all HPLC fractions. For the hPTH (79-84) assay, this was accomplished by comparing the amount of immunoreactivity recovered by this assay with that of the two other PTH assays.

Radioactive protein sequencing

Localization of the metabolically labeled residues was determined by automated amino-terminal Edman degradation, using Applied Biosystems sequenator (Model Procise 494 cLC) (Foster City, CA, USA) operated according to the manufacturer's protocol. The only modification was the addition of narrow-bore tubing linking the outlet of valve 39 to a programmable fraction collector. In the sequencing program, all steps involving transfer to the conversion flask as well as functioning of the conversion flask were eliminated or disabled. Transfer of the butyl chloride (S3) extract was done directly from the reaction cartridge to the fraction collector with functions 119 and 122 each activated for a 45-second duration. Finally, prior to initiating sequencing, two complete degradation cycles, where all additions of phenylisothiocyanate (PITC, R1) were omitted to condition the sample, were programmed, followed by a further cycle with no trifluoroacetic acid addition, permitting double coupling of PITC to the amino terminus in the first cycle. The HPLC-purified and vacuum-dried sample was redissolved in 50 μL of water containing 1 μL of an apomyoglobin solution (75 pmol/μL). The reconstituted sample was then serially added (7 μL at a time) on the 6 mm glass fiber filter on which 7.5 μL of standard Biobrene solution had previously been added. Radioactivity at each cycle was measured after mixing each S3 extract with 3.0 mL of scintillation liquid cocktail (UniverSol, ICN Biomedicals, Irvine, CA, USA) for 10 minutes' duration in Beckman Counter Model LS-8100.

Results are means ± SD. Planimetric evaluation of the various HPLC profiles was accomplished with Origin 7.5 software (Origin Lab Corporation, MA, USA). All HPLC profiles are corrected to 100% recovery and to the patient basal PTH value expressed in pmol/L.

RESULTS

Figure 1 illustrates the immunoreactive characteristics of the three PTH assays used in this study. The Human Bioactive PTH ELISA Kit reacted only with hPTH (1-84) and not with hPTH (7-84). Its revealing antibody could be saturated with hPTH (1-34) but not with hPTH (13-34) or hPTH (18-48). The Human PTH ELISA reacted equally well with hPTH (1-84), hPTH (7-84), half as well with [tyr^{34}] hPTH (19-84) and not at all with other C-PTH fragments, including [tyr^{34}] hPTH (24-84). Its revealing antibody could be completely saturated with hPTH (1-34), about 80% with hPTH (13-34) but only 50% with hPTH (18-48). The hPTH (79-84) RIA reacted slightly better with hPTH (39-84) than with hPTH (1-84), hPTH (7-84), or hPTH (53-84), the latter three being similarly reactive. hPTH (53-83) was only about half as reactive as hPTH (53-84) while hPTH (64-84) was even less reactive.

The biochemical and demographic characteristics of the six patients investigated in this study are depicted on Table 1. Patients with primary hyperparathyroidism had more severe hypercalcemia and were hypophosphatemic. Their AP levels varied from the upper normal limit to frankly elevated concentrations while their PTH levels were moderately elevated. Patients with secondary hyperparathyroidism had normal or slightly elevated calcium levels while being hyperphosphatemic. Their AP levels were higher than those of patients with primary hyperparathyroidism and their PTH levels were frankly elevated.

Figure 2 illustrates the HPLC profiles of circulating and parathyroid cell-secreted PTH in a patient with primary (left) and secondary (right) hyperparathyroidism. Three regions of interest were identified by the two PTH ELISAs. The first, region 38 to 42, corresponded to the elution position of hPTH (1-84) and was recognized equally well by both PTH assays. The second, region 32 to 35, corresponded to an amino-terminal form of PTH (N-PTH) which reacted less in the hPTH (12-23) assay

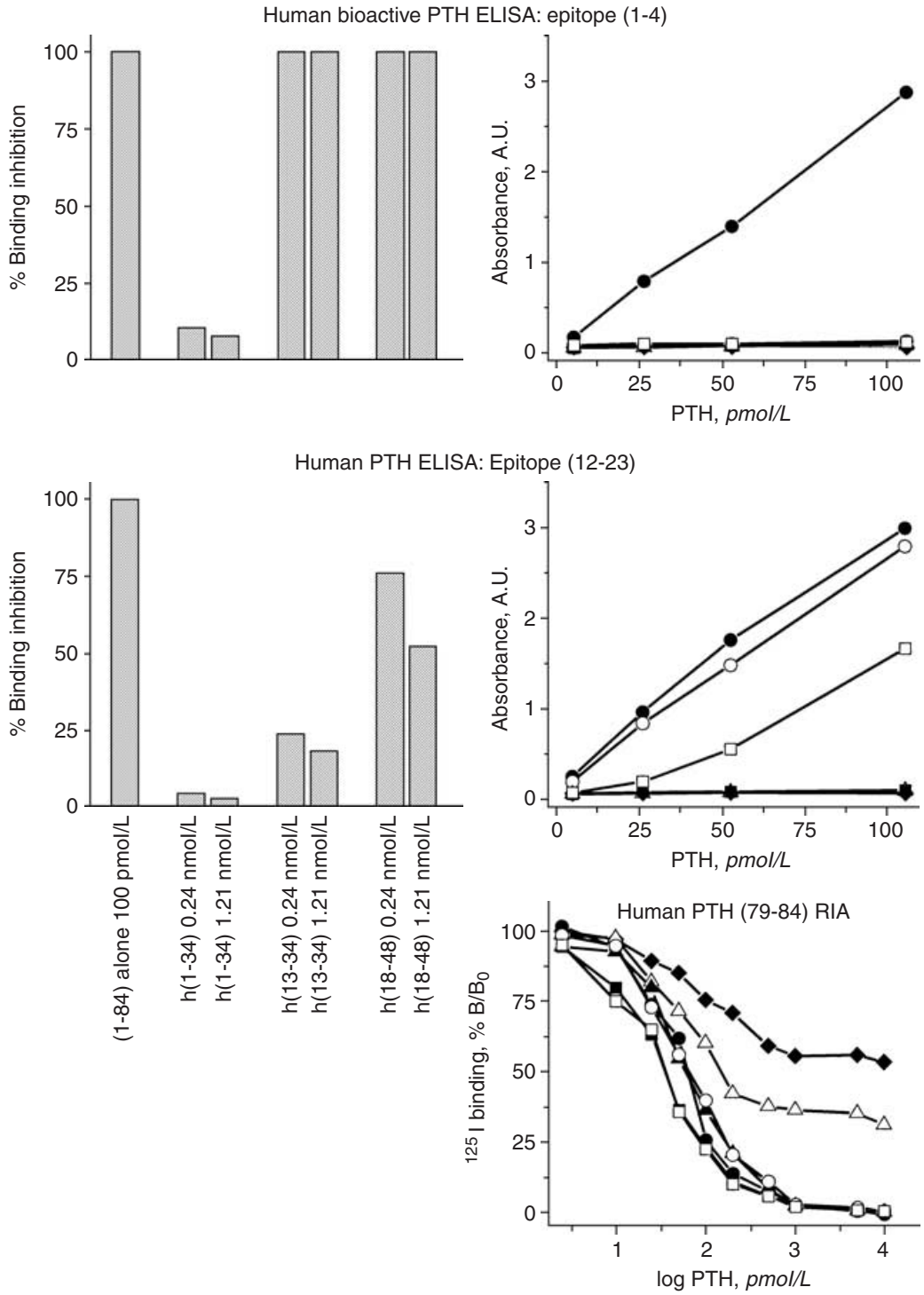

Fig. 1. Immunoreactivity of the three parathyroid hormone (PTH) assays defined with the use of various human PTH calibrators and through saturation analysis of the revealing antibody. (●) hPTH (1-84); (○) hPTH (7-84); (■) hPTH (39-84); (▲) hPTH (53-84); (△) hPTH (53-83); (◆) hPTH (64-84); (□) [tyr^{34}] hPTH (19-84); (*) [tyr^{34}] hPTH (24-84). AU is arbitrary units.

in most HPLC profiles. Finally, the third region, region 21 to 28, corresponded to non-(1-84) PTH fragments and reacted better in the hPTH (12-23) assay. This region was arbitrarily divided into two regions, 3 (21 to 25) and 4 (26 to 28), for sequence analysis because region 3 had significant hPTH (1-4) immunoreactivity while region 4 almost none.

Table 2 summarizes the planimetric evaluation of HPLC profiles performed on serum or parathyroid cells supernatant. The percent of immunoreactivity

Table 1. Characteristics of patients with primary hyperparathyroidism (PHP) and secondary hyperparathyroidism (SHP)

			Biochemical measurements						
Groups	Number	Gender	Ca^{++} (1.19-1.34 mmol/L)	Ca_t (2.11-2.56 mmol/L)	PO_4 (0.77-1.4 mmol/L)	Creatinine (38-115 μmol/L)	Alkaline phosphatase (16-101 U/L)	PTH (1-4) (0.5-4.1 pmol/L)	PTH (12-23) (1.5-7.0 pmol/L)
PHP	1	F	1.88	2.88	0.47	56	172	24.4	42.5
	2	F	1.7	3.19	0.67	73	104	17.6	18.8
	3	M	1.84	3.02	0.57	91	116	24.2	33.5
Mean ± SD			1.81	3.03	0.57	73	130.7	22.1	31.6
			0.09	0.16	0.10	18	36.30	3.9	12
SHP	1	M	1.35	2.32	1.55	654	346	117.8	176.6
	2	M	1.21	2.22	1.73	781	163	102.2	114.5
	3	M	1.3	2.41	2.14	573	129	100.4	166.6
Mean ± SD			1.29	2.32	1.81	669	212.7	106.8	152.6
			0.07	0.10	0.30	105	116.7	9.6	33.3

corresponding to hPTH (1-84), N-PTH, and non-(1-84) PTH regions are given as well as the% immunoreactivity recovery with the two PTH assays in each HPLC profile. The last was better than 80% for both PTH assays and in all profiles, one excepted at 71.9%. With the hPTH (12-23) assay, there was a relatively good concordance between the % of each HPLC region in serum and parathyroid cells supernatant in both populations, with the exception of N-PTH which was higher in the parathyroid cells supernatant of patients with secondary hyperparathyroidism than it was in serum. Furthermore, the percentage of non-(1-84) PTH fragments was similar in serum and parathyroid cells supernatant in both populations. With the PTH (1-4) assay, the amount of N-PTH varied greatly among patients with primary hyperparathyroidism, from 3.3% to 45.5% in serum and from 4.5% to 38.5% in parathyroid cells supernatant, affecting other region results. This problem still existed but to a lesser extend in patients with secondary hyperparathyroidism.

The ^{35}S-methionine counts corresponding to HPLC regions 1, 2, 3, and 4 (Fig. 2) were submitted to sequence analysis. The results for one patient with primary hyperparathyroidism and one with secondary hyperparathyropidism are illustrated in Figure 3. These data are also summarized for all patients in Table 3. Peak 1, corresponding to the elution position of hPTH (1-84), disclosed ^{35}S-methionine residues at cycles 8 and 18, corresponding to a single peptide starting at position 1 in all cases. Peak 2 gave similar results in all cases with a dominant peptide starting at position 1, but also weaker signals at positions 5 and 15 in two cases and 2 and 12 in two other cases, corresponding to peptides starting at positions 4 and 7, respectively. Peak 3 gave signals at positions 8 and 18, 2 and 12, as well as four corresponding to peptides starting at positions 1, 7, and 15. Positions 8 and 18 were the most important quantitatively in four cases while 2 and 12 in two cases. Peak 4 gave signals corresponding to peptides starting at positions 1, 7, 8, 10, and 15. The most important signal corresponded to a peptide starting at position 7 in all cases.

Figure 4 summarizes our effort to study the carboxyl-terminal structure of these peaks, with a RIA that reacted better with hPTH (53-84) than with hPTH (53-83), in a patient with primary hyperparathyroidism as well as in a patient with secondary hyperparathyroidism. The amount of PTH recovered by the hPTH (1-4) and (79-84) assays was similar for peaks 1 and 2. For the non-(1-84) region (regions 3 and 4), the amount of immunoreactivity recovered by the hPTH (79-84) assay was only 61.3% (primary hyperparathyroidism type 2) and 83.6% (secondary hyperparathyroidism type 2) of the amount recovered by the hPTH (13-23) assay.

DISCUSSION

This study was planned to elucidate the N-sequence of non-(1-84) PTH fragments. The importance of these fragments in PTH physiology has been evaluated indirectly through the use of hPTH (7-84), a synthetic surrogate for these fragments (8-11). Recent data suggest that these fragments start prior to position 19 of the PTH structure [7]. We decided to sequence internally labeled non-(1-84) PTH fragments because this has been demonstrated to be the most sensitive method in the past [15–17]. The alternative, that is, sequencing purified nonradioactive non-(1-84) PTH fragments, would have required liters of serum from patients with very high PTH levels and liters of medium from parathyroid cell incubations.

We initially worked with small amounts of parathyroid tissue (less than 500 mg) and soon discovered that the amount of internally labeled secretion products was too small to work with efficiently. This is why we started to identify patients with primary or secondary hyperparathyroidism who had larger tumors to enroll them in the protocol. The more severe biochemical status of these patients reflected the greater amount of parathyroid tissue.

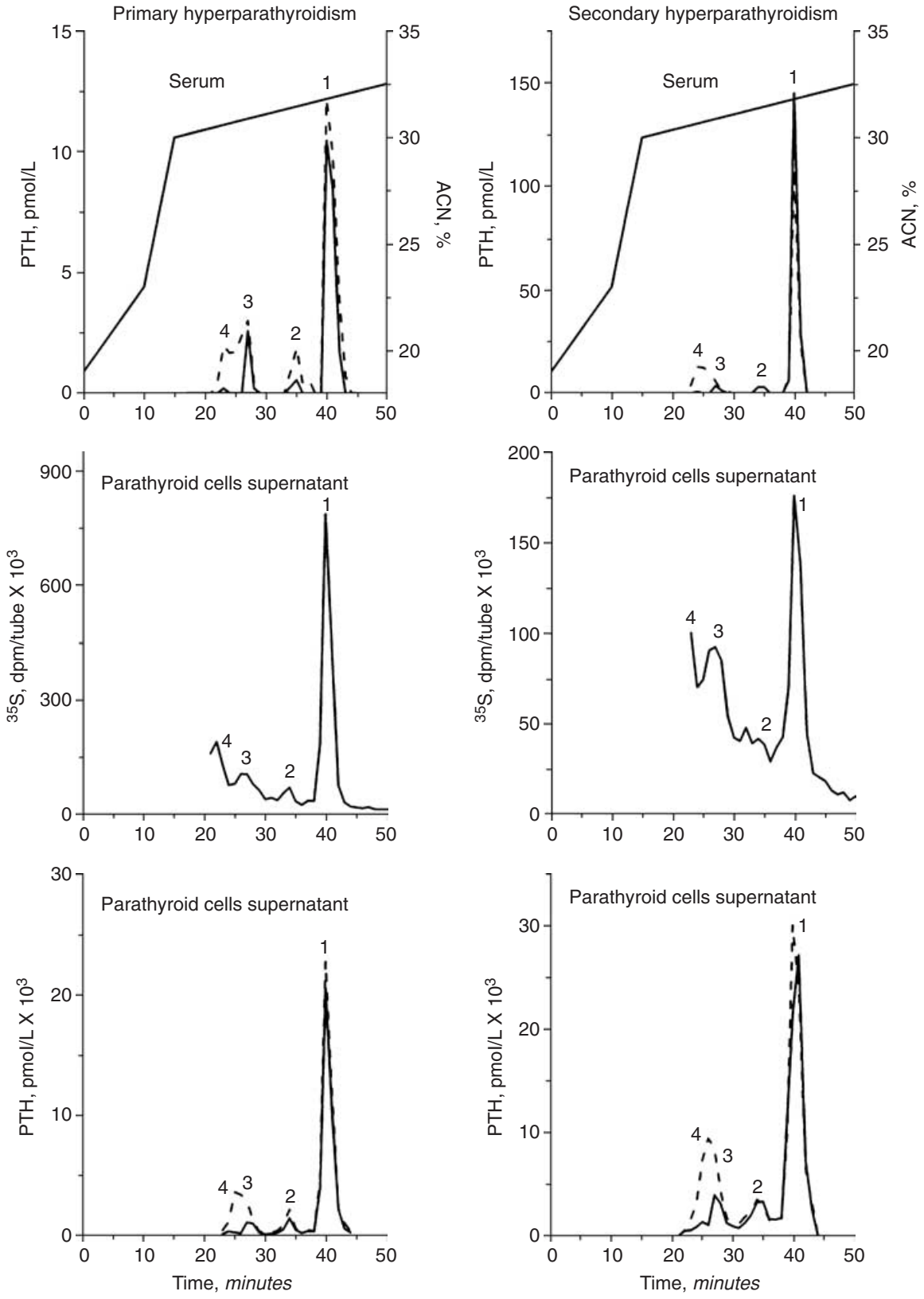

Fig. 2. High-performance liquid chromatography (HPLC) profiles of circulating and secreted parathyroid hormone (PTH) in a patient with primary (left) and a patient with secondary (right) hyperparathyroidism. Immunoreactivity (top and bottom graphs) was analyzed with PTH assays having (1-4) (.....) and (12-23) (-----) epitopes. The middle graph, in each case, represents ^{35}S-methionine counts corresponding to immunoreactivity in the bottom graphs. Regions 1, 2, 3, and 4 corresponding to hPTH (1-84), amino-terminal PTH and non-(1-84) PTH fragments (3 and 4) were submitted to sequence analysis (see Fig. 3).

Table 2. Planimetric evaluation of serum and parathyroid cell medium high-performance liquid chromatography (HPLC) profiles

Milieu	Groups	PTH (1-4) *pmol/L*	HPLC profile (% of total) (1-84)	N-PTH	Non-(1-84)	Recovery	PTH (12-23) *pmol/L*	HPLC profile (% of total) (1-84)	N-PTH	Non-(1-84)	Recovery
	PHP 1	24.4	84.5	3.3	12.2	100.9	42.5	62.8	9.1	28.2	82.1
	PHP 2	17.6	62.4	25.0	12.7	85.1	18.8	71.9	4.7	23.4	71.9
	PHP 3	24.2	49.4	45.4	5.2	81.6	33.5	45.1	11.3	43.6	81.1
Serum	Mean ± SD	22.1	65.4	24.6	10.0	89.2	31.6	59.0	8.4	31.7	78.4
		3.8	17.7	21.1	4.2	10.3	12	13.6	3.4	10.5	5.6
	SHP 1	117.8	84.4	9.0	6.6	85.2	176.6	71.0	0.9	28.1	112.9
	SHP 2	102.2	87.7	6.5	5.8	90.3	114.5	69.7	4.7	25.6	95.4
	SHP 3	100.4	84.3	5.9	9.8	94.1	166.6	67.2	0.0	32.8	117.1
	Mean ± SD	106.8	85.5	7.1	7.4	89.9	152.6	69.3	1.9	28.8	108.5
		9.6	1.95	1.6	2.11	4.5	33.3	1.9	2.5	3.7	11.5
Supernatant	PHP 1	2288	91.0	4.5	4.6	99	3078	75.0	5.1	20.0	99
	PHP 2	3413	81.9	13.4	4.7	103	3667	72.9	7.7	19.4	99
	PHP 3	880	59.5	38.5	2.0	101.6	1215	41.2	9.7	49.1	86.9
	Mean ± SD	2194	77.5	18.8	3.8	100.9	2653	63.0	7.5	29.5	94.7
		1269	16.2	17.6	1.5	2	1280	18.9	2.3	17	6.7
	SHP 1	14487	71.2	20.3	8.5	113.1	22109	53.6	19.4	27.0	112.3
	SHP 2	9330	81.4	10.1	8.5	98.5	12600	63.9	8.1	28.0	97.6
	SHP 3	380	86.5	9.9	3.6	100.6	408	76.6	4.5	18.9	97.8
	Mean± SD	8066	79.7	13.4	6.9	104.1	11706	64.7	10.7	24.6	102.6
		7138	7.8	5.9	2.8	7.9	10878	11.5	7.8	5	8.4

Results are means ± SD. Recovery of immunoreactivity in all tubes of the HPLC profile compared to the amount loaded.

The PTH assays used for these studies corresponded to a second generation and a third generation PTH assay. Based on our results and those obtained by another study [12], the Human Bioactive PTH ELISA has a (1-4) epitope its revealing antibody being completely saturable with hPTH (1-34), about 50% with hPTH (2-34), 4% with hPTH (3-34) and less than 0.1% with hPTH (4-34) [12]. The Human PTH ELISA probably has several epitopes in the region (12-23), about 50% of them being proximal to position 18 while 50% distal to position 18. The ending position 23 is based on non reactivity of [tyr^{34}] hPTH (24-84) in the assay while position 12 on the fact that even if hPTH (13-34) was used to immunoaffinity purify the antibody it could not completely saturate the antibody. We also developed a RIA with an imunoaffinity purified (79-84) antibody and ^{125}I [tyr^{53}] hPTH (53-84) as tracer in which hPTH (53-84) reacted better than hPTH (53-84) suggesting an influence of the last amino acid. The fact that hPTH (69-84) was even less reactive than hPTH (53-83) also suggest that the antibody may be sensitive to some tertiary structure element present in hPTH (53-83) but at least partially absent in hPTH (69-84). This assay was mainly used to access the C-terminal structure of non-(1-84) PTH fragments. Although, it does not constitute the perfect tool, it remains the only one available to date to address this question.

The HPLC profiles of circulating and secreted PTH were relatively similar in all patients, with three main regions of immunoreactivity corresponding to hPTH (1-84) (region 1), N-PTH (region 2) and non-(1-84) PTH (regions 3 and 4). The non-(1-84) PTH region was divided into two different regions because hPTH (1-4) immunoreactivity was usually present in region 3 but absent in region 4. Region 1 reacted equally in all three PTH assays and contained a single peptide starting at position 1. The elution position of this peptide was identical to the one of standard hPTH (1-84), indicating similar hydrophobic properties. Region 2 reacted usually better in the hPTH (1-4) and (79-84) assays than in the hPTH (12-23) assay. This is expected because N-PTH is believed posttranslationally modified in region (15-20) [7]. Since the hPTH (12-23) assays has a fair amount of epitopes in that region, one would expect decreased immunoreactivity, which is not complete because epitopes proximal to position 15 and distal to position 20 are also present. The sequence of this peak revealed a major peptide starting at position 1 in all six cases, but also minor peptides starting at position 4 in two cases and at position 7 in two other cases. The last could contribute to some of the immunoreactivity detected by the hPTH (12-23) assay in these patients. Slightly more immunoreactivity was usually detected by hPTH (12-23) and hPTH (79-84) assays than by the hPTH (1-4) assay in region 3. Despite this, the major peptide in that region, the one with the most important ^{35}S-methionine signal, was a peptide starting at position 1 in 4 out of six cases. A peptide starting at position 7 had the most important signal in two cases, while a minor peptide starting at position 15 was also present in all patients. It is difficult to speculate on the structure of the peptide starting at position 1. It is either missing part of the C-terminal end structure or modified posttranslationally differently than N-PTH (peak 2) is. There might be a slight difference between results obtained with the hPTH (79-84) and the hPTH (12-23) PTH assays in favor of the latter, suggesting that all non-(1-84) PTH fragments may not have a structure going to position 84. More data

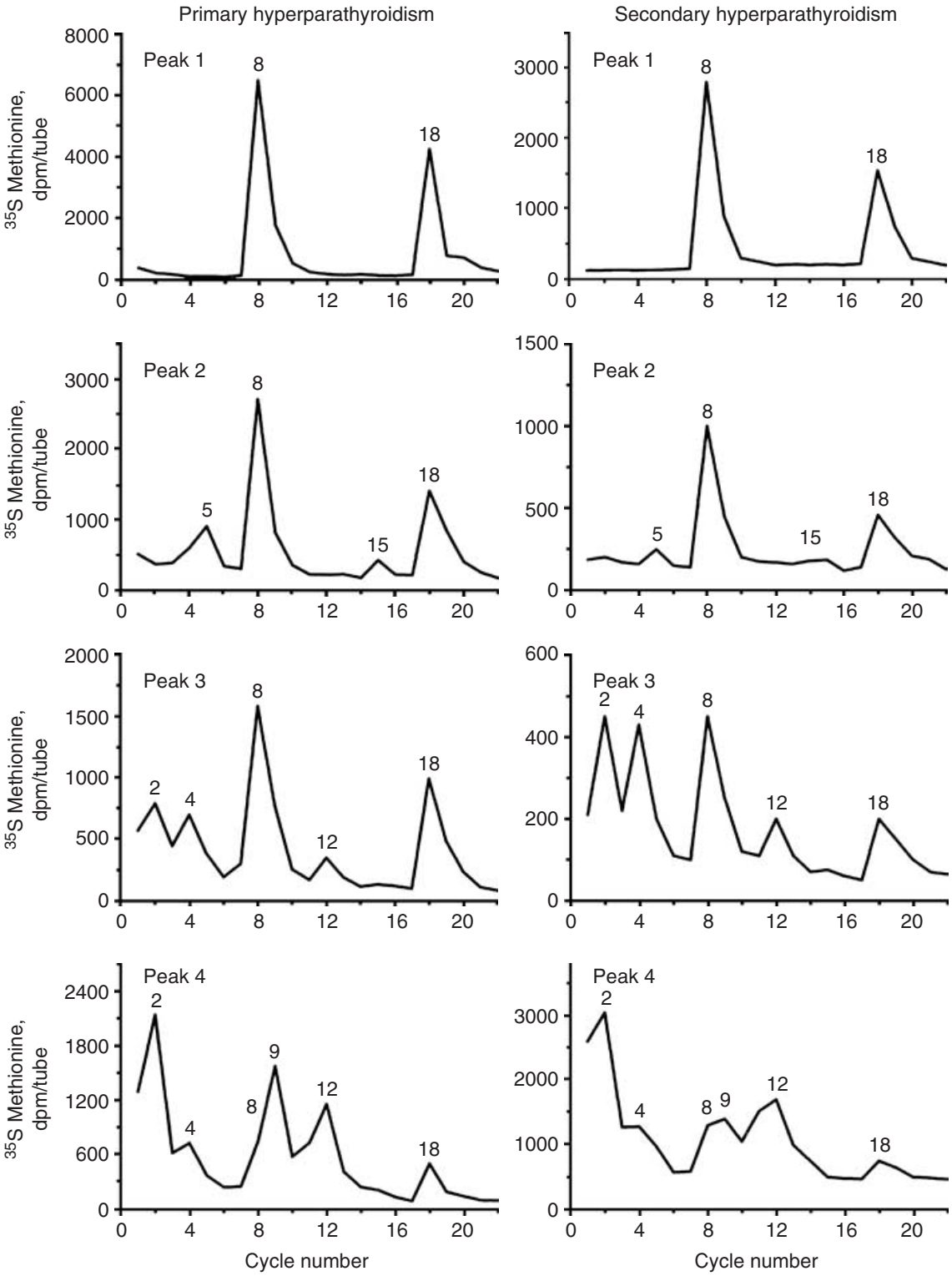

Fig. 3. Sequence analysis of internally labeled parathyroid hormone (PTH) molecular forms secreted by the parathyroid cells of a patient with primary hyperparathyroidism (left) and of a patient with secondary hyperparathyroidism (right). Peaks 1, 2, 3 and 4 refer to immunoreactive peaks identified in Figure 2 and corresponding to hPTH (1-84) (peak 1), N-PTH (peak 2), and non-(1-84) PTH (peaks 3 and 4).

are requested to address this point. Region 4 reacted both with the hPTH (12-23) and (79-84) assays but little with the hPTH (1-4) assay, as expected. Again immunoreactivity with the hPTH (12-23) assay may be slightly more important than with the hPTH (79-84) assay. Sequence analysis revealed the presence of a major signal corresponding to a peptide starting at position 7 in all cases. A minor signal was also present for a peptide starting at

Table 3. Summary of sequence analysis results

HPLC peaks	Sequence analysis of ^{35}S-residues	Starting position	Major signal (no/6)	Signal (no/6)
1	8 18 SVSEIQLMHNLGKHLNSMERV...	1	6/6	6/6
2	8 18 SVSEIQLMHNLGKHLNSMERV...	1	6/6	6/6
	5 15 EIQLMHNLGKHLNSMERV...	4	0/6	2/6
	2 12 LMHNLGKHLNSMERV...	7	0/6	2/6
3	8 18 SVSEIQLMHNLGKHLNSMERV...	1	4/6	6/6
	2 12 LMHNLGKHLNSMERV...	7	2/6	6/6
	4 LNSMERV...	15	0/6	6/6
4	8 18 SVSEIQLMHNLGKHLNSMERV...	1	0/6	6/6
	2 12 LMHNLGKHLNSMERV...	7	6/6	6/6
	1 11 MHNLGKHLNSMERV...	8	0/6	2/6
	9 HNLGKHLNSMERV...	10	0/6	3/6
	4 LNSMERV...	15	0/6	6/6

HPLC is high-performance liquid chromatography. 1, 2, 3, and 4 refer to HPLC peaks identified on HPLC profiles (Figure 2). Major signal indicates the most important signal by quantity of radioactivity. Signal indicates in how many patients studied the signal was present.

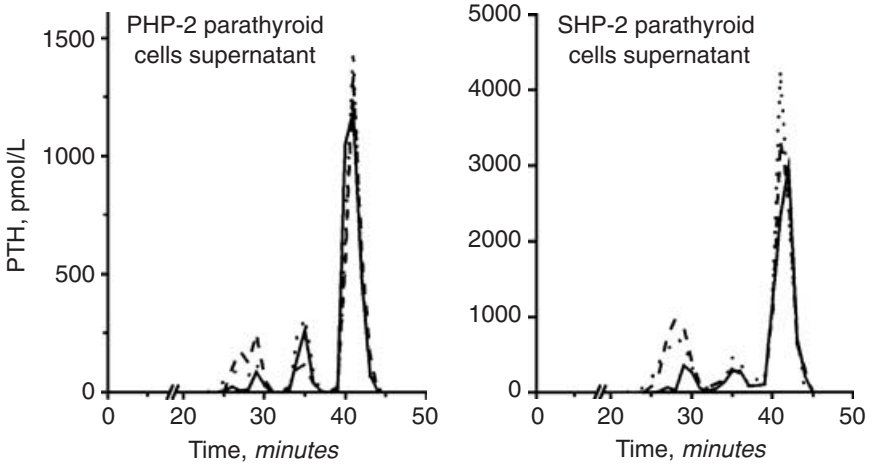

Fig. 4. Immunoreactivity of parathyroid hormone (PTH) molecular forms secreted by parathyroid cells and separated by high-performance liquid chromatography (HPLC) in three different PTH assays with (1-4) (.....), (12-23) (-----), and (79-84) (●●●●) epitopes.

position 15 in all cases, and for minor peptides starting at position 8 and 10 in two and three cases, respectively. We are very confident of sequence analysis results for peptides having two ^{35}S-methionine signals (those starting at positions 1, 4, 7, and 8) but less for those having a single signal (starting positions 10 and 15). Although unlikely, we cannot completely eliminate the presence of another ^{35}S-methionine labeled peptide interference in the last cases.

Overall, our results suggest that non-(1-84) PTH fragments constitute a family of fragments which have an N-structure starting at positions 4, 7, 8, 10, and 15, the

peptide starting at position 7 being the major fragment. We cannot be specific as to how exactly they are produced. It could be both by specific cleavage at given positions or progressive cleavage from the earlier to the latest position as seen during the peripheral metabolism of various ^{125}I-PTH preparations [4, 15].

The structure of non-(1-84) PTH fragments, as defined here, points to a likely interaction with the C-PTH receptor. This is based on the interaction of hPTH (7-84) and [tyr^{34}] hPTH (19-84) with the C-PTH receptor in various cell lines [8, 9, 18, 19]. It is more than likely that the biological data already obtained with these peptides (8-11) are applicable to the PTH peptides described here. This, in turn, justifies further studies to elucidate the role of these fragments in PTH biology.

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