THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 270, No. 16, Issue of April 21, pp. 9517–9525, 1995
Printed in U.S.A.

Proparathyroid Hormone Is Preferentially Cleaved to Parathyroid Hormone by the Prohormone Convertase Furin

A MASS SPECTROMETRIC STUDY*

(Received for publication, July 6, 1994, and in revised form, January 6, 1995)

Geoffrey N. Hendy‡§¶, Hugh P. J. Bennett‡, Bernard F. Gibbs||, Claude Lazure‡‡, Robert Day‡‡§§, and Nabil G. Seidah§§**

*From the Departments of ‡Medicine and §Physiology, McGill University and Royal Victoria Hospital, Montréal, Québec, H3A 1A1, ||Biotechnology Research Institute, Montréal, Québec, H4P 2R2, **Neuropeptides Structure and Metabolism Laboratory, and the §§J. A. DeSève Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montréal, University of Montréal, Québec, H2W 1R7 Canada*

Parathyroid hormone (PTH), an 84-amino acid peptide, is the major regulator of blood calcium homeostasis. Its mRNA, in addition to encoding the mature peptide, also encodes a "pre" sequence of 25 amino acids and a basic "pro" hexapeptide. To assess which of the subtilisin-like prohormone convertases can process proPTH to PTH we coinfected cells with a vaccinia virus construct expressing human preproPTH and vaccinia virus constructs expressing furin, PC1 or PC2. BSC-40 cells, having a constitutive secretory pathway, and GH4C1 cells, having a regulated secretory pathway, were used. PTH biosynthetic products in cell extracts and media were purified by high performance liquid chromatography, identified by radioimmunoassay, and unambiguously defined as either proPTH or PTH by ion-spray mass spectrometry. In both cell types, furin was the most effective in processing proPTH to PTH. In all cases only PTH was released into the medium. In addition, partially purified furin and PC1 were tested for their ability to appropriately cleave a tridecapeptide spanning the prohormone cleavage site found in proPTH. Here too furin was much more effective at cleaving at the correct site. Northern blot analysis and *in situ* hybridization showed that furin and preproPTH mRNA are co-expressed in the parathyroid, whereas PC1, PC2, and PC5 are not and PACE4 is expressed only at very low levels. Taken together these studies strongly suggest that furin is the enzyme responsible for the physiological processing of proPTH to PTH.

Parathyroid hormone (PTH),[1] an 84-amino acid polypeptide, is the major regulator of blood calcium homeostasis. Its messenger RNA, in addition to encoding the mature peptide, also encodes a "pre" or signal sequence of 25 amino acids and a basic "pro" peptide of 6 amino acids (1). The signal sequence facilitates entry of the nascent peptide chain into the endoplasmic reticulum and following entry into the intracisternal space this peptide sequence is cleaved. ProPTH is then transported to the *trans*-Golgi network where the propeptide is removed followed by packaging of PTH into secretory granules (2). The enzyme or enzymes responsible for this latter cleavage are unknown. However, several mammalian subtilisin-like serine endoproteases have recently been described which can function to process proproteins by cleaving at pairs of basic residues. These include furin (PACE) (3, 4) PC1 (PC3) (5–7), PC2 (5, 8, 9), PACE4 (10), PC4 (11, 12), and PC5 (PC6) (13, 14). Furin has a neutral pH optimum and contains a transmembrane region and is thought to function in the *trans*-Golgi network, whereas PC1 and PC2 have more acidic pH optima and are thought to act within secretory granules.

In order to assess which of these enzymes can process proPTH to PTH we coinfected cultured cell lines with a vaccinia virus construct expressing human (h) preproPTH and vaccinia virus constructs expressing either furin, PC1 or PC2. BSC-40 cells, having a constitutive secretory pathway, and GH4C1 cells which, additionally, have a regulated secretory pathway, were used. PTH biosynthetic products were purified by HPLC and identified by mass spectroscopy.

In addition, partially purified preparations of furin and PC1 were tested for their ability to appropriately cleave a 13-amino acid peptide spanning the prohormone cleavage site in proPTH. To assess which of the processing enzymes are expressed in the parathyroid, Northern blot analysis and *in situ* hybridization were conducted on parathyroid tissue.

The coinfection studies revealed that in both cells of the constitutive and regulated pathway furin was the most effective at processing proPTH to PTH. Colocalization studies showed that furin is expressed in the parathyroid whereas PC1 and PC2 are not. Moreover, hfurin processes the −6 to +7 sequence of hproPTH very efficiently *in vitro*. Therefore, furin is most probably the enzyme responsible for the physiological processing of proPTH to PTH.

EXPERIMENTAL PROCEDURES

Vaccinia Virus Constructs

The purified recombinant vaccinia viruses (VV) used were as follows. The vaccinia virus recombinant of human proPTH (VV:hPTH) was prepared using a full-length human (h) preproPTH cDNA (15), VV:mPC1 used the full-length cDNA insert of mouse PC1 (6), VV:mPC2 was constructed from the full-length cDNA insert of mouse PC2 (5). The recombinant VV:hfurin was prepared using the cDNA of human furin (a

* This work was supported in part by Medical Research Council of Canada Grants MT-9315, MT-6733, and PG-11474. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

A preliminary report of this work was presented at the 76th Annual Meeting of the Endocrine Society, Anaheim, CA. June 15–18, 1994.

‡‡ Scholar of the Fonds de la recherche en Santé du Québec (FRSQ).

¶ Scientist of the Medical Research Council of Canada. To whom correspondence should be addressed: Calcium Research Laboratory, Rm. H4.67, Royal Victoria Hospital, 687 Pine Ave. West, Montreal, QC, H3A 1A1 Canada. Tel.: 514-843-1632; Fax: 514-982-0872.

[1] The abbreviations used are: PTH, parathyroid hormone; proPTH, proparathyroid hormone; preproPTH, preproparathyroid hormone; h, human; m, mouse; r, rat; i, immunoreactive; POMC, proopiomelanocortin; RP, reversed-phase; HPLC, high performance liquid chromatography; C_3F_7COOH, heptafluorobutyric acid; CF_3COOH, trifluoroacetic acid; MCA, 4-methylcoumaryl-1-amide; AMC, 7-amino-4-methylcoumarin; CgA, chromogranin A.

gift from Dr. A. Rehemtulla, Genetics Institute) subcloned into the pVV3 transfer vector as reported for VV:mPC1 and VV:mPC2 (16). The vaccinia virus recombinant of mouse proopiomelanocortin (VV: mPOMC) was a gift from Dr. G. Thomas (Vollum Institute, Portland, OR).

Vaccinia Virus Infections

Cells, either BSC-40, an African green monkey epithelial-like cell line, or GH4C1, a rat pituitary tumor cell line, were infected with a mixture of VV:hPTH and either VV:mPOMC (control), VV:hfurin, VV: mPC1, or VV:mPC2, as described previously (17). After the infection period, the inoculum was replaced with Dulbecco's modified Eagle's medium and cells were incubated for 17 h at 37 °C. The cells were then incubated in Dulbecco's modified Eagle's medium containing 0.01% bovine serum albumin for 3 h after which cells and medium were harvested for further analysis.

Reversed-phase High Performance Liquid Chromatography of Culture Media and Cell Extracts

Reversed-phase high performance liquid chromatography (RP-HPLC) of culture media and cell extracts was performed using C_{18} μ-Bondapak (Waters) and C_{18} Vydac TP201 (Cole Parmer, Chicago) columns, as described previously (18).

Both culture media and cell extracts were separately subjected to an initial RP-HPLC step using the μBondapak column which was eluted over 1 h with a linear gradient of 28–48% aqueous acetonitrile containing 0.13% (v/v) heptafluorobutyric acid (C_3F_7COOH) at a flow rate of 1.5 ml/min. This gradient was chosen since PTH and all known parathyroid cell derived fragments of PTH elute from the column under these conditions (19). The presence of immunoreactive PTH in column fractions was determined by radioimmunoassay with a goat antiserum raised against hPTH-(1–84) and employing ^{125}I-Tyr^{43}-hPTH-(44–68) as tracer. ProPTH and PTH cross-react identically in this radioimmunoassay. We previously determined that both proPTH and PTH are recovered with 80–85% efficiency through successive RP-HPLC steps (20, 21). In the present studies, culture media derived from BSC-40 and GH4C1 cells generated chromatograms in which immunoreactive PTH coincided with single peaks of UV absorbance. For these experiments fractions were dried and subjected to mass spectrometric analysis without further purification. For all cell extracts further RP-HPLC procedures were required to obtain material of sufficient purity for mass spectrometry. For these experiments fractions containing immunoreactive PTH were first subjected to RP-HPLC using the Vydac column which in each case was eluted over 1 h with a linear gradient of 8–48% aqueous acetonitrile containing 0.1% (v/v) CF_3COOH at a flow rate of 1.5 ml/min. At this stage all samples derived from BSC-40 cell extracts yielded chromatograms where PTH immunoreactivity was associated with single peaks of UV absorbance. These materials were subjected to mass spectrometric analysis. Further chromatography of material with PTH immunoreactivity derived from extracts of GH4C1 cells was required prior to mass spectrometry. In each case the PTH immunoreactivity could be resolved into two fractions which were subjected separately to RP-HPLC on the μBondapak column which was in each case eluted over 1 h with a linear gradient of 16–56% aqueous acetonitrile containing 0.1% CF_3COOH at a flow rate of 1.5 ml/min.

Ion Spray Mass Spectrometry

Ion spray mass spectra of purified peptides were obtained using an API III triple stage mass spectrometer (Sciex, Thornhill, Ontario, Canada). Mass estimations were done essentially as described previously (22). The lyophilized peptide samples were redissolved in 10% acetic acid and infused through a stainless steel capillary (100-μm internal diameter) at a flow rate of 1 μl/min. A stream of air was introduced to assist in the formation of submicron droplets (23). These droplets were evaporated at the interface by nitrogen gas, producing a series of multiply charged ions which are mass analyzed. Simple algorithms correlate the charges produced by peptides and proteins to their molecular weights. The mass to charge ratio (m/z) of each of these ions produces a molecular weight estimate. These estimates are averaged to give an observed mass.

In Vitro Cleavage of Human ProPTH Peptide

Recombinant hPC1 and hfurin—The two human endoproteases, hPC1 and hfurin, were obtained from the medium of somatomammotroph GH4C1 cells following infection with the respective recombinant vaccinia virus as described elsewhere (24). The two proteases were partially purified using anion exchange chromatography prior to use and the enzymatic activity of each recombinant enzyme was assayed using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA (Peptides International, Louisville, KY).

Digestion of Synthetic Human ProPTH Peptide by hPC1 and hfurin—The synthetic tridecapeptide (proPTH(−6-+7)), corresponding to amino acids −6 to +7 of the human proPTH molecule (with +1 designating the first amino acid of the mature 84-amino acid molecule), was kindly synthesized and purified by Drs. S. Sakakibara and T. Kimura (Peptide Institute, Osaka, Japan). A 100-μg sample was incubated for various time intervals with 18 μl of purified hfurin (containing an activity of 50 nmol/h of released AMC from pGlu-Arg-Thr-Lys-Arg-MCA (70 μM)) at 25 °C in 300 μl of 50 mM sodium acetate, pH 7.0, containing 2 mM $CaCl_2$ and 1 μM guanidinoethylmercaptosuccinic acid (Calbiochem Corp., La Jolla, CA). Similarly, incubations were also done using 50 μl of purified hPC1 (corresponding to an activity of 50 nmol/h of released AMC from pGlu-Arg-Thr-Lys-Arg-MCA (70 μM)) at 25 °C in 300 μl of 50 mM sodium acetate, pH 6, containing 5 mM $CaCl_2$ and 1 μM guanidinoethylmercaptosuccinic acid. All fluorescence measurements were made with a Perkin-Elmer MPF-3L spectrofluorometer using an excitation wavelength set at 370 nm and an emission wavelength set at 460 nm in order to measure the AMC released, as described previously (25).

Following incubation for 0, 30, 60, 90, 120, and 240 min, identical aliquots were taken, acidified with 50 μl of acetic acid, and analyzed on an Exsil 300A/ODS column (25 cm × 0.46 cm). The buffer system consisted of an aqueous 0.1% (v/v) CF_3COOH solution and an organic phase containing 0.1% (v/v) CF_3COOH in acetonitrile. Elution was carried out by using a linear gradient of 1% organic phase/min following a 5-min initial isocratic step; the flow rate was 1 ml/min. The elution pattern was monitored by measuring the UV absorbance at 225 nm. The peptides detected were automatically collected and kept at −20 °C until characterized by amino acid analysis as described previously (26).

Determination of Kinetic Constants K_m and V_{max}—In order to compare these constants between small fluorogenic peptidyl substrates and those obtained with the tridecapeptide, a fluorogenic substrate corresponding to benzylocarbonyl (*Z*)-Val-Lys-Lys-Arg-MCA (Enzyme System Products, Livermore, CA) was used. Incubations for determination of kinetic constants were typically carried out in the presence of various concentrations of fluorogenic peptide substrates from 20 μM to 2 mM for each enzyme in the above mentioned buffers at 25 °C for 4 h before stopping the reaction by the addition of 50 μl of acetic acid. The amount of AMC released was determined by spectrofluorometry.

Determination of kinetic constants for the proPTH peptide was accomplished by reversed-phase HPLC analysis followed by integration of the peak area corresponding to the released COOH-terminal fragment Ser-Val-Ser-Glu-Ile-Gln-Leu. Various concentrations of the proPTH peptide ranging from 10 μM to 400 μM were incubated in the presence of either hPC1 or hfurin in a total volume ranging from 400 to 30 μl keeping the enzyme concentration constant. Following incubation for 90 min (hPC1) or 15 min (hfurin), the amount of the released COOH-terminal fragment, Ser-Val-Ser-Glu-Ile-Gln-Leu, was determined after RP-HPLC, as described above, and by amino acid analysis. The K_m and V_{max} values were determined after curve-fitting using Sigmaplot wv 1.02 software (Jandel Scientific, San Rafael, CA).

Northern Analysis

Total RNA was prepared from bovine parathyroid glands by the guanidinium isothiocyanate/cesium chloride method. Ten-microgram aliquots of RNA were electrophoresed on 1.1% agarose-formaldehyde gels, blotted onto Nylon membranes, and hybridized with random primer ^{32}P-labeled probes as described elsewhere (27). The probes used were a 2.2-kilobase *Sma*I-*Sal*I restriction fragment encoding a human furin cDNA (gift of Dr. A. Rehemtulla, Genetics Institute), a 325-base pair rat PC1 cDNA corresponding to the segment 715-1039 of mPC1 (5, 6), a 753-base pair rat PC2 cDNA corresponding to the segment 559-1326 of mPC2 (5), and a 534bp rPACE4 cDNA corresponding to the segment 1153–1687 of hPACE4 (10).

In Situ Hybridization

Rat thyroids including the parathyroid glands were frozen in isopentane at −35 °C. Frozen 10-μm sections were cut on a cryostat, thaw-mounted on polylysine-coated glass slides, and stored at −70 °C until processed for *in situ* hybridization as described previously (28). Hybridization was carried out with a digoxigenin-labeled UTP cRNA probe (PTH) or with ^{35}S-UTP-labeled cRNA probes (prohormone convertases). Antisense cRNA probes consisted of a 430-nucleotide rat (r) PTH probe (29) kindly provided by Dr. G. Heinrich, a 590-nucleotide rat (r) PC1 probe equivalent to segment 1841–2430 in mouse (m) PC1 (6); a 425

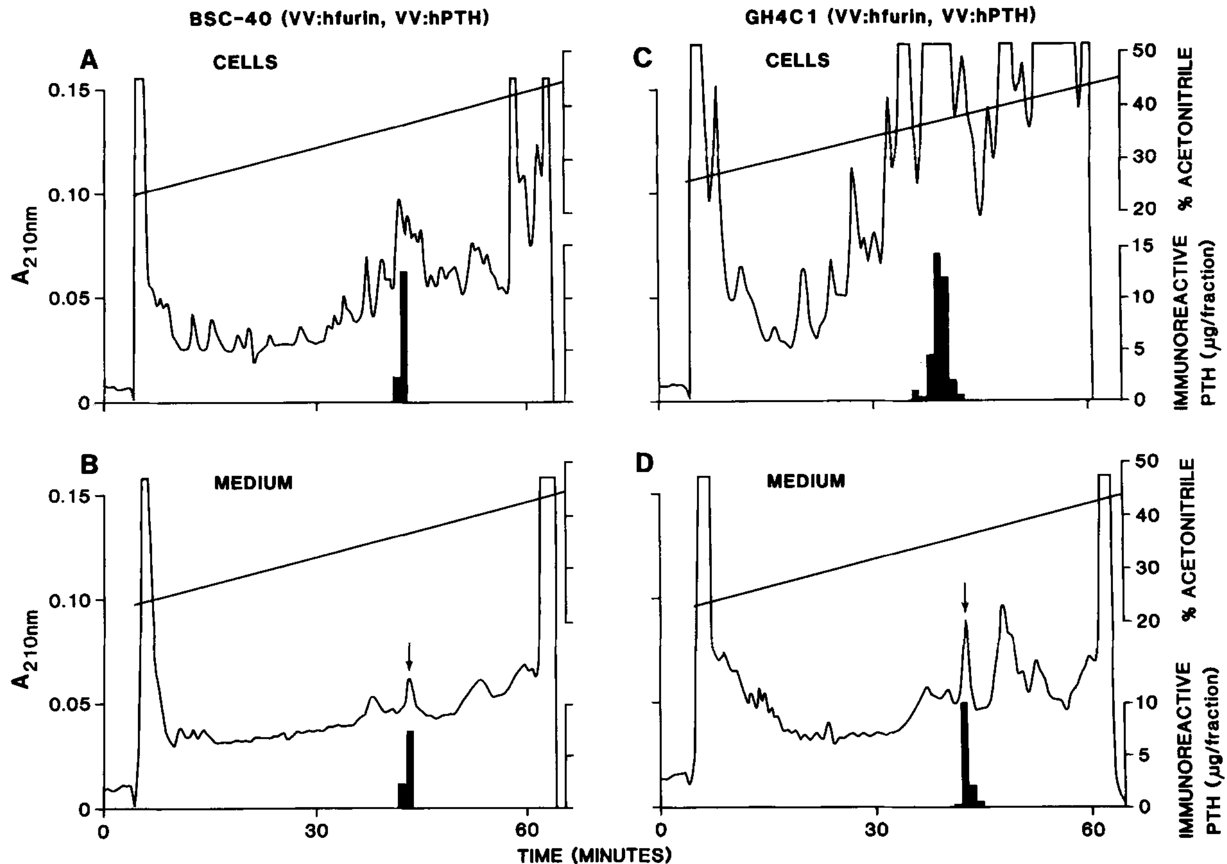

FIG. 1. **Reversed-phase HPLC of cell extracts and media derived from cultures of BSC-40 cells (*Panels A and B*) and GH4C1 cells (*Panels C and D*) infected with VV:hfurin and VV:hPTH.** In each instance the column was eluted with a linear gradient of aqueous acetonitrile containing 0.13% (v/v) C_3F_7COOH throughout. Column eluates were monitored for UV absorbance at 210 nm (*continuous line*) and column fractions were assayed for the presence of immunoreactive hPTH (*solid bars*). Note that for the elution profiles of media samples (*Panels B* and *D*), a peak of PTH immunoreactivity coincided with a single peak of UV absorbing material (*arrow*) which was positively identified as hPTH using mass spectrometric analysis (see Fig. 2).

nucleotide rPC2 probe equivalent to segment 1574–1998 in mPC2 (5); a 1232-nucleotide rfurin probe equivalent to segment 823-2053 in human (h) furin (4); a 534-nucleotide rPACE4 probe equivalent to the segment 1153–1687 of hPACE4 (10); and a 837 nucleotide rPC5 segment 1089–1925 (13).

The hybridization conditions were as follows. The cRNA probes were diluted in hybridization buffer (75% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate, pH 7.4, 1 × Denhardt's solution, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml salmon sperm DNA) with the addition of 1 mM dithiothreitol. The slides were incubated for 16 h at 55 °C. After hybridization the coverslips were removed and the sections were treated with RNase A (40 μg/ml) for 45 min at 37 °C and then washed sequentially in 2 ×, 1 × and 0.5 × SSC for 10 min, followed by a 1-h wash in 0.1 × SSC at 60 °C. After dehydration the sections were air-dried and subsequently dipped in emulsion, and exposure times ranged from 10 to 15 days. Slides to which digoxigenin-labeled UTP cRNA probe was hybridized were treated as follows. Each section was incubated 2 h with a solution of 2 × SSC, 0.05% Triton X-100, 2% normal sheep serum. The sections were washed in 100 mM Tris, pH 7.5, 150 mM NaCl followed by a 5-h incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (diluted 1:1000 in 100 mM Tris, pH 7.5, 150 mM NaCl, 0.3% Triton X-100, 1% normal sheep serum). The sections were washed two times for 10 min each in 100 mM Tris, pH 7.5, 150 mM NaCl and once in 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. This was followed by an overnight incubation in chromogen solution, consisting of 45 ml of nitro blue tetrazolium (75 mg/ml in dimethylformamide), 35 ml of 5-bromo-4-chloro-3-indolyl-phosphate, and 2.4 mg of levamisole per 10 ml of 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. Incubation was performed in humidified chambers in the dark. The chromagen reaction was stopped in 10 mM Tris, pH 8.0, 1 mM EDTA. Controls for specificity of hybridization were carried out using sense strand cRNA probes of similar length and specific activity or by pretreatment of the sections with RNase A.

RESULTS

Coexpression of ProPTH and Prohormone Convertases in BSC-40 and GH4C1 Cells, Effect of Furin, PC1, and PC2 on ProPTH Processing—Purification of immunoreactive (i) PTH moieties from both cell and media extracts of BSC-40 and GH4C1 cells was readily accomplished by reversed phase RP-HPLC. On initial chromatography using an acetonitrile/ C_3F_7COOH solvent system for both cell extracts and media of BSC-40 cells, iPTH eluted as a major peak at 38% acetonitrile (Fig. 1, *A* and *B*). This is the expected elution position of both proPTH and PTH in this system. A similar elution pattern was seen when GH4C1 cell extracts and media were purified using the same solvent system (Fig. 1, *C* and *D*). Insignificant amounts of iPTH eluted either before or after this major peak. This was observed in cells and medium for both the control (coinfection with VV:mPOMC) and experimental (coinfection with VV:hfurin, VV:mPC1 and VV:mPC2) situations, indicating that none of the enzymes tested caused significant fragmentation of the PTH molecule at potential internal cleavage sites. With medium from BSC-40 cells (Fig. 1*B* and data not shown), a single absorbance peak coeluted with the major iPTH peak. These fractions were pooled and subjected to mass spectrometric analysis (Fig. 2). In all cases only PTH itself was found to be released from the cells; *i.e.* no proPTH was detectable in the medium.

In the case of BSC-40 cell extracts, the fractions obtained from the initial RP-HPLC using C_3F_7COOH as counterion, and which correspond to the major peak of iPTH, were pooled and

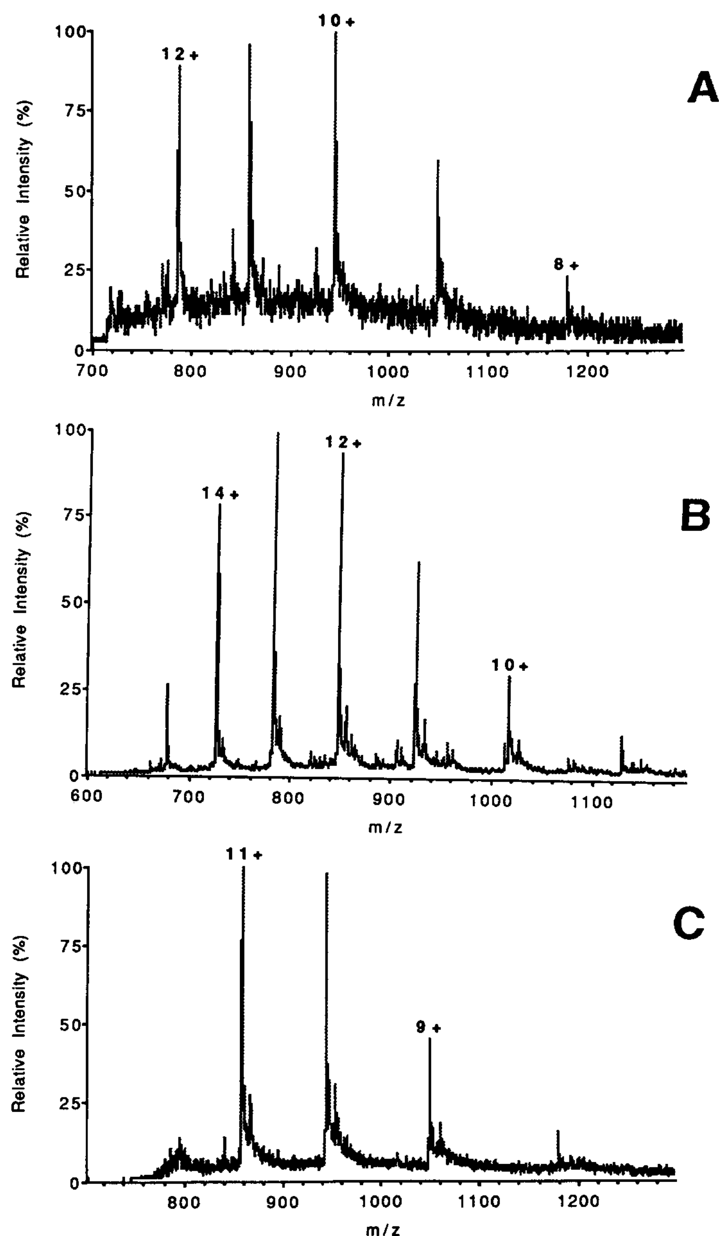

FIG. 2. **Selected examples showing ion-spray mass spectra of immunoreactive PTH purified from extracts of the medium (*A*) and cells (*B* and *C*) derived from cultured GH4C1 cells infected with vaccinia virus constructs bearing PTH and POMC (control) cDNAs.** All major immunoreactive species isolated from both BSC-40 and GH4C1 cells and media were identified as either proPTH or PTH by mass spectrometric analysis as described under "Experimental Procedures." This allowed unequivocal assignment of the molecular forms to be made. All values agreed within two mass units of the theoretical values for hproPTH (10,151 Da) and hPTH (9,424 Da) (15). *Panel A* shows the mass spectrum of PTH from the culture medium (M_r = 9,424.8). *Panels B* and *C* show the mass spectra of proPTH (M_r = 10,152.4) and PTH (M_r = 9,425.9), respectively, purified from cell extracts. In each case the molecular weight (M_r) was calculated from the observed mass values of multiply charged ion signals using the equation $M_r = nm - n$, where n is the number of positive charges and m is the mass signal bearing n positive charges.

rechromatographed using a solvent system containing C_3F_7COOH. Absorbance peaks which were positive by radioimmunoassay were individually collected and analyzed as described above. This defined the absorbance peaks as representing either proPTH or PTH (Fig. 2). In BSC-40 cell extracts, both proPTH and PTH were present (Fig. 3). In the BSC-40 cells coinfected with either VV:POMC (control) or VV:PC2, the least amount of processing was observed (Table I). Processing of proPTH in cells coinfected with VV:PC1 was not different from that in the control, however, in cells coinfected with furin, processing was markedly stimulated being almost three times that in control cells (Table I).

In profiles derived from the GH4C1 medium (Fig. 4), as for BSC-40 medium, a single absorbance peak coeluted with the major peak of iPTH. These fractions were pooled and subjected to mass spectrometric analysis (Fig. 2). Again, as in the case of medium derived from BSC-40 cells, only the PTH molecular species was found in the medium and no proPTH. It was of interest to note in the medium of both BSC-40 and GH4C1 cells infected with the VV:POMC construct an absorbance peak eluting at an acetonitrile concentration of 45% and having endorphin immunoreactivity (see Fig. 4). This corresponds to POMC itself.

For the GH4C1 cell extracts, after the first chromatography step using a C_3F_7COOH solvent system (see Fig. 1*C*), and a second step using solvents containing CF_3COOH (not shown), two pools corresponding in elution positions to proPTH and PTH were separately rechromatographed using the CF_3COOH solvent system (data not shown). Absorbance peaks were individually collected and those positive in the PTH radioimmunoassay were analyzed as described and defined as either proPTH or PTH (Fig. 2). In GH4C1 cells little processing was evident in VV:PC2 coinfected cells, the ratio of PTH to proPTH being similar to that of control VV:POMC coinfected cells. However, this ratio was approximately twice that of the control in cells coinfected with VV:PC1, and more than 400% of control in cells coinfected with VV:furin (Table I).

Thus in cells having a constitutive secretory pathway only (*i.e.* BSC-40) and in cells having both constitutive and regulated secretory pathways (*i.e.* GH4C1), exogenous expression of furin markedly stimulated processing of proPTH to PTH (Table I). In addition, exogenous expression of PC1 stimulated

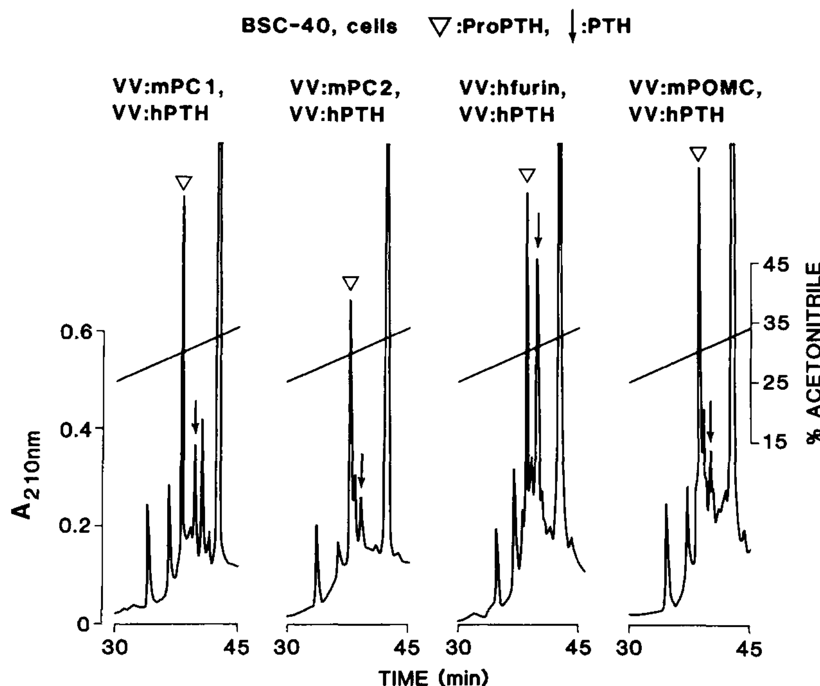

FIG. 3. **Final reversed-phase HPLC of extracts of BSC-40 cells infected with VV:hPTH and (*a*) VV:PC-1, (*b*) VV:PC-2, (*c*) VV:furin, or (*d*) VV:POMC (control).** Cell extracts were subjected to initial reversed-phase chromatography using solvents containing 0.13% (v/v) C_3F_7COOH. Fractions containing PTH immunoreactivity were subjected to further chromatography (shown here). In each instance the column was eluted with a linear gradient of aqueous acetonitrile containing 0.1% (v/v) CF_3COOH throughout and column eluates were monitored for UV absorbance at 210 nm (*continuous line*). Column fractions were assayed for the presence of immunoreactive hPTH and in each case two peaks of immunoreactive hPTH were found to correspond to two peaks of UV absorbing material. These corresponded in elution position to hproPTH (*open arrowhead*) and hPTH (*arrow*), and were identified as such by mass spectrometric analysis (see Fig. 2).

TABLE I
Conversion of proPTH to PTH

Cells	Ratio of PTH to proPTH in VV:hPTH			
	+VV:mPOMC	+VV:hfurin	+VV:mPC1	+VV:mPC2
BSC-40	100[a]	286	110	86
GH4C1	100	417	183	108

[a] All values are expressed relative to the PTH/proPTH ratio in control (VV:hPTH; VV:mPOMC) cells which is set at 100%. For BSC-40 (VV:hPTH; VV:mPOMC) PTH/proPTH equals 0.29 and for GH4C1 (VV:hPTH; VV:mPOMC) PTH/proPTH equals 0.12.

proPTH processing in GH4C1 cells.

In Vitro Cleavage of ProPTH Peptide with hPC1 and hfurin—In order to study the cleavage of proPTH by hfurin and hPC1 further, the tridecapeptide proPTH(−6→+7) as well as a small related synthetic fluorogenic substrate carbobenzoxy-Val-Lys-Lys-Arg-MCA were used as model peptides. As shown in Table II, this small synthetic substrate, lacking the basic residue at P6 present in the tridecapeptide, failed to discriminate between hfurin and hPC1 as, in both cases, similar V_{max}/K_m values were obtained. Thus, despite representing the sequence from P4 to P1 of proPTH, it was less efficiently cleaved by either enzyme than the pGlu-Arg-Thr-Lys-Arg-MCA substrate as shown previously (24). This is not unexpected as it does not contain the P4 Arg residue present in the latter sequence.

In contrast, digestion of the synthetic proPTH peptide by equal amounts (as assessed using the same fluorogenic substrate) of hfurin or hPC1 enzymatic activity clearly demonstrated as shown in Fig. 5 that the full proPTH recognition sequence is very efficiently cleaved by hfurin as compared to hPC1. Whereas the half-maximal value of appearance of the COOH-terminal fragment was 154 min with hPC1, under the same conditions this was only 28 min for hfurin. In order to assess whether this was due to a better recognition of the substrate, or an increase in catalytic efficiency or both, the kinetic constants were determined with each enzyme. As shown in Fig. 6, purified hPC1 exhibited an apparent K_m value of 79 ± 14 μM and V_{max} value of 34.8 ± 2.5 nmol of the COOH-terminal fragment/h, whereas, purified hfurin exhibited apparent values of 23 ± 8 μM and 129 ± 10 nmol of the COOH-terminal fragment/h, respectively. As shown in Table II, the major difference observed lies in the significant increase of the V_{max} observed upon incubation of the tridecapeptide with hfurin; indeed, this value is 3–100-fold higher than that for all other substrates analyzed with either hfurin or hPC1. Thus, compared to hPC1 the increased catalytic efficiency of hfurin with the proPTH tridecapeptide, appears to be due to both an increase in the apparent affinity for the substrate (lower K_m), and an increase in the rate of processing of the substrate (higher V_{max}).

Expression of Prohormone Convertase mRNAs in Parathyroid Tissue Assessed by Northern Blot Analysis—As shown in Fig. 7 furin transcripts were identified in both parathyroid (bovine parathyroid gland) and thyroid (medullary thyroid cell line (TT)), whereas, only thyroid contained PC1 and PC2 transcripts, with them being absent in parathyroid. PACE4 mRNA was expressed at low levels in both parathyroid and thyroid.

Expression of Prohormone Convertase mRNAs in Parathyroid Tissue Assessed by in Situ Hybridization—As shown in Fig. 8 furin mRNA is colocalized in PTH-expressing cells in the rat parathyroid gland. These cells do not express PC1, PC2, or PC5 mRNA, but do express, at a very low level, PACE4 mRNA.

DISCUSSION

The isolation and characterization of several mammalian subtilisin-like serine endoproteases which cleave carboxyl-terminal to pairs of basic amino acids has added a new dimension to the analysis of proprotein processing (30–34). These enzymes include furin and PC1 (PC3) and PC2. Furin has a wide tissue distribution, and is localized in the *trans*-Golgi network (35). It has a neutral pH optimum and functions within the constitutive secretory pathway. In contrast, PC1 and PC2 function within the regulated secretory pathway. These latter enzymes are expressed only in endocrine and neural tissues and have been localized to the secretory granule (36). These proteases display acidic pH optima consistent with the secretory granule environment.

Although proPTH was one of the first prohormones to be described (37, 38) and its processing to PTH has been extensively analyzed, no study to date has addressed the issue of which of the processing enzymes is responsible for its cleavage.

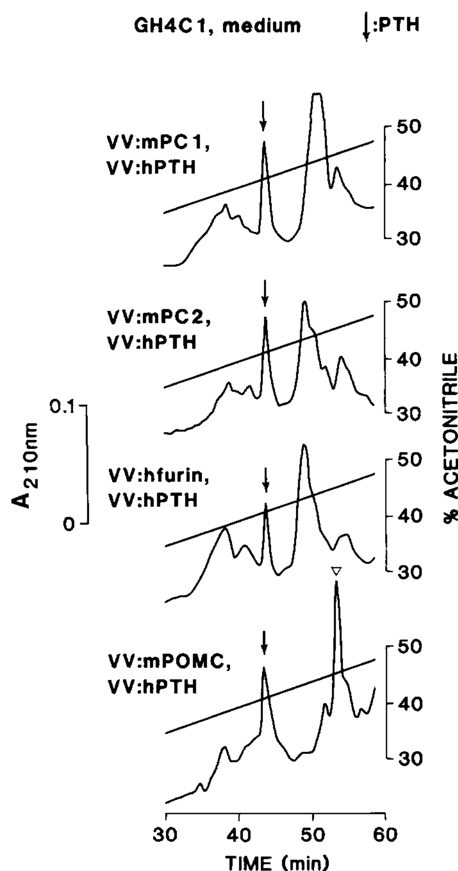

FIG. 4. **Reversed-phase HPLC of media derived from cultured GH4C1 cells infected with VV:hPTH together with (*a*) VV:PC-1, (*b*) VV:PC-2, (*c*) VV:furin, or (*c*) VV:POMC (control).** The column was eluted in each instance with a linear gradient of aqueous acetonitrile containing 0.13% (v/v) C_3F_7COOH and column eluates were monitored for UV absorbance at 210 nm (*continuous line*). Column fractions were assayed for the presence of immunoreactive hPTH and in each case a peak of hPTH immunoreactivity was found to coincide with a single peak of UV absorbing material (*arrow*) which was positively identified as hPTH using mass spectrometric analysis (see Fig. 2). In the control panel the peak of endorphin immunoreactivity corresponding to POMC is indicated by the *open arrowhead*.

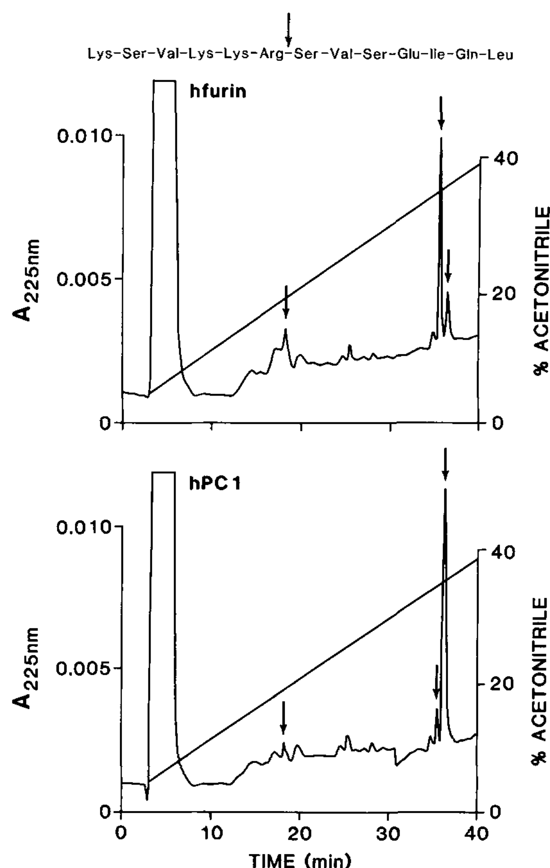

FIG. 5. **RP-HPLC chromatogram of the products obtained following proteolytic digestion of proPTH(−6-+7) by partially purified hfurin or hPC1.** Incubation of the tridecapeptide for 60 min with either hfurin (*upper panel*) or hPC1 (*lower panel*) enzymatic activity was carried out as described under "Experimental Procedures." Identification of the peaks was made by amino acid analysis. The peak eluting at 18 min is Lys-Ser-Val-Lys-Lys-Arg, the peak eluting at 35 min is the Ser-Val-Ser-Glu-Ile-Gln-Leu fragment, and the peak eluting at 36 min is the undigested proPTH(−6-+7) peptide.

TABLE II
Kinetic constants for cleavage of various flurogenic peptide substrates and synthetic hproPTH (−6-+7) with hPCl and hfurin

Compound[a]	K_m	V_{max}	V_{max}/K_m	Enzyme
	μM	*nmol/h*		
acRSKR-MCA	45	3.1	0.07	hPC1[b]
bocRVRR-MCA	572	18.6	0.03	hPC1[b]
pERTKR-MCA	20	2.2	0.11	hPC1[b]
CbzVKKR-MCA	125	2.2	0.02	hPC1[c]
hproPTH(−6-+7)	79	34.8	0.44	hPC1[c]
bocRVRR-MCA	52			hfurin[d]
pERTQR-MCA	176			hfurin[d]
pERTKR-MCA	6			hfurin[d]
CbzVKKR-MCA	70	1.3	0.02	hfurin[c]
hproPTH(−6-+7)	23	129.4	5.60	hfurin[c]

[a] ac, acetyl; boc, *t*-butoxycarbonyl; p, pyro; Cbz, carbobenzoxy.
[b] Jean *et al.* (24).
[c] This study.
[d] Hatsuzawa *et al.* (42).

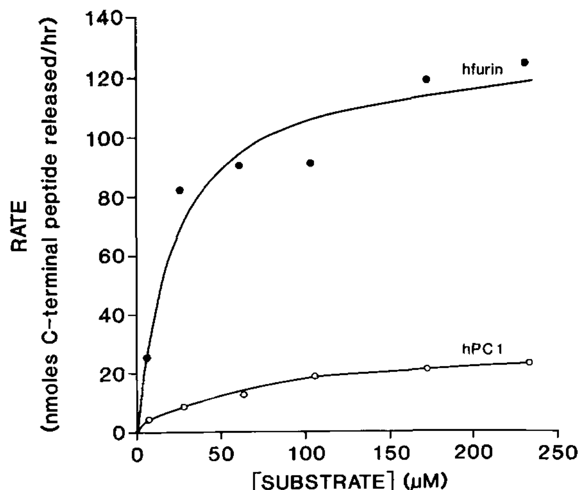

FIG. 6. **Graphical determination of the kinetic constants K_m and V_{max} of hfurin and hPC1 using the synthetic hproPTH(−6→+7) tridecapeptide as substrate.** The rate of release of the COOH-terminal fragment was determined as described under "Experimental Procedures."

The conversion of endogenously labeled proPTH to PTH was previously shown to take place within 15 min of synthesis in the *trans*-Golgi in cultured bovine parathyroid gland slices *in vitro* (2). Complementary DNA encoding preproPTH was stably introduced into rat fibroblast NIH 3T3 cells and the rat pituitary cell line GH4C1 with a recombinant retrovirus (39). In both these cell-lines proPTH was appropriately cleaved to PTH, and only PTH was released into the medium. Therefore, it

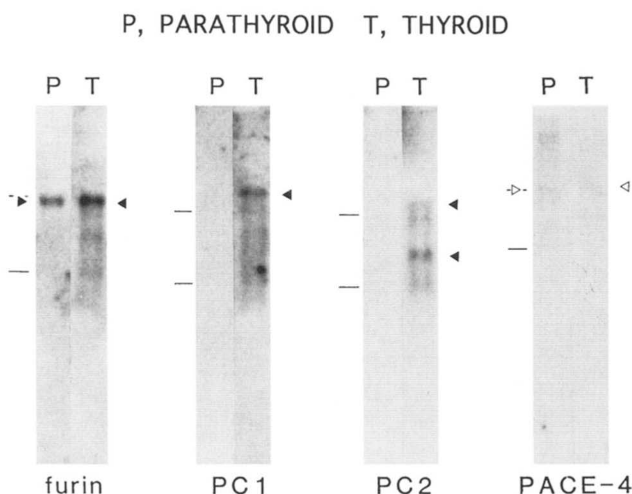

FIG. 7. **Northern blot analysis of total RNA from bovine parathyroid gland (*P*) and a human medullary thyroid carcinoma TT cell line (*T*).** Blots were hybridized with furin, PC1, PC2, or PACE4 probes as described under "Experimental Procedures."

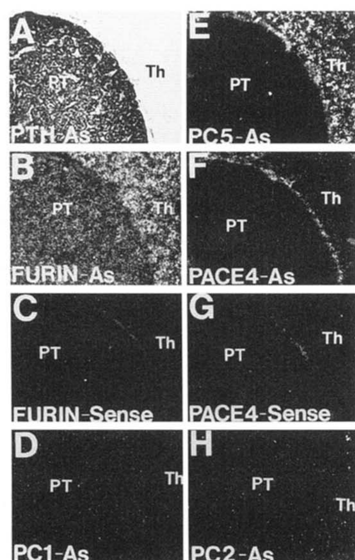

FIG. 8. **In situ hybridization histochemistry of the rat parathyroid gland with neighboring thyroid tissue shown for comparison.** Localization of *A*, PTH; *B*, furin; *D*, PC1; *E*, PC5; *F*, PACE4; and *H*, PC2 mRNAs were made in adjacent sections of parathyroid gland using antisense cRNA probes as described under "Experimental Procedures." Sense-strand controls are shown in *C*, furin-sense, and in *G*, PACE4-sense. Note that the PTH cRNA probe shown in *A* was labeled with digoxigenin, whereas the other probes were labeled with ^{35}S-UTP. Thus, a positive signal in *A* is shown by the *dark areas*, but in all other frames (*B–H*) is represented by the *white* autoradiographic grains shown against a *dark background*. Abbreviations: *PT*, parathyroid; *Th*, thyroid. Magnification is ×50.

could be speculated on two counts, that the enzyme responsible for cleaving proPTH to PTH was furin-like. First, proPTH is processed in the *trans*-Golgi rather than in secretory granules, and second, proPTH is appropriately processed in cells of the constitutive as well as the regulated secretory pathway type.

We demonstrate here that preproPTH expressed using a vaccinia virus system in either BSC-40 cells or GH4C1 cells led to proPTH being accurately cleaved to PTH and that the processed PTH molecule only was released from the cell. In the BSC-40 cells coinfection of preproPTH and furin constructs led to a clear increase in processing of proPTH to PTH. In contrast, coinfection of preproPTH with either PC1 or PC2 did not alter the rate from that of basal. In GH4C1 cells, whereas coinfection with PC2 again was ineffective in stepping up the rate of conversion, furin expression led to an even more marked processing than that observed in the BSC-40 cells and PC1 coexpression caused a conversion that was intermediate between that found for furin and PC2. Thus, our studies confirm that proPTH cleavage takes place in cells exhibiting either a constitutive or regulated pathway. Exogenous furin expression causes an enhancement of this in both cell types, and exogenous PC1 is somewhat effective in a cell type with a regulated secretory pathway which therefore provides the appropriate environment for it to be active (17). The finding that furin, an enzyme normally associated with the constitutive pathway, processes proteins destined for the regulated pathway does have precedents. For example, the precursor to the neuroendocrine protein, 7B2, is processed by furin within endocrine cells (40). Thus, although it is generally accepted that furin cleaves proteins destined for the constitutive pathway, our results emphasize that exceptions do exist.

The ability of PC1 to process proPTH is consistent with our previous finding that partially purified PC1 can correctly cleave a tridecapeptide spanning the proPTH cleavage site (24). Here, we have extended those studies to show that partially purified furin also appropriately cleaves this peptide, but in a much more efficient manner. The increased catalytic efficiency of furin appears to be due to both an increase in affinity of the substrate and an increase in the rate of processing of the substrate. Based upon the results obtained with several different fluorogenic substrates (see Table II), one can speculate that the apparent increase in affinity of the tridecapeptide could be due to the presence of the basic residue occupying position P6 which essentially plays a role similar to the more frequently encountered P4 Arg residue (41). On the other hand, we cannot rule out the possibility that the COOH-terminal extension could also be responsible for the increase in processing rate of the tridecapeptide which in turn results in an increase in catalytic efficiency. This conclusion, if warranted, could not have been obtained by studies employing only the usual small peptidyl fluorogenic substrates. It suggests that there are multiple subsites in the active site cleft of convertases which are likely to interact with substrate residues located on both sides of the bond to be processed. Along these lines, it is of interest to note that in both the activation sequence of the proenzyme forms of several of the convertases (30, 42), as well as in numerous sequences of various precursors cleaved by furin (43), one can observe the presence, as in the tridecapeptide sequence of proPTH, of a hydrophobic aliphatic residue at position P2′. Clearly, based upon these observations, further studies are warranted to delineate the structural requirements of the recognition sites of convertases.

Our present studies confirm those of others that previously demonstrated that no proPTH is released from cells (or indeed, found circulating in the blood) (44). This would indicate that the prosequence exclusively serves an intracellular function. It has been suggested that the prosequence can be considered as part of a functional unit responsible for transport and cleavage of the precursor (the preproPTH molecule) upon its entry into the secretory pathway. Evidence for such a role came from studies demonstrating that amino acids carboxyl-terminal to the signal peptide play a role in the efficiency of cleavage of the signal peptide and the sequence of the propeptide is compatible with this cleavage, whereas the NH_2-terminal sequence of PTH which is constrained by its critical role in conferring bioactivity, is incompatible with efficient cleavage of the prepeptide (45). Hence the need for the prosequence as a spacer between the pre- and PTH sequences.

Interestingly, no increase in the amount of PTH released into

the medium was observed in cells expressing exogenous furin *versus* control cells. This would indicate that in control cells overexpressing PTH the available granules are already being maximally utilized and when PTH production is increased by exogenous expression of furin the secretory apparatus is unable to respond by increasing its capacity further. It remains unknown whether this is characteristic of normal cells or whether the use of the vaccinia expression system influenced the outcome. Since, after a period of time, normal cellular protein synthesis is shut down by the vaccinia infection, this may have disrupted the formation of functional new secretory granules.

In the parathyroid chief cell the amount of available intact PTH is thought to be regulated in part by a blood calcium controlled intracellular degradative pathway. Thus, under low extracellular levels of calcium little degradation takes place (46). However, under high extracellular calcium conditions, degradation of the hormone takes place and fragments, predominantly comprising the middle and carboxyl-terminal regions of the molecule are formed and released. The enzyme(s) responsible for this metabolism of PTH is (are) unknown. It has been estimated that the concentration of fragments within the parathyroid gland is approximately 9–16% of the total content of PTH moieties (47). We found no evidence for significant fragmentation of the PTH molecule itself in any of the conditions examined, either by overexpressing PTH alone or with any of the convertases tested. The chromatography methods used and the midmolecule radioimmunoassay employed would have been capable of detecting fragments. Therefore, it is unlikely that this particular class of enzyme plays a role in the intraglandular degradation of PTH.

By both Northern blot and *in situ* hybridization analysis only furin (and very low levels of PACE4), but not PC1, PC2, or PC5, was found to be expressed in the parathyroid gland. This, taken together with the results of the coinfection studies, points to furin as the physiological mediator of proPTH processing. It remains to be determined if the expression of furin is regulated in the parathyroid, for example, by the major regulators of parathyroid gland activity, calcium and 1,25-dihydroxyvitamin D. Studies have shown that the conversion of proPTH to PTH is not regulated by extracellular calcium (46) at least not in the short term, and therefore it would be anticipated that parathyroid furin expression may not be regulated by changes in this parameter.

The finding of furin, but not of PC1, PC2, or PC5 in the parathyroid, also bears upon the processing of the other major secretory product (besides PTH) of this gland, namely, chromogranin A (CgA). CgA, which contains several pairs of basic residues, but no consensus furin site, is expressed widely in neuroendocrine cells and is cleaved to generate active peptides such as pancreastatin, which modulate hormone secretion in an autocrine or paracrine manner. The extent of processing of CgA varies from one type of neuroendocrine cell to the next. In the parathyroid the amount of processing of CgA, although it does occur (48), is slight in comparison to that in other neuroendocrine cells, *e.g.* the β-cell of the pancreas (49). Therefore, the modest cleavage of CgA in the parathyroid is probably related to the lack of expression of PC1 and PC2. The precise roles played by furin and PACE4 in CgA processing in the parathyroid remain to be determined.

In summary, we have shown that furin is the most effective convertase in processing proPTH to PTH, but is probably not involved in the further intraglandular metabolism of the PTH molecule. Furin and preproPTH mRNAs are coexpressed in the parathyroid, whereas PC1, PC2, and PC5 are not. Therefore, the available data strongly suggest that furin is the convertase responsible for the physiological processing of proPTH to PTH. This is the only example characterized thus far of biosynthesis of a peptide hormone which is dependent upon the action of furin.

Acknowledgments—We thank Weijia Dong, Dany Gauthier, Susan James, Normand Rondeau, Diane Savaria, and Jasloveleen Sohi for their help with various aspects of this study, and Judith Marshall and Karuna Patel for preparation of the manuscript.

REFERENCES

1. Kemper, B., Habener, J. F., Mulligan, R. C., Potts, J. T., Jr., and Rich, A. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 3731–3735
2. Habener, J. F., Amherdt, M., Ravazzola, M., and Orci, L. (1979) *J. Cell Biol.* **80**, 715–731
3. Van de Ven, W. J. M., Voorberg, J., Fonttijn, R., Pannekoek, H., Van den Ouweland, A. M., Van Duijnhoven, H. L., Roebroek, A. J., and Siezen, R. J. (1990) *Mol. Biol. Rep.* **14**, 265–275
4. Barr, P. J., Mason, O. B., Landsberg, K. E., Wong, P. A., Kiefer, M. C., and Brake, A. J. (1991) *DNA Cell Biol.* **10**, 319–328
5. Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M., and Chrétien, M. (1990) *DNA Cell Biol.* **9**, 415–424
6. Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M., and Chrétien, M. (1991) *Mol. Endocrinol.* **5**, 111–122
7. Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J., and Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 340–344
8. Smeekens, S. P., and Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 2997–3000
9. Hakes, D. J., Birch, N. P., Mezey, A., and Dixon, J. E. (1991) *Endocrinology* **129**, 3053–3063
10. Kiefer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D., and Barr, P. J. (1991) *DNA Cell Biol.* **10**, 757–769
11. Nakayama, K., Kim, W. S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T., and Murakami, K. (1992) *J. Biol. Chem.* **267**, 5897–5900
12. Seidah, N. G., Day, R., Hamelin, J., Gaspar, A., Collard, M. W., and Chrétien, M. (1992) *Mol. Endocrinol.* **6**, 1559–1569
13. Lusson, J., Vieau, D., Hamelin, J., Day, R., Chrétien, M., and Seidah, N. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6691–6695
14. Nakagawa, T., Hosaka, M., Torji, S., Watanabe, T., Murakami, K., and Nakayama, K. (1993) *Biochem. J.* **113**, 132–135
15. Hendy, G. N., Kronenberg, H. M., Potts, J. T., Jr., and Rich, A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 7365–7369
16. Benjannet, S., Rondeau, N., Day, R., Chrétien, M., and Seidah, N. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3564–3568
17. Benjannet, S., Reudelhuber, T., Mercure, C., Rondeau, N., Chrétien, M., and Seidah, N. G. (1992) *J. Biol. Chem.* **267**, 11417–11423
18. Bennett, H. P. J., Browne, C. A., and Solomon, S. (1981) *Biochemistry* **20**, 4530–4538
19. Bennett, H. P. J., Solomon, S., and Goltzman, D. (1981) *Biochem. J.* **197**, 391–400
20. Rabbani, S. A., Yasuda, T., Bennett, H. P. J., Sung, W. L., Zahab, D. M., Tam, C. S., Goltzman, D., and Hendy, G. N. (1988) *J. Biol. Chem.* **263**, 1307–1313
21. Rabbani, S. A., Kaiser, S. M., Henderson, J. E., Bernier, S. M., Mouland, A. J., Roy, D. R., Zahab, D. M., Sung, W. L., Goltzman, D., and Hendy, G. N. (1990) *Biochemistry* **29**, 10080–10089
22. James, S., Gibbs, B. F., Toney, K., and Bennett, H. P. J. (1994) *Anal. Biochem.* **217**, 84–90
23. Covey, T. R., Bonner, R. F., Shushen, B. I., and Henion, J. (1988) *Rapid Commun. Mass Spectrom.* **2**, 249–256
24. Jean, F., Basak, A., Rondeau, N., Benjannet, S., Hendy, G. N., Seidah, N. G., Chrétien, M., and Lazure, C. (1993) *Biochem. J.* **292**, 891–900
25. Castillo, M. J., Nakajima, K., Zimmerman, M., and Powers, J. C. (1979) *Biochemistry* **99**, 53–64
26. Jean F., Basak, A., Chrétien, M., and Lazure, C. (1991) *Anal. Biochem.* **194**, 399–406
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Day, R., Schäfer, M. K. H., Watson, S. J., Chrétien, M., and Seidah, N. G. (1992) *Mol. Endocrinol.* **6**, 485–497
29. Heinrich, G., Kronenberg, H. M., Potts, J. T., Jr., and Habener, J. F. (1984) *J. Biol. Chem.* **259**, 3320–3329
30. Seidah, N. G., and Chrétien, M. (1992) *Trends Endocrinol. Metabol.* **3**, 133–140
31. Van de Ven, W. J. M., and Roebroek, A. (1993) *Crit. Rev. Encog.* **4**, 115–136
32. Lindberg, I. (1991) *Mol. Endocrinol.* **5**, 1361–1365
33. Barr, P. J. (1991) *Cell* **66**, 1–3
34. Steiner, D. F., Smeekens, S. P., Ohagi, S., and Chan, S. J. (1992) *J. Biol. Chem.* **267**, 23435–23438
35. Molloy, S. S., Thomas, L, VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994) *EMBO J.* **13**, 18–23
36. Malide, D., Seidah, N. G., Chrétien, M., and Bendayan, M. (1995) *J. Histochem. Cytochem.* **43**, 11–19
37. Cohn, D. V., MacGregor, R. R., Chu, L. L. H., Huang, D. W. Y., Anast, C. S., and Hamilton, J. W. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 1521–1525
38. Kemper, B., Habener, J. F., Potts, J. T., Jr., and Rich, A. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 643–647
39. Hellerman, J. C., Cone, R. C., Potts, J. T., Jr., Rich, A., Mulligan, R. C., and Kronenberg, H. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 5340–5344
40. Paquet, L., Bergeron, F., Boudreault, A., Seidah, N. G., Chrétien, M., Mbikay, M., and Lazure, C. (1994) *J. Biol. Chem.* **269**, 19279–19285
41. Watanabe, T., Murakami, K., and Nakayama, K. (1993) *FEBS Lett.* **320**, 215–218

42. Hatsuzawa, K., Nagahama, M., Takahashi, S., Takada, K., Murakami, K., and Nakayama, K. (1992) *J. Biol. Chem.* **267,** 16094–16099
43. Hosaka, M., Nagahama, M., Kim, W-S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K., and Nakayama, K. (1991) *J. Biol. Chem.* **266,** 12127–12130
44. Habener, J. F., Stevens, T. D., Tregear, G. W., and Potts, J. T., Jr. (1976) *J. Clin. Endocrinol. Metab.* **42,** 520–530
45. Wiren, K. M., Potts, J. T., Jr., and Kronenberg, H. M. (1988) *J. Biol. Chem.* **263,** 19771–19777
46. Habener, J. F., Kemper, B., and Potts, J. T., Jr. (1975) *Endocrinology* **97,** 431–441
47. MacGregor, R. R., Jilka, R. L., and Hamilton, J. W. (1986) *J. Biol. Chem.* **261,** 1929–1934
48. Drees, B. M., and Hamilton, J. W. (1994) *Endocrinology* **134,** 2057–2063
49. Hutton, J. C., Davidson, H. W., and Peshavaria, M. (1987) *Biochem. J.* **244,** 457–464