Purification and Amino Acid Sequence of a Noncalcitonin Secretory Peptide Derived from Preprocalcitonin*

Roger S. Birnbaum‡§, Walter Mahoney¶, and Bernard A. Roos§

From the ‡Department of Medicine, Veterans Administration Medical Center and School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106 and the ¶Division of Biomedical Research, Immuno Nuclear Corporation, Stillwater, Minnesota 55082

A previous report from this laboratory (Birnbaum R. S., O'Neil, J. A., Muszynski, M., Aron, D. C., and Roos, B. A. (1982) J. Biol. Chem. 257, 241-244) provided immunochemical and biochemical evidence for the existence of a secretory peptide derived from the noncalcitonin region of rat preprocalcitonin. By a variety of criteria, we demonstrated that this naturally occurring peptide was similar, if not identical, to a synthetic peptide which consisted of the NH2-terminal 16 residues of the calcitonin mRNA translation product. We have now purified this peptide from rat medullary thyroid carcinoma and sequenced it.

A rat tumor of the 1-2-4 tumor series was extracted in 0.1 N HCl yielding 900 µg of immunoreactive peptide. The peptide was purified to homogeneity by: 1) trichloroacetic acid precipitation of contaminating protein; 2) gel filtration; and finally, 3) reverse phase high pressure liquid chromatography. Overall yield was approximately 24%. Amino acid analysis and sequencing of the peptide yielded a composition and sequence identical with that of the synthetic peptide.

Calcitonin, a 32-residue amided peptide hormone, is produced in C cells of the thyroid by post-translational processing of larger precursors (1-6). While the complete amino acid sequence of the precursor awaits elucidation, an inferred amino acid sequence has been proposed (6, 7). Based upon information obtained from the nucleic acid sequence of a cDNA derived from calcitonin mRNA, it appears that the mature calcitonin hormone is nestled within a 136-residue precursor. Within this precursor, the first residue of mature calcitonin is preceded by the pair of amino acids, Lys-Arg, which are in turn preceded by 82 additional amino acids. On the distal side of the mature hormone is a 20-residue sequence beginning with the tetrapeptide Gly-Lys-Lys-Lys (Fig. 1). This tetrapeptide sequence is also found in precursors of α-MSH (8) and in which it separates α-MSH (ACTH[1-13]amide)

from corticotropin-like intermediate lobe protein (ACTH[18-39]). By analogy to α-MSH synthesis, we suggested that a peptide corresponding to the final hexadecapeptide constitutes a noncalcitonin secretory peptide derived from preprocalcitonin (9).

In our initial studies (9) to detect this hexadecapeptide, termed CCAP for calcitonin-carboxyl-adjacent peptide, we showed that a peptide with size and isoelectric point indistinguishable from synthetic CCAP was detected by an immunoassay based on the synthetic peptide. We also showed that the CCAP and calcitonin content of calcitonin-containing tissues were approximately equimolar. Higher molecular weight immunoreactive forms could also be demonstrated. However, the possibility existed that the low molecular weight immunoreactive peptide detected in calcitonin-producing tissues was not identical with the synthetic peptide. Post-translational modification of a slightly larger peptide, for example acetylation of an Arg-CCAP (Fig 1), could result in chromatographic and electrophoretic behavior similar to synthetic CCAP. In addition, there was a need to confirm the amino acid sequence for this important portion of preprocalcitonin. We also noted reports in the literature of discrepancies between the cDNA-derived amino acid sequence of human β-lipotropin in preproopiomelanocortin (11) and the actual sequence of human pituitary β-lipotropin (12, 13). According to previous reports, we proposed to determine the amino acid sequence.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic CCAP was obtained as previously reported (9). Sephadex G-25 (Fine) was obtained from Pharmacia. Acetonitrile (UV grade) was purchased from Burdick and Jackson Laboratories, Inc. Sequanal-grade trifluoroacetic acid, Quadrol, and Polybrene were obtained from Pierce Chemical Co.

**Radioimmunoassay**—The assay for CCAP was performed as described previously (9) except that phase separation was accomplished using a formolin-treated heat-denatured preparation of *Staphylococcus aureus*, Cowan I strain (Iggsorb, The Enzyme Center) (14). The assay standards were based on amino acid analysis of synthetic CCAP.

**Tissue Extraction**—The starting material for the purification of tumor CCAP was 29 g of nonencrusted tissue of the I-2-4 rat medullary thyroid carcinoma tumor series (15). The tissue was cut into large chunks and dispersed in 6 volumes of ice-cold 0.1 N HCl using a Brinkmann Polytron. All subsequent steps were performed at 4 °C. The extract was centrifuged for 10 min at 2200 × g. Trichloroacetic acid was added slowly to the supernatant to a final concentration of 5%. The suspension was centrifuged as before and the resulting supernatant extracted 3 times with equal volumes of water-saturated diethyl ether. The solution was then lyophilized.

**Gel Filtration**—A column (0.3 × 114 cm) of Sephadex G-25 (Fine)

CCAP was abbreviated CAP in the previous communication from this laboratory (9); it has also been called CCP, for COOH-terminal cleavage peptide (6) and the analogous human peptide has been designated PDN-21 (10).
Preprocalcitonin-derived Noncalcitonin Peptide

allowed us to begin purification of the peptide from a larger amount of tissue than could conveniently be obtained using rat thyroids. The 1-2-4 series tumor selected as starting material contained greater than 10-fold more immunoreactive CCAP per g of tissue than normal thyroid (9). Thus 29 g of tissue from a single 1-2-4 series tumor was extracted in hydrochloric acid yielding 900 g of immunoreactive CCAP in 1.44 g of protein. The second factor was the solubility of the peptide in 5% trichloroacetic acid. Approximately two-thirds of the immunoreactive CCAP remained in the supernatant after acid treatment, whereas 75% of the protein was precipitated.

The resulting lyophilized residue was applied to a Sephadex G-25 column (Fig. 2). Immunoreactive CCAP eluted with a Kd of 0.2 and was separated from the bulk of the protein which eluted at the void volume. The specific activity of the peak tubes of immunoreactive CCAP was about 700 μg/mg of protein. Even though synthetic CCAP gives a value of 840 μg/mg of protein in the Lowry assay, the protein values, except for the void volume fractions, probably did not reflect the true amount of contaminating protein. The impurity of this material became apparent when sequencing attempts yielded essentially all Pth derivatives in the first sequenator cycle.

The peak tubes of immunoreactive CCAP from Sephadex gel filtration were pooled and lyophilized. The residue was dissolved in 1% trichloroacetic acid (16) and subjected to reverse phase chromatography. Initially we used the 60 Å-pore size C18 µBondapak column; later the 300 Å-pore size C18 Protesil column was substituted (23, 24). The protein and immunoassay profiles were very similar for the two columns; however, only a run using the C18 column is depicted in Fig. 3. The only significant difference between the two columns was the yield of immunoreactive CCAP. Approximately 50-60% was recovered using the C18 column while recovery was essentially 100% for the C18 column.

Two peaks of immunoreactive CCAP were observed, which corresponded to two of the UV-absorbing peaks seen with synthetic CCAP (not shown). The major immunoreactive peak seen in Fig. 3 eluted with the same retention time as that of the major peak of the synthetic material, 25 min. The minor peak eluting at about minute 18 has been tentatively identified as methionine-sulfoxide CCAP based upon: 1) its shorter retention time; 2) its similarity in size to the species eluting at minute 25; 3) its reduction with 2.9 M mercaptoethanol at 37 °C for 42 h which shifted the retention time.

RESULTS

Two factors facilitated the purification of CCAP. The availability of large transplantable medullary thyroid carcinomas...
to that of the major CCAP peak; and 4) its amino acid analysis following acid hydrolysis showed no differences from the major peak. Rechromatography of the major peak under isocratic conditions (17.5% acetonitrile) revealed no contaminating material (not shown). Overall recovery of CCAP from tumor extract to HPLC-purified material was about 24%. The purification is summarized in Table I.

The amino acid content was determined by analysis of an acid hydrolysate of the major CCAP peak (Table II). No differences in amino acid content were noted from that predicted from the cDNA sequence or from amino acid analysis of synthetic CCAP. In particular, there was only a single lysine and no arginine, ruling out the possibility that the naturally occurring peptide contained the sequence of the synthetic material with an NH2-terminal extension.

Sequence analysis was performed on 19 nmol of purified CCAP. The identity and yield of the 16 amino acids found are shown in Table III. No Pth derivative was detected at Cycle 17. Extrapolation of the yield to the initial amount (or Cycle 0) gives approximately 19 nmol, indicating that the sequence of only one peptide form of CCAP was present.

**DISCUSSION**

The CCAP-like peptide isolated from the calcitonin-producing rat medullary thyroid carcinoma is identical with the synthetic hexadecapeptide and the amino acid sequence predicted from the cDNA structure. Nothing was observed during purification or analysis to suggest that CCAP was either larger than the synthetic peptide (due to an NH2-terminal extension) or had undergone such post-translational modifications as phosphorylation, glycosylation, acetylation, or sulfation. We cannot, however, rule out the possibility that certain minor forms of CCAP might be so modified.

The detection, characterization, and subsequent isolation of CCAP resulted from predictions of the processing of preprocalcitonin based upon a sequence inferred from analysis of a cDNA (6, 7). Our success confirms this approach to identifying novel secretory peptides, first demonstrated with the y-MSH region of preproopiomelanocortin (8, 25, 26). However, the

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**Table I**

**Summary of the purification of rat tumor CCAP**

| Step                      | Total iCCAP* µg | Total protein mg | Specific activity µg/mg | Recovery % | Purification % |
|---------------------------|-----------------|------------------|-------------------------|------------|----------------|
| 0.1 N HCl extract         | 900             | 1438             | 0.63                    | 100        |                |
| Trichloroacetic acid supernatant | 578             | 238              | 1.66                    | 64         | 2.63           |
| Post-G-25 chromatography  | 400             | 0.09             | 97.8                    | 44         | 155            |
| Post-HPLC                 | 216             | 0.26             | 840                     | 24         | 1333           |

*Immunoreactive CCAP.

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**Table II**

**Amino acid composition of purified tumor CCAP**

| Amino acid | Predicted | Observed |
|------------|-----------|----------|
| Aspartic acid/asparagine | 4 | 3.62 |
| Threonine | 1 | 0.93 |
| Glutamic acid/glutamine | 1 | 1.22 |
| Proline | 1 | 0.98 |
| Glycine | 1 | 1.04 |
| Alanine | 1 | 0.99 |
| Methionine | 1 | 0.86 |
| Leucine | 1 | 0.98 |
| Tyrosine | 1 | 0.83 |
| Phenylalanine | 1 | 0.81 |
| Histidine | 2 | 1.72 |
| Lysine | 1 | 1.04 |

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**Table III**

**Yields and identification of products generated by automated Edman degradation**

| Cycle | Residue | Yield* nmol |
|-------|---------|-------------|
| 1     | Asp     | 17.3        |
| 2     | Met     | 16.0        |
| 3     | Ala     | 14.1        |
| 4     | Lys     | 14.8        |
| 5     | Asp     | 11.1        |
| 6     | Leu     | 10.6        |
| 7     | Gln     | 7.7         |
| 8     | Thr     | 7.0         |
| 9     | Asn     | 7.3         |
| 10    | His     | 6.8         |
| 11    | His     | 7.3         |
| 12    | Pro     | 6.9         |
| 13    | Tyr     | 6.5         |
| 14    | Phe     | 6.2         |
| 15    | Gln     | 6.4         |
| 16    | Asn     | 5.0         |

*Only 25% of each product generated by the sequencer was analyzed. Yields listed above are normalized to 100% injection.
intermediate lobe of the pituitary. From the known or predicted structures of amidated secretory peptide precursors is analogous to the processing of residues was not initially appreciated as an indication of a novel secretory products. A glycine residue preceding the two arginine residues was also based upon analysis of a cDNA derived from human medullary thyroid carcinoma, decreased blood calcium in rats but had no effect on phosphate excretion. The sequence, therefore, contains no obvious clues to a bioactive role. However, CCAP's coelomation with calcitonin does suggest the possibility of some function associated with skeletal and mineral homeostasis. Recently, a report has appeared that the analogous human synthetic peptide, whose sequence was also based upon analysis of a cDNA derived from human medullary thyroid carcinoma, decreased blood calcium in rats but had no effect on phosphate excretion.

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TABLE IV

| Amidated secretory peptide | Amidated residue | Codon | Amino terminal residue* | Reference |
|---------------------------|-----------------|-------|-------------------------|-----------|
| Calcitonin (rat or human)  | Pro             | Gly-Lys-Lys-Arg | Asp | 6, 7, 28 |
| α-MSH (all mammalian)     | Phe             | Gly-Lys-Lys-Arg | Arg | 29 |
| Gastrin (porcine)         | Phe             | Gly-Arg-Arg    | Ser | 30 |
| γ-MSH (bovine, human, rat, and porcine) | Phe | Gly-Lys-Arg | Asn | 8, 10, 31, 32 |
| Vasopressin (bovine)      | Gly             | Gly-Lys-Arg    | Ala | 33 |

* The residue at the NH2 terminus after enzymatic removal of the "codon."
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