Immunological and biochemical characterization of processing products from the neurotensin/neuromedin N precursor in the rat medullary thyroid carcinoma 6-23 cell line

Jean-Noël BIDARD,* François de NADAI,* Carole ROVERE,* Danielle MOINIER,* Jeanine LAUR,† Jean MARTINEZ,† Jean-Claude CUBER‡ and Patrick KITABGI*§

*Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, Université de Nice–Sophia Antipolis, Sophia Antipolis, 660 Route des Lucioles, 06560 Valbonne,
†Centre CNRS–INSERM de Pharmacologie–Endocrinologie, 34094 Montpellier Cedex,
and ‡INSERM Unité 45, Hôpital E. Herriot, Pavillon Hbis, 69437 Lyon Cedex 03, France

Neurotensin (NT) and neuromedin N (NN) are two related biologically active peptides that are encoded in the same precursor molecule. In the rat, the precursor consists of a 169-residue polypeptide starting with an N-terminal signal peptide and containing in its C-terminal region one copy each of NT and NN. NN precedes NT and is separated from it by a Lys-Arg sequence. Two other Lys-Arg sequences flank the N-terminus of NN and the C-terminus of NT. A fourth Lys-Arg sequence occurs near the middle of the precursor and is followed by an NN-like sequence. Finally, an Arg-Arg pair is present within the NT moiety. The four Lys-Arg doublets represent putative processing sites in the precursor molecule. The present study was designed to investigate the post-translational processing of the NT/NN precursor in the rat medullary thyroid carcinoma (rMTC) 6-23 cell line, which synthesizes large amounts of NT upon dexamethasone treatment. Five region-specific antisera recognizing the free N- or C-termini of sequences adjacent to the basic doublets were produced, characterized and used for immunoblotting and radioimmunoassay studies in combination with gel filtration, reverse-phase h.p.l.c. and trypsin digestion of rMTC 6-23 cell extracts. Because two of the antigenic sequences, i.e. NN and the NN-like sequence, start with a lysine residue that is essential for recognition by their respective antisera, a micro-method by which trypsin specifically cleaves at arginine residues was developed. The results show that dexamethasone-treated rMTC 6-23 cells produced comparable amounts of NT, NN and a peptide corresponding to a large N-terminal precursor fragment lacking the NN and NT moieties. This large fragment was purified. N-Terminal sequencing revealed that it started at residue Ser^{23} of the prepro-NT/NN sequence, and thus established the Cys^{22}–Ser^{23} bond as the cleavage site of the signal peptide. Two other large N-terminal fragments bearing respectively the NN and NT sequences at their C-termini were present in lower amounts. The NN-like sequence was internal to all the large fragments. There was no evidence for the presence of peptides with the NN-like sequence at their N-termini. This shows that, in rMTC 6-23 cells, the precursor is readily processed at the three Lys-Arg doublets that flank and separate the NT and NN sequences. In contrast, the Lys-Arg doublet that precedes the NN-like sequence is not processed in this system. The tools and methods developed here which allow detection of precursor forms at the fentomolar level will be useful for the study of the post-translational processing of the NT/NN precursor in tissues that express the NT/NN gene.

INTRODUCTION

Neurotensin (NT) and neuromedin N (NN) are two structurally related brain and gut regulatory peptides. The cloning and sequencing of cDNAs from canine intestine and bovine brain, and of the rat gene encoding the NT precursor, have revealed that NT and NN are products of the same precursor [1,2]. Because NT and NN are encoded by the same exon [2], it may be expected that the two peptides will be co-localized in the tissues and cell lines that express their common precursor. Co-localization of NT and NN has been immunohistochemically demonstrated in the rat intestine [3], and has been suggested in brain tissues by radioimmunoassay (RIA) studies showing co-distribution [4,5] and co-release [6] of the two peptides.

In all species where it has been cloned, the NT/NN precursor molecule exists as a highly conserved polypeptide of 169 (rat) or 170 amino acids, starting with an N-terminal signal peptide and containing one copy each of NT and NN in its C-terminal region. Figure 1 depicts the organization of the rat NT/NN precursor. The NN sequence precedes the NT sequence and is separated from it by a Lys-Arg sequence. Two other Lys-Arg sequences flank the N-terminal lysine of NN and the C-terminal leucine of NT. A fourth Lys-Arg pair occurs in the middle of the precursor. This doublet and the one at the N-terminus of NN delimit a 54-residue-peptide which starts with an NN-like sequence (Lys-Leu-Pro-Leu-Val-Leu, designated K6L) and ends with an acidic sequence (Glu-Lys-Glu-Glu-Val-Ile, designated E6I). Finally, an Arg-Arg sequence occurs within the NT molecule. The four Lys-Arg dibasic sequences are thought to represent putative cleavage sites for proteolytic processing enzymes, thus suggesting that the precursor may be processed to generate various sets of peptides in addition to NT and NN. Furthermore, peptide combinations may vary from tissue to tissue if differential tissue-specific processing occurs, as has been described for other multi-peptide

Abbreviations used: NT, neurotensin; NN, neuromedin N; NN-KR, NN-Lys-Arg; the abbreviations for synthetic tyrosine-extended or unmodified precursor peptide fragments, i.e. K6L, K7Y, E6I, Y7I, K25R and E17Y, contain a first letter corresponding to the single letter code of the first amino acid, a middle number indicating the number of amino acid residues in the peptide and a final letter corresponding to the single letter code of the last amino acid; iNT, iNN, iK6L, iE6I and iNT-(1–8), immunoreactive NT, NN, K6L, E6I and NT-(1–8) respectively; RIA, radioimmunoassay; CTU, citraconylation/trypsin/unblocking procedure; rMTC, rat medullary thyroid carcinoma.

§ To whom correspondence should be addressed.

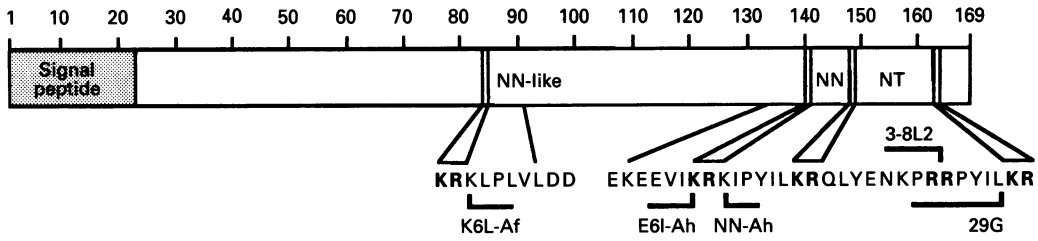

Figure 1 Diagrammatic representation of the rat NT/NN precursor and of region-specific antisera

The position of the four Lys-Arg doublets are indicated by vertical lines. Relevant amino acid sequences are given together with the regions recognized by each of the antisera used in the present study.

precursors [7]. Evidence suggests that the NT/NN precursor is differentially processed in brain compared with intestinal tissues [8,9], and among different brain structures [10]. However, little is known about the extents to which the four putative processing sites are cleaved, and hence about the processing intermediates and combinations of peptides that are produced in the various tissues and cell lines that express the NT/NN precursor.

In the present paper we report on the development of immunological and biochemical tools and techniques to identify and quantify the various polypeptides that may theoretically result from maturation of the NT/NN precursor. Five region-specific antisera recognizing either the free N- or C-termini of sequences adjacent to dibasic sites (Figure 1) were used for immunoblotting analysis and RIA. High-molecular-mass precursor forms were cleaved at dibasic sites in order to generate and quantify internal immunoreactive sequences. Because two of those sequences, i.e. NN and the NN-like sequence, start with a lysine residue immediately following the Lys-Arg dibasic site (Figure 1), a micro-method in which trypsin specifically cleaves peptides after arginine residues was used. These tools and techniques were applied to the rat medullary thyroid carcinoma (rMTC) 6-23 cell line, which produces large amounts of NT in response to dexamethasone [11,12] and hence is expected to contain high concentrations of NT/NN-precursor-derived peptides. Finally, the most abundant large precursor fragment that resulted from precursor processing in the rMTC 6-23 cell line was purified and N-terminally sequenced, which, together with immunological data, allowed us to deduce its structure and to determine the exact cleavage sites of the precursor signal sequence.

MATERIALS AND METHODS

Synthetic peptides

NT and NN were purchased from Neosystem (Strasbourg, France). The peptides Lys-Leu-Pro-Leu-Val-Leu-Tyr (designated K7Y), Tyr-Glu-Asn-Lys-Pro-Arg [NT-(3–8)] and Tyr-Glu-Lys-Glu-Glu-Val-Ile (designated Y7I) were custom-synthesized by Neosystem. K7Y and Y7I represent C- and N-terminally tyrosine-extended K6L and E6I sequences respectively (Figure 1), and were therefore amenable to iodination. The maleoyl peptides maleoyl-Glu-Lys-Glu-Glu-Val-Ile (maleoyl-E6I) and Lys-Leu-Pro-Leu-Val-Leu-maleoyl (K6L-maleoyl) were synthesized according to [13]. The peptides Lys-Ile-Pro-Tyr-Ile-Leu-Lys-Arg (NN-Lys-Arg; designated NN-KR), Gln-Leu-Tyr-Glu-Asn-Lys-Pro-Arg {[Gln^1]NT-(1–8)} and Lys-Leu-Pro-Leu-Val-Leu-Asp-Asp-Phe-Ser-Leu-Glu-Ala-Leu-Leu-Thr-Val-Phe-Gln-Leu-Gln-Lys-Ile-Cys-Arg (designated K25R) were synthesized and purified by Dr. Solange Lavielle (Université Paris VII). The latter peptide corresponded to amino acids 87–111 of the NT/NN precursor, i.e. the NN-like sequence with a 19-residue C-terminal extension. The C-terminal heptadecapeptide (amino acids 153–169) of the NT/NN precursor, i.e. Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-Lys-Arg-Ala-Ser-Tyr-Tyr-Tyr (designated E17Y), corresponding to NT-(4–13) with a 7-residue C-terminal extension, was synthesized and purified by Dr. H. Boussetta in the laboratory of Professor Paul Cohen (Université Pierre et Marie Curie, Paris, France). Other synthetic peptides used in this work were purchased from Neosystem and Peninsula, or were obtained as previously reported [14].

Immunization

In order to obtain anti-NT-(1–8) antibodies that would cross-react only minimally with NT, the production of antibodies directed toward the C-terminus of NT-(1–8) was favoured by coupling NT-(3–8) to ovalbumin exclusively through its N-terminal tyrosine residue using the bisdiazobenzidine method [15]. Similarly, in order to direct the production of antibodies toward the C-terminus of E6I and the N-terminus of K6L (see Figure 1), the maleoyl groups of maleoyl-E6I and K6L-maleoyl were reacted with free thiol groups incorporated in BSA, as previously reported [13]. Rabbits were immunized with these conjugates as previously described [5].

Iodinations

NT, NT-(1–8), NN, K7Y and Y7I (1 nmol) were iodinated with Na^{125}I (0.5 nmol; 1 mCi; Amersham) and chloramine T (16 nmol) in 10 mM sodium phosphate buffer, pH 7.4. The reaction was stopped after 1 min by addition of $Na_2S_2O_5$ (16 nmol) and BSA (50 μg). The monoiodinated derivatives were purified by reverse-phase h.p.l.c. (RP18 Lichrosorb column; Merck) on a Waters apparatus with automatic recording of both the absorbance at 230 nm (Waters model 450 detector) and the radioactivity (Beckman radioisotope detector). Elutions were carried out in 0.1 % trifluoroacetic acid and 0.05 % triethylamine. For all peptides except NT, a linear gradient from 10 to 60 % acetonitrile over 70 min at a flow rate of 1 ml/min was applied. Retention times of unlabelled, monoiodinated and di-iodinated derivatives were, respectively, 17, 24.2 and 29 min for NT-(1–8); 34.4, 41.2 and 46 min for NN; 36.5, 43 and 48 min for K7Y; and 20, 25 and 29.5 min for Y7I. For NT, which contains Tyr residues in positions 3 and 11, a linear gradient from 10 to 30 % acetonitrile over 28 min was followed by a second linear gradient from 30 to 35 % acetonitrile over 20 min. Unlabelled NT eluted at 33 min, monoiodo-[^{125}I-Tyr^3]NT and monoiodo-[^{125}I-Tyr^{11}]NT at 36 and 37 min respectively, and di-iodinated

Table 1 Main characteristics of RIAs

Antiserum...	29G	NN-Ah	3-8L2	K6L-Af	E6I-Ah
Immunoreactivity detected	iNT	iNN	iNT-(1–8)	iK6L	iE6I
^{125}I-labelled ligand	^{125}I-NT	^{125}I-NN	^{125}I-NT-(1–8)	^{125}I-K7Y	^{125}I-Y7I
Unlabelled competitor	NT	NN	NT-(1–8)	K7Y	Y7I
Antiserum dilution	1:50000	1:40000	1:500	1:60000	1:7500
Detection limit (fmol/tube)	0.5	0.5	15	5	15
EC_{50} (fmol/tube)	5	5	150	50	170
Region recognized in unlabelled competitor	C-Terminal	N-Terminal	C-Terminal	N-Terminal	C-Terminal

derivatives at 40–41 min. Monoiodo-[^{125}I-Tyr^3]NT and monoiodinated NT-(1–8), NN, K7Y and Y7I were obtained at a specific radioactivity of 2000 Ci/mmol. They were suspended in a solution containing 0.5% BSA and stored frozen.

Radioimmunoassays

All RIAs, except the E6I RIA, were done in phosphate-buffered saline (50 mM Na_2HPO_4 and 140 mM NaCl, pH 7.5) containing 0.1% gelatin and 0.01% Triton X-100. The E6I RIA was carried out in 50 mM Tris/HCl buffer, pH 8.6, containing 0.1% BSA. RIAs were incubated for 24–72 h at 4 °C in a final volume of 500 μl containing 5000–6000 c.p.m. of tracer and various concentrations of the appropriate standard or various dilutions of synthetic peptides or unknown samples. Separation of bound from free peptide was performed with charcoal [16]. The main characteristics of the RIAs used here are summarized in Table 1. Further details on the specificity of each antiserum are given in the Results section.

Cell culture and extracts

The rMTC 6-23 cell line (kindly provided by Dr. Eleni Dicou, INSERM U298, Angers, France) was established in culture from a transplantable rat medullary thyroid carcinoma [11]. The cells were cultured and propagated in H-21 medium containing 15% horse serum and 2.5% fetal calf serum as described in [11]. For dexamethasone treatment, cells plated in 100 mm diameter dishes were grown until they reached 60–70% confluency. The culture medium was replaced by H-21 medium containing 2% horse serum and 1 μM dexamethasone. After 72 h, the medium was removed and the cells were washed with PBS and extracted in ice-cold 0.1 M HCl. The extract was centrifuged and the supernatant was placed in boiling water for 10 min and then kept frozen until use. In some experiments, cells were extracted in acetone/0.1 M HCl (4:1, v/v). After centrifugation, the acetone acid extract was kept at −30 °C until use. The protein content of the extracts was determined using the Bio-Rad protein assay reagent, following the procedure recommended by the manufacturer.

Gel-permeation chromatography

Acid extracts from rMTC 6-23 cells were applied on to a 95 cm × 2 cm column of Sephadex G-50 (medium grade) equilibrated in 0.1 M acetic acid. The column was eluted at a flow rate of 25 ml/h and fractions of 4 ml were collected. Portions from each fraction were assayed for their immunoreactive NT, NN, E6I, K6L and NT-(1–8) [iNT, iNN, iE6I, iK6L and iNT-(1–8)] content. The fractions were then pooled as indicated. Portions of the pools were lyophilized in low-protein-absorption tubes and stored frozen until use. The column was calibrated with Blue Dextran (V_0), soybean trypsin inhibitor (22 kDa), bovine heart cytochrome *c* (12.3 kDa), *Naja mossambica* cardiotoxin CT 41 (6.7 kDa) and *Anemonia sulcata* toxin II (5 kDa).

Phosphate/urea SDS/PAGE and Western blot analysis

Acid acetone extracts of rMTC 6-23 cells or portions of the G-50 column pools were evaporated to dryness and taken up in sample buffer consisting of 1% SDS, 8 M urea, 1% 2-mercaptoethanol and 0.01 M H_3PO_4 adjusted to pH 6.8 with Tris base. The samples were resolved by phosphate/urea SDS/PAGE on a 12% gel in 6 M urea [17]. Molecular mass markers ranging from 2512 to 16950 Da were from Pharmacia. Gels were transferred to nitrocellulose paper (Hybond C; Amersham) using a semi-dry transfer apparatus (Semi-Dry Electroblotter A; Ancos). Blotting efficiency was monitored by staining the nitrocellulose sheet with Ponceau Red and the transferred gel with Coomassie Blue R-250. After rinsing with water and washing with Tris/saline buffer (20 mM Tris, pH 7.4, 140 mM NaCl) containing 0.2% (w/v) Tween 20, the blot was incubated overnight at 4 °C in Tris/saline buffer containing 5% (w/v) non-fat dry milk, and then for 4 h at room temperature in Tris/saline buffer containing the primary antibodies at the appropriate dilutions. After 5 × 5 min washes in Tris/saline buffer containing 0.2% Tween 20 and one 5 min wash in Tris/saline buffer, the blot was incubated at room temperature for 45 min in Tris/saline buffer containing 5% (w/v) BSA and a 1:7500 dilution of alkaline phosphatase-coupled goat anti-(rabbit immunoglobulins) (Promega Kit W3930). After washing as above, the blot was revealed according to the procedure recommended by the manufacturer. The primary antibodies used here were the anti-NT (29G) antiserum at a 1:500 dilution and the anti-E6I (E6I-Ah) antiserum at a 1:600 dilution.

Citraconic anhydride treatment, digestion with trypsin and unblocking procedure

Two of the four Lys-Arg pairs of basic residues in the NT/NN precursor are followed by sequences that start with a lysine residue, i.e. the NN-like sequence K6L and NN itself (Figure 1). These lysine residues are essential for the recognition of K6L and NN by their respective antibodies. Therefore, in order to cleave the NT/NN precursor at the C-terminal side of Lys-Arg pairs without removing the adjacent lysine, the amine function of lysine side chains was reversibly protected by treatment with citraconic anhydride [18]. Trypsin could then be used to cleave the citraconylated peptides specifically at the C-terminal sides of arginine residues. RIAs were performed after unblocking the lysine residues.

Dried samples containing less than 0.2 mg of protein were dissolved in 250 μl of 0.05% (w/v) Triton X-100. Then, 20 μl of citraconic anhydride (5 mM final concentration), dissolved in dimethyl sulphoxide, and 40 μl of 1 M Hepes/NaOH buffer, pH 8.6, were added to the samples. The reaction was carried out at room temperature for 10 min, during which time the pH dropped to 8.1 and remained stable thereafter. Ethanolamine [20 μl of a 1% (v/v) solution] was added to hydrolyse any excess citraconic anhydride. Tryptic digestion of the mixture was performed with 50 units of diphenylcarbamyl chloride-treated trypsin/ml (8300 BAEE units/mg of solid; Sigma) in the presence of 2 mM $CaCl_2$ for 60 min at room temperature in a final volume of 400 μl. The reaction was terminated by adding 10 μg of soybean trypsin inhibitor (Sigma). The citraconylated amine functions were unblocked by bringing the pH to 2.8 with HCl and incubating for 10 min at 95 °C in a final volume of 400 μl. The unblocking reaction was stopped by neutralization with NaOH in PBS/gelatin/Triton X-100. The samples were then assayed for their iNT, iNN, iK6L and iNT-(1–8) contents. In preliminary experiments, these conditions for the citraconylation/trypsin/unblocking (CTU) procedure were found to generate maximal amounts of immunoreactive materials from rMTC 6-23 cell extracts containing high-molecular-mass precursor-derived peptides (results not shown). It was also confirmed, using synthetic peptides, that all steps of the CTU procedure proceeded with yields close to 100% (results not shown).

Reverse-phase h.p.l.c.

Samples that had been submitted to the CTU procedure were injected on to a 4 mm × 250 mm RP18 Lichrosorb column. Elution was carried out in 0.1% trifluoroacetic acid/0.05% triethylamine with a linear gradient from 10 to 60% acetonitrile over 70 min at a flow rate of 1 ml/min. Fractions 1–40 were lyophilized and reconstituted in 500 μl of PBS/gelatin/Triton X-100, followed by sonication for 10 min, and fractions 41–80 were kept in the h.p.l.c. solvent. The fractions were assayed for their iNT, iE6I, iNN, iK6L and iNT-(1–8) contents.

Purification of high-molecular-mass iE6I

Acid extracts of dexamethasone-treated rMTC 6-23 cells were adjusted to pH 2.2 with NaOH and centrifuged at 100000 *g* for 45 min. The supernatant (264 ml) was kept frozen until use. All chromatographic steps were performed with a Beckman h.p.l.c. apparatus equipped with a diode array detector and a system 'Gold' computer. A 44 ml portion of cell extract was filtered on a 0.45 μm-pore-size Millipore filter and applied to a reverse-phase Nucleosil C-4 column (5 μm particle size; 33 nm pore-size; 10 mm × 250 mm) mounted in series with a C-18 precolumn (40–63 μm particle size; 30 nm pore size; 8 mm × 40 mm). Solvent A was 0.1% trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The column was eluted at 4 ml/min and equilibrated in 10% B. At 40 min after sample application, a 105 min linear gradient was run from 10% to 40% B, followed by a 75 min linear gradient to 50% B. Fractions of 8 ml were collected, directly assayed for their iE6I content and kept at 4 °C. This step was performed six times. The immunoreactive fractions (eluting at ~ 41% B) were pooled and lyophilized. The dry residue was resuspended in 10 ml of 0.04% Tween 20, 0.1% trifluoroacetic acid and 25% acetonitrile, and centrifuged at 5000 *g* for 15 min.

The supernatant was submitted to cation-exchange h.p.l.c. following the procedure reported for the purification of large NN [19], with minor modifications. A 5 ml portion was applied to a Bio-Gel TSK-sulphopropyl 5PW column (7.5 mm × 75 mm) equilibrated at 1.5 ml/min with 0.5 M acetic acid and 25% (v/v) acetonitrile (solvent C). Solvent D was 1.9 mM acetic acid and 18.75% (w/v) guanidine-HCl (pH 3.8) in 25% acetonitrile. The column was eluted with a 15 min linear gradient to 30% D, followed by a 10 min isocratic elution and by a 40 min linear gradient to 70% D. Fractions of 1 ml were collected and assayed for their iE6I content. The immunoreactive fractions (eluting at ~ 30% D, between fractions 34 and 38) from two runs were pooled and diluted twice with 0.1% trifluoroacetic acid, yielding 24 ml of solution.

This solution was applied to a reverse-phase Nucleosil C-4 column (5 μm particle size; 33 nm pore size; 4.6 mm × 25 mm). The column was equilibrated at 1 ml/min in 12% solvent B (see above); at 30 min after injection, three linear gradients were successively run for 11, 14 and 30 min to 30%, 40% and 50% B respectively. Fractions of 1 ml were collected and assayed for their iE6I content. The active material (eluting at ~ 42% B) was concentrated in a Speed-Vac concentrator, diluted to 200 μl with 0.05% Tween 20, 0.1% trifluoroacetic acid and 10% acetonitrile, and centrifuged at 10000 *g* for 15 min.

The supernatant was applied on to a reverse-phase Aquapore RP-300 column (10 μm particle size; 30 nm pore size; 2.1 mm × 220 mm). Solvent A was 0.1% trifluoroacetic acid (as above) and solvent E was 0.1% trifluoroacetic acid in 70% acetonitrile. The column was equilibrated at 0.2 ml/min in 20% E, and 5 min after injection, a 125 min linear gradient was run to 80% E. Fractions of 0.2 ml were collected in low-absorption tubes and assayed for their iE6I content. The peak fractions (nos. 99–100, eluting at 45.6% E), were pooled, concentrated, taken up in 100 μl of 0.1% Tween 20, 0.1% trifluoroacetic acid and 10% acetonitrile, and re-run on the same column using a slightly different program which involved two successive 80 min linear gradients to 56% and 70% solvent E respectively.

The peak fractions (nos. 97 and 98, eluting at ~ 45% E) from the latter chromatography were divided into several portions, which were subjected to N-terminal sequencing using an Applied Biosystems Sequenator (model 477 A) equipped with an on-line phenylthiohydantoin analyser (model 120 A).

RESULTS

Specificity of the antisera

Table 2 presents a detailed characterization of the specificity of the antisera used in the present study. The data can be summarized as follows.

(1) The anti-NT antiserum 29G was directed towards the C-terminal heptapeptide portion of NT and did not cross-react with N-terminal NT fragments. Furthermore, it did not recognize NN, which shares a common C-terminal tetrapeptide sequence with NT. Importantly, antiserum 29G did not cross-react with C-terminally amidated or extended forms of NT such as NT-(8–13) amide or peptide E17Y. These results demonstrate that the free C-terminal sequence of NT is an absolute requirement for recognition by antiserum 29G.

(2) The anti-NT-(1–8) antiserum 3-8L2 was fully specific for the free C-terminal portion of NT-(1–8), as it cross-reacted very weakly with NT, NT-(1–11), NT-(4–13) and NT-(7–13). In addition, it reacted poorly with tuftsin, which shares a common C-terminal tripeptide sequence with NT-(1–8).

(3) The anti-NN antiserum NN-Ah was strictly specific for the free intact N-terminal portion of NN, as it cross-reacted barely if at all with NN-(2–6), NT, K7Y (NN-like sequence), Lys-Arg-Pro-Tyr-Ile-Leu or Lys-Asn-Pro-Tyr-Ile-Leu, the latter two peptides differing from NN by the residue at position 2. Finally,

Table 2 Cross-reactivity of synthetic peptides with the antisera used in this study

Peptide	Sequence	Antiserum...	Cross-reactivity (%) NT-29G	3-8L2	NN-Ah	K6L-Af	E6I-Ah
NT	pELYENKPRRPYIL		100	0.025	< 0.0001	< 0.001	< 0.01
NT-(6–13)	KPRRPYIL		75				
NT-(8–13)	RRPYIL		12				
NT-(8–13) amide	RRPYIL-NH_2		< 0.05				
E17Y	ENKPRRPYILKRASYYY		< 0.05				
NT-(1–12)	pELYENKPRRPYI		0.05				
NT-(1–8)	pELYENKPR		< 0.001	100		< 0.001	< 0.01
[Gln^1]NT-(1–8)	ELYENKPR			100			
NT-(1–11)	pELYENKPRRPY			0.055			
NT-(4–13)	ENKPRRPYIL			0.13			
Tuftsin	TKPR			0.03			
Gly-Pro-Arg	GPR			< 0.01			
NN	KIPYIL		< 0.001		100	0.01	< 0.01
NN-(2–6)	IPYIL				< 0.01		
[Arg^2]NN	KRPYIL				0.0001		
[Asn^2]NN	KNPYIL				0.0003		
[Arg^0]NN	RKIPYIL				0.46		
NN-KR	KIPYILKR				130	1.0	
K7Y (K6L-Tyr)	KLPLVLY				0.05	100	< 0.01
K6L (NN-like)	KLPLVL					52	
K6L-(2–6)	LPLVL					0.018	
K6L-(3–6)	PLVL					< 0.003	
[Arg^0]K6L	RKLPLVL					1.0	
K25R	KLPLVLDD---ICR*					75	
Y7I (Tyr-E6I)	YEKEEVI						100
E6I	EKEEVI						75
E6I-(1–5)	EKEEV						< 0.003
E6I-(1–4)	EKEE						< 0.003
E6I-Lys	EKEEVIK						0.021

* The full sequence of K25R is KLPLVLDDFSLEALLTVFQLQKICR, and corresponds to amino acids 87–111 of the NT/NN precursor.

extending the C-terminus of NN, as in NN-KR, resulted in a fully reactive peptide, whereas extending the N-terminus, as in [Arg^0]NN, markedly reduced the peptide cross-reactivity.

(4) The anti-K6L antiserum K6L-Af did not cross-react with NT or NT-(1–8), and reacted weakly with NN and NN-KR. Shortening or extending the N-terminus of K6L resulted in a dramatic loss of reactivity. Conversely, extending the C-terminus of K6L, as in K25R, had only a slight effect on cross-reactivity. Thus antiserum K6L-Af requires the free intact N-terminal sequence of K6L for recognition of the peptide.

(5) The anti-E6I antiserum E6I-Ah did not cross-react with NT, NN or K7Y. Shortening or extending the C-terminus of E6I virtually destroyed the immunoreactivity, demonstrating that antiserum E6I-Ah was strictly specific for the free intact C-terminal sequence of E6I.

In conclusion, the coupling strategies used here to generate antibodies directed towards free terminal sequences adjacent to the basic doublets in the NT/NN precursor were quite efficient. In particular, it can be inferred from the above results that iNT and iE6I will be detected only if the basic doublets adjacent to the C-terminus of NT and E6I in precursor products have been removed. Similarly iNN and iK6L will be detected only if the doublets adjacent to the N-terminus of NN and K6L have been processed.

Gel filtration of rMTC 6-23 cell extracts

Acid extracts of dexamethasone-treated rMTC 6-23 cells contained 15–30 and 20–40 pmol/mg of protein of iNT and iNN respectively, whereas iNT and iNN amounted to 2–5 and 3–8 pmol/mg of protein respectively in extracts from untreated cells. No iK6L (NN-like sequence) could be detected in extracts of either untreated or dexamethasone-treated cells. Extracts of dexamethasone-treated cells (20 ml; 8.6 mg of protein) were fractionated by gel filtration on Sephadex G-50, and the fractions were assayed for their iNT, iNN and iE6I content (Figure 2). A major peak of iNT eluted near the total volume of the column, slightly ahead of the single peak of iNN. A smaller peak of iNT was recovered in the 20 kDa region. In addition, a large peak of iE6I eluted in the same region as the high-molecular-mass iNT peak. The fractions did not contain any detectable amounts of iK6L or iNT-(1–8) (results not shown). The fractions were pooled as indicated in Figure 2 so that the high-molecular-mass iNT and most of the iE6I were contained in pool 2, and the low-molecular-mass iNT and iNN were in pool 5. Reverse-phase h.p.l.c. analysis of portions of pool 5 showed that more than 90% of low-molecular-mass iNT and iNN were recovered as single peaks co-eluting with synthetic NT and NN respectively (results not shown).

Western blot analysis

When pool 2 was subjected to phosphate/urea SDS/PAGE and Western blot analysis, the anti-NT antiserum (29G) and the anti-E6I antiserum (E6I-Ah) each specifically immunostained only one polypeptide band, with apparent molecular masses of

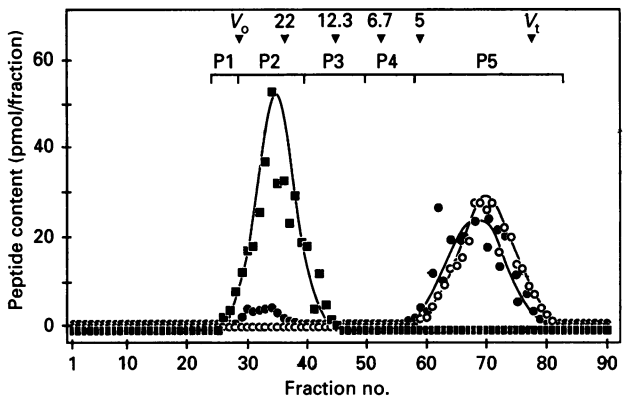

Figure 2 Gel filtration of rMTC 6-23 cell extract

An acid extract of rMTC 6-23 cells was applied to a Sephadex G-50 column (see the Materials and methods section). The fractions were assayed for their iNT (●), iNN (○) and iE6I (■) contents. Recovery of immunoreactivity was > 85% with all three assays. The fractions were pooled as shown in the figure (P1–P5). The void (V_0) and total (V_t) volumes of the column and the elution positions of molecular mass markers (in kDa) are indicated by arrowheads.

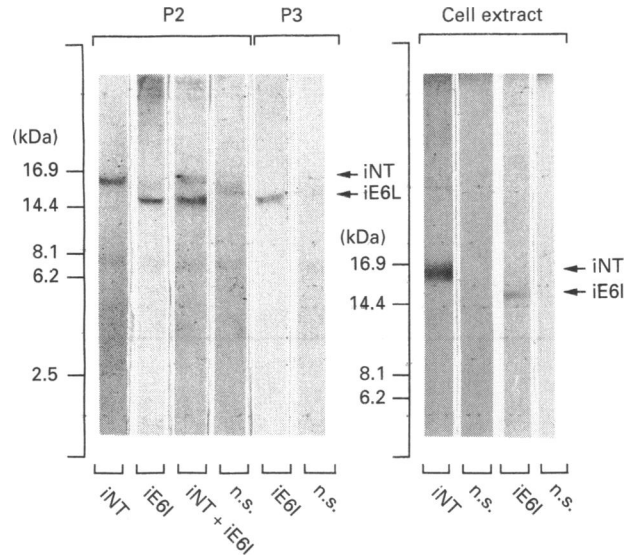

Figure 3 Western blot analysis of rMTC 6-23 cell extract and Sephadex G-50 pools 2 and 3

Portions of pools 2 and 3 (P2 and P3) and cell extract (20 μg, 58 μg and 100 μg of protein respectively) were subjected to phosphate/urea SDS/PAGE and Western blot analysis with either anti-NT (iNT) or anti-E6I (iE6I) antisera or with a mixture of both antisera (iNT + iE6I) as described in the Materials and methods section. Non-specific labelling (n.s.) was assessed by preincubating the antisera with their respective synthetic antigens, i.e. NT and Y7I, at 10 μM. Arrows depict the specifically labelled bands. The positions of the molecular mass markers are indicated.

16.5 kDa and 15 kDa respectively (Figure 3). These bands were not stained when the antisera were preincubated with their respective synthetic antigen in excess. The 16.5 kDa and 15 kDa bands were also specifically labelled by antisera 29G and E6I-Ah respectively in unfractionated extracts of dexamethasone-treated rMTC 6-23 cells (Figure 3), indicating that the immunoreactive peptides in pool 2 were not generated during Sephadex G-50 fractionation. Finally, pool 3 stained positively for the E6I-immunoreactive 15 kDa band (Figure 3), but was devoid of NT-immunoreactive 16.5 kDa material (results not shown).

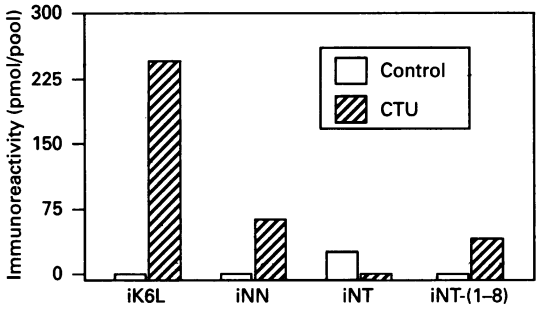

Figure 4 CTU treatment of Sephadex G-50 pool 2

Portions of pool 2 were assayed for iNN, iK6L, iNT-(1–8) and iNT prior to (control) or after CTU treatment. The data have been scaled up to represent total pool content in immunoreactive material.

Table 3 Concentrations of immunoreactive material in untreated and CTU-treated extracts from rMTC 6-23 cells

The data for high-molecular-mass peptides were obtained from assays of Sephadex G-50 pools 2 and 3 (see Figure 2) before (untreated) and after CTU treatment. The CTU-generated iNN material was further fractionated by h.p.l.c. into two components that co-migrated with synthetic NN and NN-KR, and had immunoreactivities of 7.3 and 3.4 pmol/mg of protein respectively (see Figure 4). The small peptides corresponded to the iNT and iNN material in pool 5 (see Figure 2) that co-eluted on h.p.l.c. with synthetic NT and NN respectively. The data were derived from one experiment; similar results were obtained in two other experiments. n.d., not detectable.

	Immunoreactivity (pmol/mg of protein in cell extract)						
	High-molecular-mass peptides					Small peptides	
	iK6L	iE6I	iNN	iNT	iNT-(1–8)	NT	NN
Untreated	n.d.	31	n.d.	2.8	n.d.	23	35
CTU-treated	37	–	11	n.d.	5.2	–	–

Application of the CTU procedure to rMTC 6-23 cell extracts

In order to unmask and quantify antigenic sequences that are contained within larger NT/NN-precursor-derived polypeptides, high-molecular-mass immunoreactive material was submitted to the CTU procedure. Figure 4 shows the relative amounts of iK6L, iNN and iNT-(1–8) that were generated upon CTU treatment of pool 2. When similar experiments were performed with pools 1, 3 and 4, it was found that CTU treatment of pool 3 generated small amounts of iK6L (4–5 times less than in pool 2), little if any iNN and no iNT-(1–8), and that pools 1 and 4 were devoid of immunoreactive material both before and after CTU (results not shown).

The quantitative RIA data obtained before and after CTU treatment of the Sephadex G-50 pools are summarized in Table 3, in which results are expressed as pmol of immunoreactive peptides/mg of protein in the initial cell extract. Prior to CTU, rMTC 6-23 cells contained comparable amounts of total iNT (large + small), iNN and iE6I, whereas they were devoid of iK6L. The high-molecular-mass iNT accounted for about 10% of the total iNT. CTU treatment of high-molecular-mass peptides generated iK6L in amounts greater than those of the iE6I present in the untreated extract. The procedure also generated iNN, but in amounts 3–4-fold less than that of iK6L. However, CTU-generated iNN was 3–4 times more abundant than the large iNT component. Finally, CTU treatment produced iNT-(1–8) in

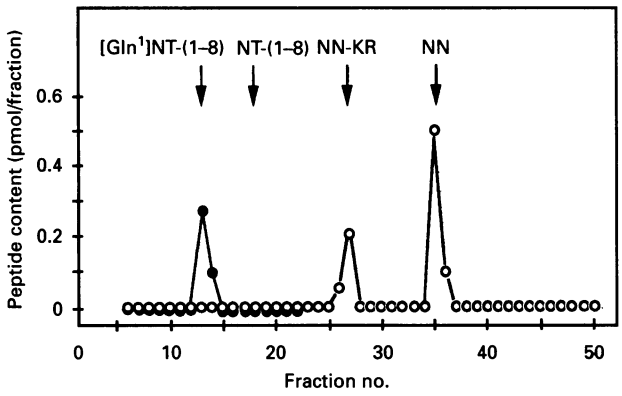

Figure 5 H.p.l.c. of CTU-treated Sephadex G-50 pool 2

Portions of CTU-treated pool 2 were applied to a C-18 h.p.l.c. column (see the Materials and methods section). The fractions were assayed for iNN (○) and iNT-(1–8) (●). Recovery of immunoreactivity was > 90%. Arrows indicate the elution positions of synthetic peptides.

Table 4 Purification of large iE6I from rMTC 6-23 cells

The protein content in the first four steps was determined using the Bio-Rad protein assay reagent. Proteins in the last two steps were estimated from the absorbance at 280 nm of the immunoreactive fractions.

Purification step	iE6I (nmol)	Yield (%)	Specific activity (nmol of iE6I/mg of protein)
Acid extract	6.1	100	0.053
C4, reverse-phase	4.5	74	2.5
SP, ion-exchange	4.0	66	7.6
C4, reverse-phase	3.3	54	8.1
1st Aquapore, reverse-phase	0.83	13.6	55
2nd Aquapore, reverse-phase	0.12	2.0	80

Table 5 N-Terminal sequencing of large iE6I

Edman degradation was performed on 40 pmol of purified iE6I taken from fractions 97 and 98 of the final purification step (see the Materials and methods section). The yield of the major phenylthiohydantoin residues at each cycle is given. Similar results were obtained with another purified sample of iE6I.

Cycle	Residue	Yield (pmol)
1	Ser	5.9
2	Asp	4.5
3	Ser	3.3
4	Glu	1.8
5	Glu	2.6
6	Asp	2.2
7	Val	2.6
8	Arg	2.0
9	Ala	1.5
10	Leu	2.5
11	Glu	1.1
12	Ala	2.7
13	Asp	1.5
14	Leu	1.7
15	Leu	2.8
16	Thr	0.8
17	Asn	2.7
18	Met	2.7
19	His	0.7
20	Ala	2.9

amounts comparable with those of the large iNT that was present before CTU.

H.p.l.c. analysis of CTU-generated immunoreactive NN and NT-(1–8)

The previous results showed that CTU treatment of pool 2 generated iNN in substantial amounts. CTU-generated iNN could theoretically arise from two types of larger NN-containing peptides: those in which the NN moiety is internal, and those in which it is present at the C-terminus. In the first case, the CTU procedure should generate NN with a C-terminal Lys-Arg extension (NN-KR), while in the second case it should generate authentic NN. In order to determine the relative contents of NN and NN-KR, portions of CTU-treated pool 2 were submitted to reverse-phase h.p.l.c. and the fractions were assayed for their iNN content (Figure 5). The CTU-generated iNN in pool 2 was resolved into two peaks, the first one co-eluting with synthetic NN-KR and the second one co-eluting with synthetic NN. The latter accounted for about two-thirds of the total iNN applied to the h.p.l.c. column. These results have been included in Table 3.

As shown above, CTU treatment of pool 2 generated iNT-(1–8). This is consistent with the presence in this pool of large NT-containing precursor form(s). Because the sequence of NT within the precursor starts with a Gln residue (Figure 1), it is expected that the CTU-generated iNT-(1–8) material should be identical to [Gln¹]NT-(1–8) rather than to NT-(1–8) with an N-terminal pyroglutamate, as in native NT. Indeed, h.p.l.c. analysis showed that this material eluted as a single peak with the same retention time as synthetic [Gln¹]NT-(1–8) (Figure 5).

Purification and N-terminal sequencing of iE6I

The high-molecular-mass iE6I material represented the most abundant large precursor fragment in rMTC 6-23 cell extracts, as shown in Table 3. Because of the strict specificity of the anti-E6I antiserum for the free (non-extended) C-terminal sequence of E6I (Table 2), it can be deduced that the E6I sequence is located at the C-terminus of the large iE6I component. However, the N-terminal sequence of this material is not known. It was therefore of interest to purify this product and to sequence its N-terminus.

Table 4 summarizes the yield and purification factor for each step of the purification procedure described in the Materials and methods section. N-Terminal sequencing of the material contained in the peak fraction of the last purification step (see the Materials and methods section) yielded a 20-residue sequence that was identical to the predicted sequence of amino acids 23–42 of the rat NT/NN precursor (Table 5). It should be noted that the purified compound showed poor solubility at neutral pH and a great tendency to stick to glassware, especially as it became purer. The use of detergent (Tween) in acidic solvent improved solubilization of this compound. These problems may explain in part the low yield in the final purification steps and in the sequencing procedure.

DISCUSSION

In the present study we have developed tools and techniques that allowed us to show that the rMTC 6-23 cell line expresses the NT/NN precursor and processes it to yield the small biologically active peptides NT and NN, and several larger polypeptides that might represent either processing intermediates or hitherto unidentified biologically active peptides. The use of several region-directed antisera of well-defined specificity proved invaluable in this regard.

In order to obtain further information regarding the nature and the relative amounts of the large processing intermediates, tryptic digestion was performed so as to unmask internal immunoreactive sequences adjacent to dibasic processing sites. This type of strategy has been applied to the study of a number of peptide precursors such as, for example, proenkephalin [20], provasopressin [21] and procholecystokinin [22]. In the case of the NT/NN precursor, two of the four Lys-Arg pairs are followed by immunoreactive sequences, i.e. NN and K6L, that themselves start with a lysine residue that is essential for binding to their respective antisera. Therefore the side-chain amino groups of lysine residues were reversibly protected by treatment with citraconic anhydride [18], carried out in order to make trypsin selective for arginine residues (CTU procedure). This method, which is usually applied to large-scale treatment of purified proteins [18], was shown here to be applicable to small amounts of cell extract and to allow detection of antigenic sequences at the fentomolar level. It might find more general application for directing cleavage of peptide precursors at the most frequently occurring dibasic processing sites (i.e. Lys-Arg or Arg-Arg) in cases where Lys residues form part of the antigenic determinant of the peptides under study.

Sephadex G-50 chromatography allowed us to identify four immunoreactive components in rMTC 6-23 cell extracts: low-molecular-mass iNT and iNN, and high-molecular-mass iNT and iE6I. The low-molecular-mass iNT and iNN co-eluted on reverse-phase h.p.l.c. with synthetic NT and NN respectively, and therefore most likely represented native endogenous NT and NN. Because our anti-NT antiserum does not recognize C-terminally extended forms of NT, it may be hypothesized that the large iNT peptide displayed the NT sequence at its C-terminus. Given an apparent molecular mass of 16.5 kDa as estimated by Western blot analysis, this N-terminally extended form of NT probably contained most of the N-terminal sequence of the NT/NN precursor, encompassing the NN, E6I and K6L sequences (the theoretical molecular mass of the rat precursor including the signal peptide is 19.5 kDa). Similar considerations lead to the conclusion that the 15 kDa iE6I polypeptide corresponded to a large N-terminally extended precursor fragment encompassing the K6L sequence and ending with the E6I sequence at its C-terminus (i.e. lacking the NN and NT moieties).

In order to determine the exact N-terminal starting point of the large iE6I precursor fragment, this material was purified and N-terminally sequenced. The results revealed that it starts with residue Ser^{23} of the rat prepro-NT/NN precursor and established the Cys^{22}-Ser^{23} bond as the cleavage site of the signal peptide. A similar cleavage site was reported for the canine prepro-NT/NN precursor [19]. Together with immunological considerations, the present sequence data are consistent with the large iE6I precursor fragment extending from residues 23 to 139 in the gene-predicted sequence of the rat prepro-NT/NN precursor [2]. The calculated molecular mass of this peptide is 13239 Da, in good agreement with the value of 15 kDa derived from Western blot experiments.

The finding that large precursor forms containing the K6L and NN sequences were present in rMTC 6-23 cells was further demonstrated by the fact that arginine-directed cleavage of high-molecular-mass peptides yielded iK6L and iNN. The iNN material could be further resolved into NN and NN-KR. The fact that NN-KR was recovered in similar amounts (3.4 pmol/mg of protein) as the large iNT (2.8 pmol/mg of protein) suggested that the latter contributed most of the CTU-generated NN-KR and that rMTC 6-23 cells contained very little unprocessed precursor. The identification of NN as a product of the CTU procedure demonstrated the existence of precursor form(s) that ended with a C-terminal NN sequence. Given the molecular mass of this material as estimated by gel filtration, it is likely that it consisted of large precursor fragment(s) encompassing the K6L sequence. The results also showed that this material was about 2–3 times more abundant (7.3 pmol/mg of protein) than material bearing a C-terminal NT (2.8 pmol/mg of protein). Interestingly, large precursor fragments with a C-terminal NN moiety were found in large amounts in canine and mouse intestine, whereas they were virtually lacking in the brain tissue of these species [8,9]. It is noteworthy that CTU treatment of the high-molecular-mass peptides generated iK6L in amounts that were comparable to the sum of large iE6I plus CTU-generated iNN (the latter is a quantitative index of the total amount of large-NN-containing precursor forms, including the large iNT material). This result further supports the conclusion based on peptide size that all the large precursor fragments identified here encompassed the K6L sequence. This was in fact directly demonstrated for the most abundant large fragment (iE6I material) by purification and N-terminal sequencing.

Altogether, the present data show that, after cleavage of the signal sequence at the Cys^{22}–Ser^{23} bond, rMTC 6-23 cells readily processed the NT/NN precursor at the Lys^{140}–Arg^{141}, Lys^{148}–Arg^{149} and Lys^{163}–Arg^{164} pairs of basic residues that flank and separate the NT and NN sequences, thus generating large amounts of the mature biologically active peptides NT and NN and of a large N-terminal peptide starting at Ser^{23} and ending with the E6I sequence. The processing also generated smaller amounts of large N-terminal peptides bearing the NT or NN sequences at the C-termini. Whether the large peptides are released and exert biological activity, or simply represent processing intermediates that accumulate inside the cell, remains to be determined. By contrast, the Lys^{85}–Arg^{86} pair that precedes the K6L (NN-like) sequence was not processed in our system. Indeed, there was no evidence for the presence of precursor-derived peptides with the K6L (NN-like) sequence at the N-termini as no iK6L could be detected prior to CTU treatment in cell extracts either directly or following gel filtration. This finding was somewhat unexpected, given the fact that Lys^{85}-Arg^{86} and Lys^{140}-Arg^{141} occur within similar amino acid sequences [1]. However, it is now recognized that conformational elements located in the vicinity of basic doublets play an important role in directing proteolytic processing enzymes [23].

In conclusion, the use of region-specific antisera directed towards various domains of the NT/NN precursor, combined with Western blot analysis, arginine-directed tryptic digestion and reverse-phase h.p.l.c., has permitted us to detect and quantify with high sensitivity (fentomolar level) various mature and intermediate peptides that result from the post-translational processing of the precursor in the rMTC 6-23 cell line. These tools and techniques will be applied in the future to other tissues and cell lines that express the NT/NN precursor. These studies will be essential in order to understand the molecular basis of the differential processing that appears to occur for the NT/NN precursor in brain and gut tissues [8–10].

We thank Marie-Thérèse Bohn and Gisèle Jarretou for expert technical assistance. We are greatly indebted to Dr. Eleni Dicou for providing the rMTC 6-23 cell line and to Dr. Solange Lavielle and Dr. Hamadi Boussetta for peptide synthesis.

REFERENCES

1 Dobner, P. R., Barber, D., Villa-Komaroff, L. and McKiernan, C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3516–3520
2 Kislauskis, E., Bullock, B., McNeil, S. and Dobner, P. R. (1988) J. Biol. Chem. **263**, 4963–4968
3 Cuber, J. C., Herrmann, C., Kitabgi, P., Bosshard, A., Bernard, C., De Nadai, F. and Chayvialle, J. C. (1990) Endocrinology (Baltimore) **126**, 1584–1592
4 Carraway, R. E. and Mitra, S. P. (1987) Endocrinology (Baltimore) **120**, 2092–2100

5 De Nadai, F., Cuber, J. C. and Kitabgi, P. (1989) Brain Res. **500**, 193–198
6 Kitabgi, P., De Nadai, F., Cuber, J. C., Dubuc, I., Nouel, D. and Costentin, J. (1990) Neuropeptides **15**, 111–114
7 Douglass, J., Civelli, O. and Herbert, E. (1984) Annu. Rev. Biochem. **53**, 665–715
8 Shaw, C., McKay, D., Johnston, C. F., Halton, D. W., Fairweather, I., Kitabgi, P. and Buchanan, K. D. (1990) Peptides **11**, 227–235
9 Carraway, R. E. and Mitra, S. P. (1990) J. Biol. Chem. **265**, 8627–8631
10 Kitabgi, P., Masuo, Y., Nicot, A., Bérod, A., Cuber, J. C. and Rostène, W. (1991) Neurosci. Lett. **124**, 9–12
11 Zeytinoglou, F. N., Gagel, R. F., Tashjian, A. H., Jr., Hammer, R. A. and Leeman, S. E. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3741–3745
12 Zeytinoglou, F. S., Brazeau, P. and Mougin, C. (1983) Regul. Peptides **6**, 147–154
13 Blache, P., Kervran, A., Martinez, J. and Bataille, D. (1988) Anal. Biochem. **173**, 151–159
14 Granier, C., Van Rietschoten, J., Kitabgi, P., Poustis, C. and Freychet, P. (1982) Eur. J. Biochem. **124**, 117–124
15 Glazer, A. N., Delange, R. J. and Sigman, D. S. (1975) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T. F. and Work, E., eds.), pp. 205–227, Elsevier, Amsterdam
16 Carraway, R. E. and Leeman, S. E. (1976) J. Biol. Chem. **251**, 7035–7044
17 Swank, R. W. and Munkres, K. D. (1971) Anal. Biochem. **39**, 462–477
18 Wilkinson, J. M. (1986) in Practical Protein Chemistry: A Handbook (Darbre, A., ed.), pp. 121–148, John Wiley and Sons, Chichester
19 Carraway, R. E. and Mitra, S. P. (1991) Biochem. Biophys. Res. Commun. **179**, 301–308
20 Cupo, A., Pontarotti, P. A., Jarry, T. and Delaage, M. (1984) Neuropeptides **4**, 375–387
21 Cupo, A., Rougon-Rapuzzi, G. and Delaage, M. (1981) Eur. J. Biochem. **115**, 169–174
22 Odum, L. and Rehfeld, J. F. (1990) Biochem. J. **271**, 31–36
23 Brakch, N., Boussetta, H., Rholam, M. and Cohen, P. (1989) J. Biol. Chem. **264**, 15912–15916

Received 25 June 1992/16 October 1992; accepted 16 November 1992