Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5784–5787, June 1994
Biochemistry

The neuroendocrine polypeptide 7B2 is an endogenous inhibitor of prohormone convertase PC2

GERARD J. M. MARTENS*†, JOANNA A. M. BRAKS*, DOUGLAS W. EIB*, YI ZHOU‡, AND IRIS LINDBERG‡

*Department of Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands; and ‡Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70112-1393

Communicated by Donald F. Steiner, March 1, 1994

ABSTRACT **The subtilisin-like prohormone convertase PC2 and the polypeptide 7B2 (an intracellularly cleaved protein of unknown function) are both selectively present in the regulated secretory pathway of neurons and endocrine cells. Here we demonstrate that intact recombinant 7B2 is a potent inhibitor of PC2 and prevents proPC2 cleavage *in vitro*, whereas the 7B2 cleavage product is virtually inactive. The PC2-related proteinase PC1/PC3 is not inhibited by 7B2. Furthermore, the carboxyl-terminal half of the 7B2 protein sequence is distantly related to the so-called potato inhibitor I family (which includes subtilisin inhibitors). Our findings indicate that 7B2 is a physiological inhibitor of PC2 and may provide alternative avenues for the manipulation of peptide hormone levels.**

Biologically active peptides are often produced by specific cleavage of precursor proteins at pairs of basic amino acid residues (1). Recent work has resulted in the characterization of the family of enzymes involved in the endoproteolytic cleavage of proproteins at dibasic pairs in the secretory pathway (for review, see refs. 2–4). This family is related to the bacterial serine proteinase subtilisin and includes the yeast enzyme Kex2 and the mammalian endoproteinases furin, PACE4, and the prohormone convertases PC1 (also known as PC3), PC2, PC4, and PC5/PC6. The proprotein cleavage enzymes have been implicated in a variety of biological processes from peptide hormone production and blood coagulation to human immunodeficiency virus proliferation. Family members such as Kex2 and furin proteolytically activate proproteins in the constitutive pathway of protein secretion, a basic feature of all secretory cells. In contrast, PC1/PC3 and PC2 cleave precursors for peptide hormones and neuropeptides, and these cleavages occur exclusively in the regulated secretory pathway of neurons and endocrine cells (2–4).

Synthetic compounds (e.g., peptidyl chloromethanes containing basic amino acid residues) have been shown to inhibit the Kex2 enzyme and furin-mediated cleavage of the human immunodeficiency viral coat protein gp160 (5, 6). In addition, the furin enzyme is inhibited by α_1-antitrypsin genetically engineered to contain the consensus cleavage site of furin at its reactive site (7) and furin is moderately inhibited by the similarly mutated turkey ovomucoid third domain (8). However, as yet, potent naturally occurring inhibitors of the proprotein cleavage enzymes have not been identified.

The neuroendocrine-specific polypeptide 7B2 was initially isolated from porcine anterior pituitary glands as a protein of ≈21 kDa (9). Biosynthesis of the ≈21-kDa 7B2 protein occurs through carboxyl-terminal processing of a ≈27-kDa precursor protein (10, 11) and only the cleaved form of 7B2 is released (10). Secretion of the 7B2 cleavage product could be regulated (10, 12, 13), establishing that, like PC1/PC3 and PC2, the polypeptide 7B2 is in the regulated secretory pathway. The 7B2 protein is highly conserved and widely distributed in the central nervous system and endocrine tissues (14–16). Both an intracellular function for 7B2 during prohormone maturation and an extracellular role for the 7B2-derived products as bioactive peptides have been suggested (15–17). In the present study, we demonstrate that the precursor form but not the processed form of 7B2 is a potent inhibitor of PC2.

MATERIALS AND METHODS

Production of Recombinant 7B2 Proteins. For functional studies, recombinant 7B2 protein was produced in *Escherichia coli* as a hexahistidine fusion protein by expression of a human 7B2 cDNA cloned into the *Bam*HI and *Hin*dIII sites of the prokaryotic expression vector pQE30 and induction of the cells with isopropyl β-D-thiogalactopyranoside. The cDNA for expression of the 21-kDa 7B2 protein consisted of nucleotides 107–562 (based on the numbering used in ref. 18) and was generated by PCR using specific primers; the 5′ primer corresponded to nucleotides 107–129 with a *Bam*HI site introduced at the 5′ end and the 3′ primer consisted of nucleotides 538–562 with an introduced 5′ stop codon and 5′ *Hin*dIII site. The recombinant 21-kDa 7B2 protein represents amino acids 1–151 of the human 7B2 protein and corresponds to the 7B2 cleavage product isolated from anterior pituitaries (9, 11). The expression plasmid for the intact 27-kDa 7B2 precursor protein was constructed by replacing the ≈0.2-kb *Kpn* I–*Hin*dIII fragment of the 21-kDa 7B2 construct by the ≈0.8-kb *Kpn* I–*Hin*dIII fragment (encoding the carboxyl-terminal half of the precursor protein) of a full-length human 7B2 cDNA clone (18). Recombinant 7B2 was purified by Ni^{2+}-NTA agarose affinity chromatography according to the instructions of the manufacturer (Qiagen, Chatsworth, CA).

Preparation of PC1/PC3 and PC2 Enzymes. Active 87-kDa PC1/PC3 was purified from medium of overexpressing CHO cells as described (19). PC2 was obtained from the conditioned medium of βTC3 cells through immunopurification (20). One hundred milliliters of 16-h conditioned βTC3 cell culture medium (containing aprotinin at 100 μg/ml) was collected, centrifuged, and concentrated 20-fold. To protect PC2 during immunopurification, enzyme inhibitors were added to the following concentrations: 1 μM *trans*-epoxysuccinic acid, 1 μM pepstatin, *N*-tosyl L-phenylalanine chloromethyl ketone (100 μg/ml), and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (50 μg/ml). Two hundred microliters of anti-PC2 antiserum (rabbit polyclonal antiserum directed against the carboxyl-terminal 10 amino acids of mouse PC2) was added and the mixture was incubated for 6–16 h at 4°C. Five hundred microliters of 50% (vol/vol) protein A-Sepharose was then added and the mixture was shaken for 30 min at 4°C. The pelleted beads were washed twice with 10 vol of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: AMC, aminomethylcoumarin.

†To whom reprint requests should be addressed.

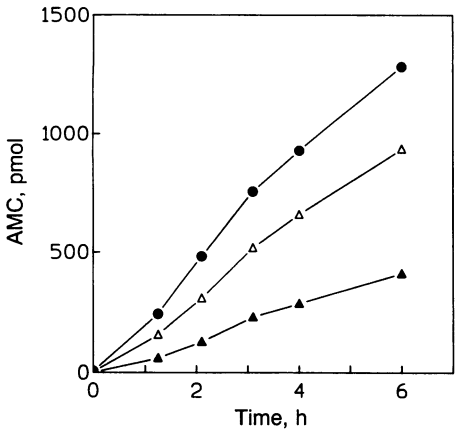

FIG. 1. Recombinant 27-kDa 7B2 inhibits PC2 enzyme activity. Immunopurified PC2 was incubated with recombinant 27-kDa 7B2 for the indicated times. ●, Control; △, 0.74 nM recombinant 27-kDa 7B2; ▲, 2.2 nM recombinant 27-kDa 7B2. PC2 enzyme activity was detected by the cleavage of a fluorogenic substrate (200 μM).

ice-cold AG buffer (0.1 M sodium phosphate, pH 7.4/1 mM EDTA/0.1% Triton X-100/0.5% Nonidet P-40/150 mM NaCl) and twice with 10 vol of ice-cold Dulbecco's PBS prior to storage in 2–3 ml of PBS containing 20% (vol/vol) glycerol at −20°C. Five to 10 μl of this suspension (10% gel) was used as the enzyme source in the enzyme assay. PC2 enzymatic activity and immunoreactivity (as assessed by Western blot analysis) are absent if either preimmune serum or antigen-blocked anti-PC2 antiserum is used in the immunopurification procedure (20).

Enzyme Assay. Duplicate reactions were performed in 0.1 M sodium acetate, pH 5.5/5 mM calcium chloride/0.2 mM fluorogenic substrate (carbobenzoxy-Arg-Tyr-Lys-Arg-AMC, where AMC is aminomethylcoumarin)/0.1% Brij/5 μg of bovine serum albumin (electrophoresis grade; Miles)/the above inhibitor mixture/recombinant 7B2 at the indicated concentrations. Reaction mixtures were preincubated with recombinant 7B2 for 15 min at room temperature before the addition of substrate. Released AMC was determined at various times in the same samples by fluorometry with emission at 460 nm and excitation at 380 nm, and values were compared to a standard curve of free AMC; <5% of the substrate was consumed during the control reaction.

Cleavage Studies. For the study of the effect of recombinant 7B2 on proPC2 cleavage, reaction mixtures contained 10 μl of PC2 enzyme suspension (see above), 0.1 M sodium acetate (pH 5.5), the inhibitor mixture described above, either 5 mM calcium chloride or 5 mM EDTA, and either 1 μg of recombinant 21-kDa 7B2 or 1 μg of 27-kDa 7B2. After incubation for 6 h at 37°C, the samples were subjected to SDS/PAGE and subsequent Western blot analysis using anti-PC2 antiserum as described (20). For the study of the *in vitro* cleavage of 7B2 by PC1/PC3 and PC2, recombinant 27-kDa 7B2 (1 μg) and 21-kDa 7B2 (1 μg) were incubated with or without 10 μl of purified PC1/PC3 or immunopurified PC2 for 6 h at 37°C under the conditions described above. Aliquots of the samples were subjected to SDS/PAGE and subsequent Western blot analysis using the anti-7B2 monoclonal antibodies MON102 and MON144 (21). Recombinant 27-kDa 7B2 and 21-kDa 7B2 were used as markers. To determine at which site recombinant 27-kDa 7B2 is cleaved by PC1/PC3, the 7B2 cleavage products were purified by reverse-phase HPLC (Vydac C_4 column), and from ≈10 pmol of the major cleavage product, a partial amino acid sequence was determined (San Diego State University Microchemical Core Facility, San Diego).

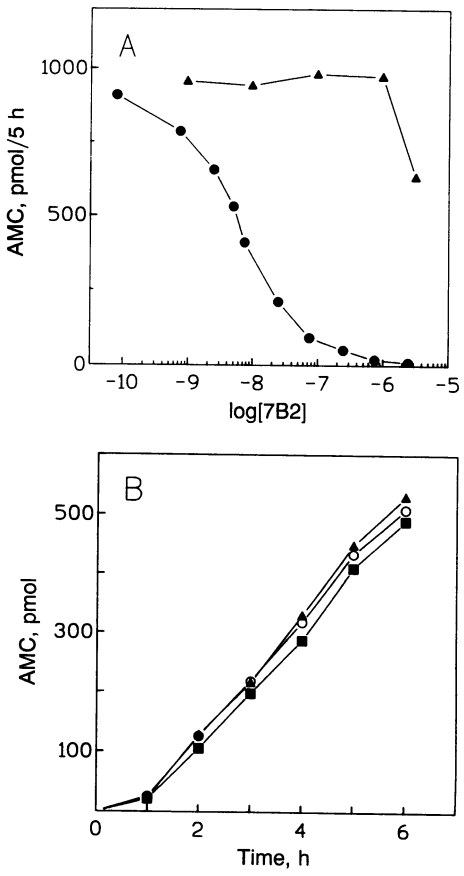

FIG. 2. Specificity of the inhibition of enzyme activity by recombinant 7B2. (*A*) At nanomolar concentrations, recombinant 27-kDa 7B2 (●) but not carboxyl-terminally truncated recombinant 21-kDa 7B2 (▲) inhibits PC2 cleavage of a fluorogenic substrate (200 μM). (*B*) Recombinant 21-kDa 7B2 and recombinant 27-kDa 7B2 do not inhibit PC1/PC3 cleavage of a fluorogenic substrate (200 μM). Purified PC1/PC3 was incubated in the presence of 3 μg (2.2 μM) of recombinant 27-kDa 7B2 (▲) or 3 μg (2.8 μM) of recombinant 21-kDa 7B2 (■) or in the absence of 7B2 (○) for the indicated time periods.

RESULTS AND DISCUSSION

Effect of Recombinant 7B2 Precursor Protein and 7B2 Cleavage Product on PC2 and PC1/PC3 Enzyme Activity. To obtain the 27-kDa precursor form of 7B2 for functional studies, human 7B2 was expressed in *E. coli* and purified by affinity metal-chelation chromatography. Recombinant carboxyl-terminally truncated 21-kDa 7B2, corresponding to the 7B2 protein initially isolated from anterior pituitaries (9), was similarly produced. Since active purified PC2 from either natural or recombinant sources is not currently available, PC2 enzyme activity was obtained through immunopurification (20) from the conditioned medium of βTC3 cells, a mouse pancreatic cell line. Enzymatic activity of the immunopurified PC2 has a pH optimum of 5–5.5 and is calcium-dependent ($K_{0.5} \approx 100$ μM). To test the effect of 7B2 on PC2 enzyme activity, a time-course analysis of the hydrolysis of a fluorogenic substrate (carbobenzoxy-Arg-Tyr-Lys-Arg-AMC; 200 μM) was performed in the presence or absence of the 27-kDa form of recombinant 7B2. The results show that 27-kDa 7B2, at nanomolar concentrations, is a potent inhibitor of immunopurified PC2 (Fig. 1). We also observed inhibition of soluble PC2 partially purified by ion-exchange chromatography from βTC3 cell medium, indicating that inhibition by 27-kDa 7B2 is not confined to immunocomplexed PC2 (results not shown).

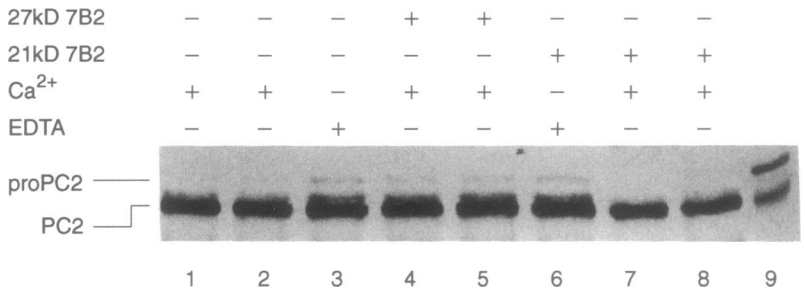

FIG. 3. Recombinant 27-kDa 7B2 protects the proform of PC2. After incubation of immunopurified PC2 with recombinant 21-kDa or 27-kDa 7B2 for 6 h at 37°C, the samples were subjected to Western blot analysis using an anti-PC2 antibody. In lane 9 an extract from βTC3 cells is used as a marker for the two forms of PC2.

Dose–response analysis confirms that 27-kDa 7B2 is a tight-binding inhibitor of PC2 with an estimated K_i of 6.7 nM. In contrast, at submicromolar concentrations, the 21-kDa form of 7B2 did not inhibit PC2 activity (Fig. 2*A*). Like the other members of the proprotein convertase family, PC2 cleaves at pairs of basic amino acid residues (2–4). The high affinity of 27-kDa 7B2 for PC2 is not due to the presence of paired basic amino acid residues in its sequence because the putative PC2 substrates proenkephalin or proinsulin (22, 23), which contain multiple paired basic residues, did not inhibit PC2 cleavage of the fluorogenic substrate when included in the reaction mixture (2 μM, final concentration). Interestingly, the neuroendocrine enzyme PC1/PC3, which in the catalytic domain exhibits 55% amino acid sequence identity with PC2 (4), was not inhibited by either form of 7B2 (Fig. 2*B*). These results indicate that 7B2 is specifically targeted toward PC2 rather than representing a general inhibitor for the family of mammalian proprotein cleavage enzymes.

Effect of Recombinant 7B2 Precursor Protein and 7B2 Cleavage Product on proPC2 Cleavage. In common with all known subtilisin-like enzymes, PC2 is synthesized as a proprotein, and cleavage of the amino-terminal pro region is thought to result in the activation of the enzyme (2–4). Western blot analysis of immunopurified PC2 obtained from conditioned medium revealed that this enzyme preparation contains a prominent band corresponding to mature PC2 and a minor band corresponding to proPC2 (Fig. 3). Incubation of the enzyme preparation in the presence of calcium resulted in the disappearance of the proform of PC2. However, when 27-kDa 7B2 was included in the reaction mixture, proPC2 was protected from cleavage whereas 21-kDa 7B2 failed to give protection (Fig. 3). This finding is consistent with the inhibitory effect of the larger, but not the smaller, form of 7B2 on PC2 enzyme activity described above. In the enzyme assay, the relative contributions of active mature PC2 vs. PC2 activity arising from proPC2 cleavage (and thus ultimately the relative levels of direct vs. indirect enzyme inhibition by 7B2) are at present not known.

The Carboxyl-Terminal Half of the 7B2 Protein Sequence Is Distantly Related to the Potato Inhibitor I Family. The fact that PC2 is a subtilisin-related proteinase (2–4) and the observation that 7B2 is an inhibitor of PC2 prompted us to search for a structural relationship between 7B2 and protein inhibitors of subtilisins. A visual search revealed that the carboxyl-terminal half of human 7B2 (18) displays 27%, 17%, 20%, and 17% amino acid sequence identity (over a stretch of 60 amino acid residues) with the following members of the potato inhibitor I family, leech inhibitor eglin c (24), Adzuki subtilisin inhibitor (25), Barley chymotrypsin inhibitor (26), and potato tuber inhibitor I (27), respectively (Fig. 4). Similar degrees of amino acid sequence identity are found when porcine, rat, mouse, salmon, or *Xenopus* 7B2 sequences (14, 29–31) are compared with the inhibitor sequences. The potato inhibitor I family encompasses a variety of sequences from plants to invertebrates (24, 28); the 7B2 protein may now extend this family to vertebrates.

***In Vitro* and *in Vivo* Cleavage of the 7B2 Protein.** Western blot analysis showed that *in vitro* PC1/PC3 effectively cleaves recombinant 27-kDa 7B2 (Fig. 5). Amino-terminal sequence analysis of the HPLC-purified carboxyl-terminal 7B2 cleavage product revealed the sequence Ser-Val-Asn-Pro/Met-Tyr, indicating that cleavage by PC1/PC3 occurred at Arg^{153}-Arg^{154} (numbering corresponds to that used in Fig. 4). The molecular mass of the amino-terminal 7B2 cleavage

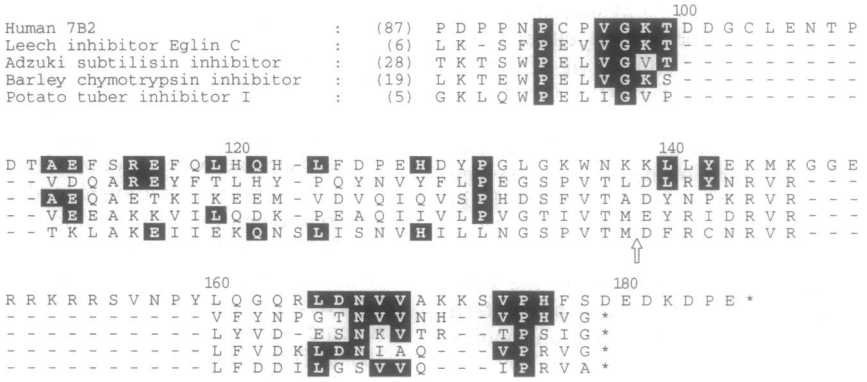

FIG. 4. Alignment of the amino acid sequence of human 7B2 with those of representative members of the potato inhibitor I family of protein proteinase inhibitors. Numbering refers to the sequence of human 7B2 (18). Residues identical between the 7B2 sequence and an inhibitor sequence are shown in white letters against a black background. Gaps introduced in the sequences to optimize the alignment are represented by dashes. Arrow indicates the scissile bond of the reactive site of the inhibitor I proteins (28). The number of amino acid residues in the amino-terminal regions of the sequences is in parentheses; asterisks denote the carboxyl-terminal end of the proteins. Amino acid sequences shown are human 7B2 (18), leech inhibitor eglin c (24), Adzuki subtilisin inhibitor (25), Barley chymotrypsin inhibitor (26), and potato tuber inhibitor I (27).

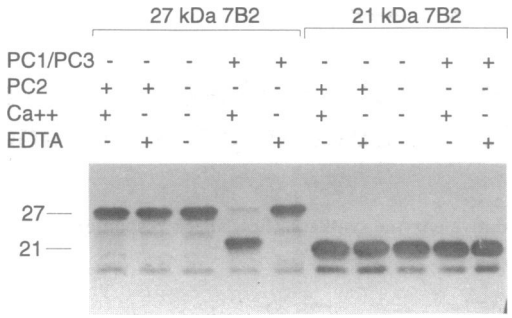

FIG. 5. Recombinant 27-kDa 7B2 is cleaved *in vitro* by PC1/PC3 but not by PC2. After incubation of recombinant 27-kDa 7B2 and 21-kDa 7B2 with PC1/PC3 or PC2, the samples were subjected to Western blot analysis using anti-7B2 monoclonal antibodies. The left portion of the blot depicts incubation of the purified enzymes with recombinant 27-kDa 7B2, and the right half of the blot shows identical incubations performed using 21-kDa 7B2. The center lane in each half represents the corresponding unincubated control recombinant 7B2 protein. Incubations were carried out for 6 h at 37°C. The enzymatic activity of PC2 measured in parallel samples lacking recombinant 7B2 was 4.5 pmol of AMC per h per μl, and the activity of PC1/PC3 was 8.4 pmol of AMC per h per μl.

product (Fig. 5) is consistent with a 21-kDa protein containing additional carboxyl-terminal basic amino acid residues (Arg^{150}-Arg-Lys-Arg-Arg^{154}). In contrast, under the conditions of the present *in vitro* incubations, recombinant 27-kDa 7B2 is not cleaved by PC2 and recombinant 21-kDa 7B2 is not cleaved by either enzyme. These observations are in line with the finding that intact 7B2 tightly binds and effectively inhibits PC2 but not PC1/PC3. *In vivo*, in the porcine anterior pituitary gland, the precursor form of 7B2 is cleaved in the region Arg^{150}-Arg-Lys-Arg-Arg^{154} to an ≈21-kDa product (11). Conversely, in the *Xenopus* intermediate pituitary gland, the cleavage of the 7B2 precursor protein to an ≈18-kDa product appears to occur at Lys^{138}-Lys^{139} (10), a site that coincides with the scissile bond of the inhibitor I proteins in the alignment shown in Fig. 4. It is not yet clear which proteinases are involved in the *in vivo* cleavage of 7B2 and whether the differential cleavage of 7B2 in the two pituitary lobes is due to species differences or to tissue-specific processing.

In conclusion, our results indicate that the neuroendocrine polypeptide 7B2 participates in the physiological regulation of the neuroendocrine-specific enzyme PC2. This important convertase has been implicated in the processing of a large number of prohormones, including those for insulin and opioid peptides (22, 23, 32). The 7B2 protein may thus represent a useful target for therapeutic regulation of PC2 enzyme activity.

We thank K. J. T. P. Van den Heuvel, A. M. Van Horssen, and J. A. M. Leunissen for assistance during this project; and C. Batie, J. P. H. Burbach, B. G. Jenks, E. W. Roubos, and W. J. M. Van de Ven for comments on the manuscript. This work was supported by a Pionier grant from the Netherlands Organization for Scientific Research (NWO) (to G.J.M.M.), a research scientist development award from the National Institute of Drug Abuse (to I.L.), European Community Grant BIOT-CT91-0302, and National Institutes of Health Grant DA 05084.

1. Douglass, J., Civelli, O. & Herbert, E. (1984) *Annu. Rev. Biochem.* **53**, 665–715.
2. Barr, P. J. (1991) *Cell* **66**, 1–3.
3. Steiner, D. F., Smeekens, S. P., Ohagi, S. & Chan, S. J. (1992) *J. Biol. Chem.* **267**, 23435–23438.
4. Seidah, N. G. & Chrétien, M. (1992) *Trends Endocrinol. Metab.* **3**, 133–140.
5. Angliker, H., Wikstrom, P., Shaw, E., Brenner, C. & Fuller, R. S. (1993) *Biochem. J.* **293**, 75–81.
6. Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H.-D. & Garten, W. (1992) *Nature (London)* **360**, 358–361.
7. Anderson, E. D., Thomas, L., Hayflick, J. S. & Thomas, G. (1993) *J. Biol. Chem.* **268**, 24887–24891.
8. Lu, W., Zhang, W., Molley, S. S., Thomas, G., Ryan, K., Chiang, Y., Anderson, S. & Laskowski, M., Jr. (1993) *J. Biol. Chem.* **268**, 14583–14585.
9. Hsi, K. L., Seidah, N. G., De Serres, G. & Chrétien, M. (1982) *FEBS Lett.* **147**, 261–266.
10. Ayoubi, T. A. Y., Van Duijnhoven, H. L. P., Van de Ven, W. J. M., Jenks, B. G., Roubos, E. W. & Martens, G. J. M. (1990) *J. Biol. Chem.* **265**, 15644–15647.
11. Lazure, C., Benjannet, S., Seidah, N. G. & Chrétien, M. (1991) *Int. J. Pept. Protein Res.* **38**, 392–400.
12. Iguchi, H., Natori, S., Nawata, H., Kato, K., Ibayahi, H., Chan, J. S. D., Seidah, N. G. & Chrétien, M. (1987) *J. Neurochem.* **49**, 1810–1814.
13. Vieau, D., Tougard, C., Rosenbaum, E., Lenne, F. & Bertagna, X. (1991) *J. Neuroendocrinol.* **3**, 229–231.
14. Waldbieser, G. C., Aimi, J. & Dixon, J. E. (1991) *Endocrinology* **128**, 3228–3236.
15. Marcinkiewicz, M., Benjannet, S., Seidah, N. G., Cantin, M. & Chrétien, M. (1987) *Cell Tissue Res.* **250**, 205–214.
16. Steel, J. H., van Noorden, S., Ballesta, J., Gibson, S. J., Ghatei, M. A., Burrin, J., Leonhardt, U., Domin, J., Bloom, S. R. & Polak, J. M. (1988) *Endocrinology* **122**, 270–282.
17. Iguchi, H., Chan, J. S. D., Seidah, N. G. & Chrétien, M. (1987) *Peptides* **8**, 593–598.
18. Martens, G. J. M. (1988) *FEBS Lett.* **234**, 160–164.
19. Zhou, Y. & Lindberg, I. (1993) *J. Biol. Chem.* **268**, 5615–5623.
20. Shen, F.-S., Seidah, N. G. & Lindberg, I. (1993) *J. Biol. Chem.* **268**, 24910–24915.
21. Van Duijnhoven, J. L. P., Verschuren, M. C. M., Timmer, E. D. J., Vissers, P. M. A. M., Groeneveld, A., Ayoubi, T. A. Y., Van den Ouweland, A. M. W. & Van de Ven, W. J. M. (1991) *J. Immunol. Methods* **142**, 187–198.
22. Breslin, M. B., Lindberg, I., Benjannet, S., Mathis, J. P., Lazure, C. & Seidah, N. G. (1993) *J. Biol. Chem.* **268**, 27084–27093.
23. Smeekens, S. P., Montag, A. G., Thomas, G., Albiges-Rizo, C., Carroll, R., Benig, M., Phillips, L. A., Martin, S., Ohagi, S., Gardner, P., Swift, H. H. & Steiner, D. F. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8822–8826.
24. Seemüller, U., Eulitz, M., Fritz, H. & Strobl, A. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1841–1846.
25. Nozaka, H., Yamagata, H., Aizono, Y., Yoshikawa, M. & Iwasaki, T. (1989) *J. Biochem.* **106**, 1003–1008.
26. Svendsen, I., Boisen, S. & Hejgaard, J. (1982) *Carlsberg Res. Commun.* **47**, 45–53.
27. Richardson, M. & Cossins, L. (1975) *FEBS Lett.* **52**, 161.
28. Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem.* **49**, 593–626.
29. Brayton, K. A., Aimi, J., Qiu, H., Yazdanparast, R., Ghatei, M. A., Polak, J. M., Bloom, S. R. & Dixon, J. E. (1988) *DNA* **7**, 713–719.
30. Mbikay, M., Grant, S. G. N., Sirois, F., Tadros, H., Skowronski, J., Lazure, C., Seidah, N. G., Hanahan, D. & Chrétien, M. (1989) *Int. J. Pept. Protein Res.* **33**, 39–45.
31. Martens, G. J. M., Bussemakers, M. J. G., Ayoubi, T. A. Y. & Jenks, B. G. (1989) *Eur. J. Biochem.* **181**, 75–79.
32. Guest, P. C., Arden, S. D., Bennet, D. L., Clark, A., Rutherford, N. G. & Hutton, J. (1992) *J. Biol. Chem.* **267**, 22401–22406.