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Differential processing of hormone precursor

independent production of somatostatins 14 and 28 in transfected neuroblastoma 2A cells

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Neuro 2A cells infected with a retroviral vector carrying human prosomatostatin cDNA expressed and processed correctly the precursor into somatostatins-14 and -28 [(1989) EMBO J. 8, 2911–2916]. In order to study the mechanisms by which the active hormone sequences arise, site directed mutagenesis was performed on either the dibasic (ArgLys) or monobasic (Arg) cleavage sites involved in the production of somatostatins-14 and -28, respectively. Radioimmunochemical analysis of the somatostatin-related products indicated that replacement of either Arg^{-2}-Lys^{-1} by Asn^{-2}-Asn^{-1} or of Arg^{-15} by Asn^{-15} resulted in the exclusive production of either somatostatin-28 or -14, respectively. Moreover only prosomatostatin[1–76] was detected and no somatostatin-28[1–12] could be measured in cell extracts. Selective suppression of either somatostatin-14 or somatostatin-28 release by mutation did not affect the level of production of the other hormone but resulted in a correlative increase of unprocessed prosomatostatin. It is concluded that in this cell type (i) somatostatin-14 is exclusively generated by dibasic cleavage at the Arg^{-2}-Lys^{-1} site of the intact precursor with concomitant production of prosomatostatin[1–76], and (ii) no direct interactions between the monobasic and dibasic processing domains occur.

Endoprotease; Somatostatin radioimmunoassay; Site-directed mutagenesis; Basic residue

1. INTRODUCTION

In mammals, prosomatostatin appears to be the product of expression of a single gene. Therefore, generation of somatostatin-14 (S-14) and somatostatin-28 (S-28) peptide sequences involves differential processing of a unique precursor by peptide bond hydrolysis at either a dibasic (RK) or a monobasic (R) cleavage site (Fig. 1). This system provides indeed a very useful model to understand how differential maturation of a single prohormone molecule can generate hormonal diversity by means of multiple cleavage loci recognition by the enzyme machinery (for a review see [1]). Gene transfer techniques offer unique possibilities to study the processing of neuropeptide precursors in cultured cell lines. Using this technology, we have shown previously [2,3] that infection of Neuro2A cells with a retrovirus carrying the human prosomatostatin cDNA resulted in the processing of the precursor into approximately equal amounts of somatostin-28 (S-28) and somatostatin-14 (S-14). In the present study, site-directed mutagenesis was performed to analyse separately the two possible prosomatostatin processing pathways. The data indicate that, in this cell type, either S-14 or S-28 appear to be produced independently and that further processing of Pro-S[1–72] into Pro-S[1–64], or of S-28 into S-28[1–12] and S-14[4–6] does not occur.

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2. MATERIALS AND METHODS

2.1. *DNA manipulations, construction of expression vectors and cell culture*

DNA modifications and subcloning, site-directed mutagenesis and construction of the pN2Som retroviral expression vectors was performed exactly as described previously [2,3]. The retroviral sequences of pN2 are derived from the Moloney murine leukemia virus [7]. It contains both LTRs and the sequences required for production of infectious viruses. In such constructs, the expression of prosomatostatin is driven by the SV40 early promoter while the bacterial gene conferring the resistance to the neomycin analog G418 is under the control of the retrovirus 5′LTR. The transcription termination/polyA addition signals are provided by the virus 3′LTR. The presence of the mutations in the expression vectors was confirmed by sequencing the mutated regions by the chain-termination method for double-stranded templates using T7 DNA polymerase [8]. Table I lists the mutants constructed for the present studies.

Table I

Mutated sequences of human prosomatostatin: the following mutated sequences were expressed after transfection in Neuro2A cells.

PROSOMATOSTATIN [Arg^{-15}-Asn^{-1}]
Arg^{-15}-Ser-Ala-Asn-Ser-Asn-Pro^{-9}-Ala-Met-Ala-Pro^{-5}-Arg-Glu-Arg^{-2}-Lys^{-1}-Ala-Gly-Cys-Lys-Asn^{-1}
MUTANT I: (Asn^{-15})
Asn-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn
MUTANT II: (Asn^{-2}-Asn^{-1})
Arg-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Asn-Asn-Ala-Gly-Cys-Lys-Asn
MUTANT III: (Gly^{-9})
Arg-Ser-Ala-Asn-Ser-Asn-Gly-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn
MUTANT IV: (Ala^{-9})
Arg-Ser-Ala-Asn-Ser-Asn-Ala-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn
MUTANT V: (Ala^{-5})
Arg-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Ala-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn
MUTANT VI: (Ala^{-9}, Ala^{-5})
Arg-Ser-Ala-Asn-Ser-Asn-Ala-Ala-Met-Ala-Ala-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn
MUTANT VII: (Ala^{-9}, Asn^{-15})
Asn-Ser-Ala-Asn-Ser-Asn-Ala-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn
MUTANT VIII: (Ala^{-9}, Asn^{-2}-Asn^{-1})
Arg-Ser-Ala-Asn-Ser-Asn-Ala-Ala-Met-Ala-Pro-Arg-Glu-Asn-Asn-Ala-Gly-Cys-Lys-Asn

The substitued residues are underlined.
Mutants III, IV, V and VI were taken from [2,3].

2.2. Analysis of somatostatin-related peptides

Subconfluent infected Neuro2A cells were harvested in Tris-buffered saline (TBS) containing 4 mM EGTA (3 ml per Petri dish). An equal volume of 2 × extraction solution (1 × extraction solution: 1 M HCl, 5% formic acid, 1% trifluoro-acetic acid (TFA), 0.2% 2-mercaptoethanol and 1% NaCl) [9] is added and the cell suspension sonicated twice for 30 s. The cell debris were pelleted and the supernatant passed through an ODS-silica cartridge (Sep-pak C18, Waters) equilibrated with 0.1% TFA. The adsorbed peptides were eluted with 40% acetonitrile in 0.1% TFA. Control experiments using S-14 and S-28[1-12] as standards allowed to determine a recovery yield for this filtration ≥80%. TFA and acetonitrile were eliminated by lyophilization and the dry peptides resuspended in 5% acetic acid. The amounts of precursor, S-28 and S-14 in the cell extracts were determined by running the samples on a C18 reverse-phase HPLC column (nucleosil 5 μ; 250 × 4 mm) eluted isocratically with 23% acetonitrile and 0.05% TFA at a flow rate of 1 ml · min^{-1} during 40 min, followed by a gradient of acetonitrile (23% to 100%) in 25 min. 1-ml fractions were collected and then assayed for the presence of immunoreactive species using a specific radioimmunoassay as described in [2]. Identification of Pro-S[1-76] was performed after the HPLC separation on the same samples using a specific RIA for S-28[1-12] [4,6]. Identification of Pro-S[1-92], S-28 and S-14 was performed similarly on the recovered HPLC fractions using a specific RIA for S-14 [2,6].

3. RESULTS AND DISCUSSION

In mammals, processing of prosomatostatin results in the production of two biologically active peptides, somatostatin-28 (S-28) and somatostatin-14 (S-14). The biogenesis of S-14 can be accomplished via two distinct processing pathways of the common precursor (pathways I and II, Fig. 1). In the first scheme, S-14 is produced directly by proteolysis of the precursor at a pair of basic amino acids (Arg^{-2}-Lys^{-1}). In the second scheme, S-28 is first generated by proteolysis of the precursor at a single arginyl residue (Arg^{-15}), followed by proteolysis of the Arg^{-2}-Lys^{-1} site resulting in the release of S-14 and S-28[1-12].

In order to separately analyse these two pathways, mutations affecting selectively either the Arg^{-15} or the Arg^{-2}-Lys^{-1} sites (mutants I and II, respectively; Table I) were thus created in the human prosomatostatin cDNA and the extracts of infected Neuro2A cells analyzed with sensitive S-14 and S-28[1-12] radioim-

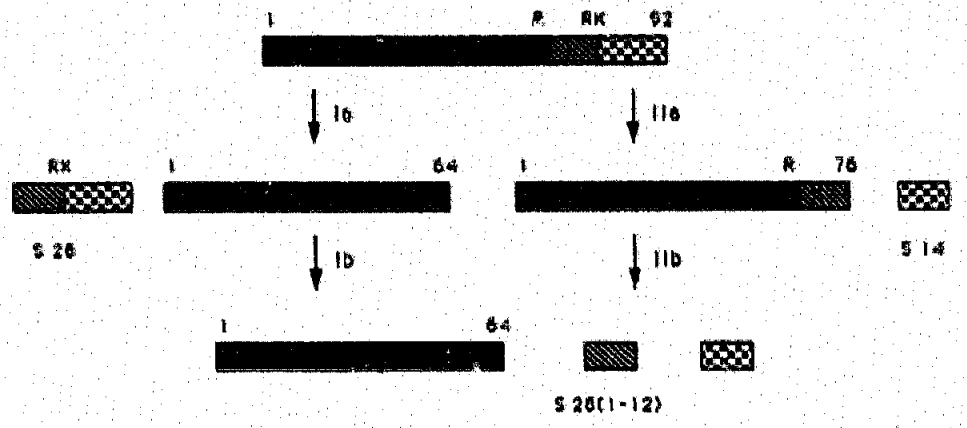

Fig. 1. Theoretical pathways of differential prosomatostatin processing. In Neuro2A cells only reactions Ia and IIa occur.

munoassays. The results presented in Table II indicate unequivocally that isosteric replacement of the basic amino acids by Asn selectively abolished either S-28 or S-14 production. In cells expressing mutant I, neither S-28 nor S-28[1–12] could be detected whereas in cells expressing mutant II, abolishment of S-14 production was accompanied by the disappearance of detectable amounts of Pro-S[1–76]. These results are in agreement with in vitro [10,11] and in vivo [12] studies indicating that the modification or the substitution of basic residues abolished recognition of the site by the processing enzymes.

Moreover, selective suppression of cleavage at either the monobasic or the dibasic site did not result in a significant increase in cleavage at the other site but rather led to a correlative increase in the ratio of unprocessed prosomatostatin (Pro-S[1–92]) detected in cell extracts (Table II). These observations could be explained by postulating the presence of two types of maturation enzymes, each one involved in the recognition of a specific site (Arg^{-2}-Lys^{-15} or Arg^{-15} site). It is also possible that these enzymes might be located in different compartments of the secretory pathway [6]. Although very unlikely, our results do not rule out the possibility that the mutations affect the folding of the precursor, retarding its exit from the rough endoplasmic reticulum and thus accounting for the increased intracellular content in the precursor. Results by others indeed suggest that the information necessary for the correct addressing of prosomatostatin is probably contained in the NH_2-terminal [1–62] portion [13], a domain which was not affected directly by the here reported mutations.

Since the yield of S-28 was similar in the extracts of cells infected with the unmutated precursor and with mutant II, one can conclude that S-28, produced by monobasic cleavage at the Arg^{-15} site, does not undergo further processing at its Arg^{-2}-Lys^{-1} site. This conclusion was supported by the observation that only Pro-S[1–76] and no S-28[1–12] could be detected in the extracts of cells infected with the unmutated cDNA. These results indicate that (i) in Neuro2A cells, S-14 is produced exclusively by the processing of Pro-S[1–92] at its dibasic Arg^{-2}-Lys^{-1} site, and (ii) production of hormonal sequences occurs independently.

In previous work [2], it was demonstrated that Pro^{-5} and Pro^{-9} residues are essential in prosomatostatin processing. In particular, replacement of Pro^{-9} (mutant IV) and Pro^{-5} (mutant V) by Ala abolished selectively S-28 and S-14 release from the precursor, an effect tentatively attributed to local disruption of an ordered

Table II

Effects of various mutations on prosomatostatin processing in transfected Neuro2A cells

Mutants	S-28	S-14	Pro-S[1–92]	Pro-S[1–76]
Unmutated	120	100	50	39
	(44)	(37)	(19)	
I	0	161	153	66
		(52)	(48)	
II	90	0	103	0
	(46)		(54)	
III	265	496	34	62
	(4)	(65)	(4)	
IV	0	68	59	11
		(53)	(47)	
V	60	0	130	0
	(31)		(69)	
VI	45	0	118	0
	(27)		(73)	
VII	0	77	115	39
		(40)	(60)	
VIII	0	0	165	0
			(100)	

The amounts of S-28, S-14, Pro-S[1–92] and Pro-S[1–76] were evaluated by RIA using antibodies against somatostatin-14 or somatostatin-28[1–12]. Results are expressed in pg of 'S-14' or 'S-28[1–12]' for the latter. Numbers in parentheses represent the fraction of respective forms calculated as percentage of total somatostatin immunoreactivity. For direct comparison, the amount of somatostatin related peptides detected per 10^6 cells is also presented. In order to allow comparisons with other mutants results for mutants III, IV, V and VI were taken from [2,3].

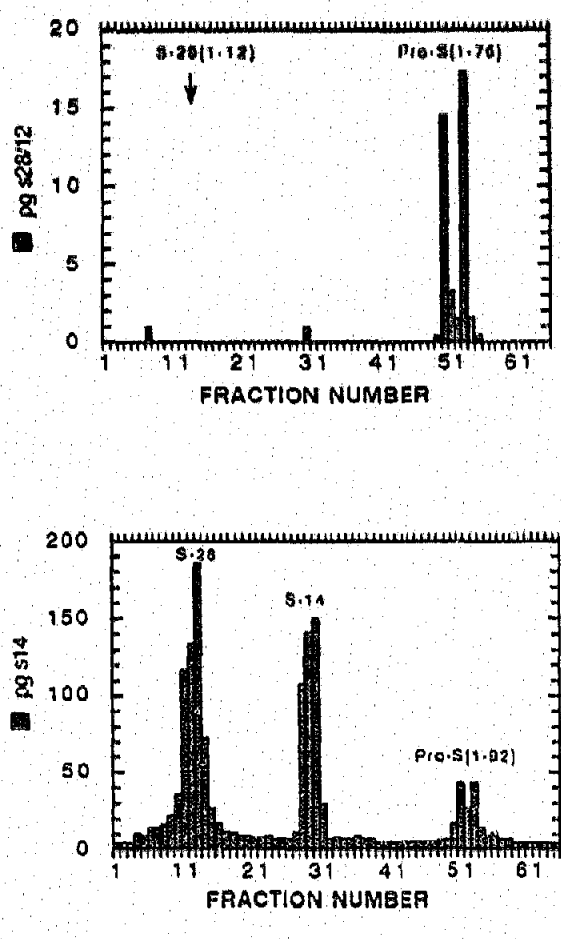

Fig. 2. HPLC analysis of prosomatostatin and of its processing fragments. Top. Immunochemical evaluation of Pro-S [1-76] and S-28 [1-12] after reverse-phase HPLC separation as described in section 2. RIA were run using anti S-28 [1-12] antibodies [3] Bottom. Immunochemical detection of Pro-S [1-92], S-28 and S-14 after HPLC separation using a specific RIA with anti S-14 antibodies [2] (see section 2).

secondary structure [2,3]. Moreover it could be shown that the importance of the proline residue at position-9 was due to its contribution to a secondary structure since replacement of Pro^{-9} by Gly^{-9} a strong β-turn former (mutant III) did not affect cleavage at the Arg^{-15} site [3]. Indeed the results obtained with the double mutant (Ala^{-9}, Ala^{-5}; i.e. mutant VI) suggested that these effects were not exclusively related to local secondary structure effects but were possibly attributed to more complex interactions occurring at the tertiary level [2,3]. In order to analyse possible effects of replacement of Pro^{-9} by Ala^{-9} on each individual processing site, mutants VII and VIII were constructed. Results obtained with the double mutant Ala^{-9}, Asn^{-15} (mutant VII) demonstrate that Ala^{-9} replacement for Pro^{-9}, which was shown previously to interfere with processing at the Arg^{-15} site, did not affect cleavage at the dibasic site. Indeed the proportion of S-14 recovered from cell extract was very similar to that found in the case of the wild type. Similarly the observation that triple mutation Ala^{-9}, Asn^{-2}-Asn^{-1} (mutant VIII) resulted in abolishment of S-28 production, reinforces the previous conclusions that the two processing motifs involved in S-14 and S-28 production by selective proteolysis processing appear to be functionally independent.

Prosomatostatin clearly constitutes a remarkable model since this particular prohormone molecule possesses intrinsic properties which allow various cell types to process this precursor according to selective differential metabolic pathways. When compared with lower organisms (like teleostean fishes [14] where two distinct and segregated precursor molecules provide the supply for different hormonal activities, the mammalian system appears as a more sophisticated one allowing for hormonal diversity by modulation of proteolytic cleavages. The question then arises as to the source of these properties. The present data obtained on a clonal cell line suggest, indeed, that differential processing of a single prohormone is achieved by two mechanisms. The first one deals with the structural properties of the prohormone molecule and the second one possibly involves subcellular segregation of the processing events and of the corresponding enzyme machinery. It can be envisioned that in situ co-localization of the processing events occurring in the Golgi apparatus [6,15,16] and of the enzyme(s) involved in such reactions will provide further insight in these important mechanisms

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REFERENCES

[1] Cohen, P., Kuks, P.F.M., Gomez, S. and Morel, A. (1988) in: Prohormones, Hormones and Their Fragments (Martinez, J., ed.) Ellis Horwood (Health Sciences Series) Verlag Chemie, pp. 83-103.
[2] Gomez, S., Boileau, G., Zollinger, L., Nault, C. Rholam, M. and Cohen, P. (1989) EMBO J. 8, 2911-2916.
[3] Brakch, N., Boileau, G., Nault, C., Joseph-Bravo, P., Rholam, M. and Cohen, P. (1991), submitted.
[4] Benoit, R., Ling, N., Alford, B. and Guillemin, R. (1982) Biochem. Biophys. Res. Commun. 107, 944-950.
[5] Gluschankof, P., Morel, A., Benoit, R. and Cohen, P. (1985) Biochem. Biophys. Res. Commun. 128, 1051-1057.
[6] Lepage-Lezin, A., Joseph-Bravo, P., Devilliers, G., Benedetti, L., Launay, J.M., Gomez, S. and Cohen, P. (1991) J. Biol. Chem. 266, 1679-1688.
[7] Armentano, D., Yu, S.F., Kantoff, P.W., von Ruden, T., Anderson, W.F. and Gilboa, E. (1987) J. Virol. 61, 1647-1650.

[8] Tabor, S. and Richardson, C.C. (1987) Proc. Natl. Acad. Sci. USA 84, 4767–4771.
[9] Bennet, H.P.J., Browne, C.A. and Solomon, S. (1981) Biochemistry 20, 4530–4538.
[10] Créminon, Ch., Rholam, M., Boussetta, H., Marrakchi, N. and Cohen, P. (1988) J. Chromatogr. 440, 439–448.
[11] Cohen, P., Gomez, S., Gluschankof, P., Clamagirand, C., Plevrakis, I., Brakch, N., Créminon, C., Lepage, A., Morel, A., Rholam, M. and Camier, M.(1989). In: The Second Forum on Peptides (A. Aubray, M. Marraud and B. Vitoux, eds) Colloque INSERM/Libbey Eurotex, Vol. 174, pp. 3–9.
[12] Docherty, K., Rhodes, J., Taylor, N.A., Shennan, K.E.J. and Hutton, J. (1989) J. Biol. Chem. 262, 18335–18339.
[13] Sevarino, K.A., Stork, Ph., Ventimiglia, R., Mandal, C and Goodman, R.H. (1989) Cell 57, 11–19.
[14] Morel, A., Kuks, P., Bourdais, J. and Cohen, P. (1988) Biochem. Biophys. Res. Commun. 151, 347–354.
[15] Bourdais, J., Devilliers, G., Girard, R., Morel, A., Benedetti, L. and Cohen, P. (1990) Biochem. Biophys. Res. Commun. 170, 1263–1272.
[16] Sossin, W.S., Fisher, J.M. and Scheller, R.H. (1989) Neuron 2, 1407–1417.