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Processing of Human Prosomatostatin in AtT-20 Cells: S-28 and S-14 Are Generated in Different Secretory Pathways

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(Abstract): Somatostatin-14 (S-14) and somatostatin-28 (S-28) are generated by differential processing of a single precursor at a dibasic (R-K) or monobasic (R) proteolytic cleavage site, respectively. To study the pathways of processing of prosomatostatin, we have expressed in AtT20 cells cDNA encoding human prosomatostatin and prosomatostatin mutated in one or the other processing site. Analysis of the peptides present in cell extracts or culture media before and after stimulation of the cells with 8-BrcAMP indicated that prosomatostatin can enter three distinct secretory pathways where it is differently processed: 1) prosomatostatin was secreted through the constitutive pathway; 2) the regulated secretory pathway generated S-14 which was released upon stimulation of the cells with 8-BrcAMP; 3) an alternative pathway, insensitive to 8-BrcAMP produced S-28 and S-14. Moreover, our results suggest that the R-K processing site used to produce S-14 is an important structural feature for targeting the precursor to the regulated secretory pathway.

Peptide hormones and neuropeptides are usually synthsized as part of large inactive precursors called polyproteins (1, 2). The bioactive peptides are then released post-translationally from the precursor by limited proteolysis occuring mostly at pairs of basic amino acid residues but also at single arginyl residues (for reviews see (1, 3)). This proteolysis is accomplished by specific endoproteases called prohormone convertases and is initiated in the trans-golgi network (TGN) (4, 5, 6, 7, 8). There is now ample evidence that the presence of basic amino acid residue(s) although necessary is not sufficient to direct recognition by endoproteases (9, 10, 11, 12, 13, 14). Fitting of the prohormone sequence to the active site of the convertase requires a

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certain specific conformation (15, 16, 17). Thus structural features of the precursor are involved in directing processing.

Similarly, targeting of the precursor to the regulated secretory pathway where limited proteolysis occurs, is thought to involve the recognition by cell-specific machinery of sequences or structure specific determinants encoded in the precursor. This was best shown by comparing in cultured neuroendocrine cells the sorting of polyproteins to that of other types of proteins such as immunoglobulins (18) or membrane proteins (19, 20): the polyproteins were targeted to the regulated secretory pathway and accumulated in vesicles at the tips of cellular extentions, while other proteins were constitutively transported and secreted by the same cells. The exact nature of the polyprotein sorting signal is yet poorly defined, but it has been proposed that they are contained in the proregion of the polyproteins (21, 22, 23). However, since cells such as fibroblasts cannot store and efficiently process precursors (24, 25, 26), cell-specific factors or structures must also be important.

Somatostatin-14 (S-14) is a 14 amino acid peptide hormone synthesized in islets of Langerhans, several regions of the brain and the gastrointestinal tract. In addition to S-14, a second form of somatostatin, somatostatin-28 (S-28), which is a 14 amino acid residues NH_2-terminal extended form of S-14, is found in small intestine. In teleostean fish two separate precursors, somatostatin I and II encoded by different genes are processed in different cells to S-14 and S-28, respectively, whereas in mammals a single gene encodes prosomatostatin (ProSOM) which undergoes tissue specific processing to yield S-14 and/or S-28. This specific processing can be explained by the presence of different processing enzymes in the cells or by the specific targeting of the precursor to different secretory pathways. In order to shed light on this question, we have expressed in AtT20 cells cDNAs encoding non-mutated and mutated ProSOM (Fig. 1) and analyzed the processing products before and after stimulation of the cells with 8-BrcAMP. Our results suggest that S-14 is the only peptide generated in the regulated secretory pathway while S-28 and S-14 are produced in an alternative pathway.

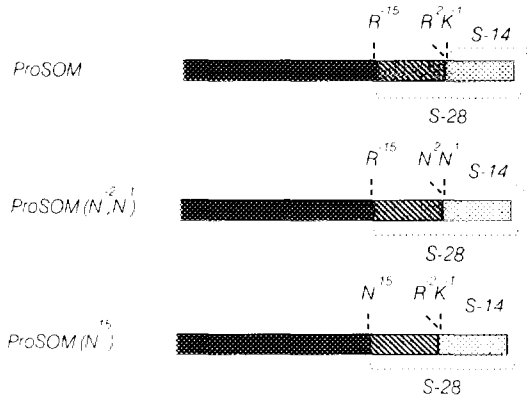

Fig. 1. Schematic representation of human prosomatostatin and mutated prosomatostatins.

MATERIAL AND METHODS

DNA manipulations and plasmid constructions

DNA manipulations were performed according to standard protocols (27). Mutation of the human prosomatostatin cDNA and construction of the expression vectors were described previously (28).

Culture and transfection of AtT20 cells

Culture conditions for AtT20 cells have been described in detail elsewhere (29). Calcium phosphate-mediated DNA transfection of AtT20 cells was basically performed as described previously by others (29). Briefly, the day before transfection cells were seeded at a density of 1 x 10^6 cells per 100 mm dish. On the day of transfection, $CaPO_4$/DNA precipitate (1 ml) containing 10 µg of the pN2-Som DNA was added to the cells and incubated for 4 hrs at 37°C. Cells were next rinsed twice with PBS (137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and cultured for 2 days in 10 ml/dish of DMEM-10% fetal bovine serum/horse serum (FBS/HS). After two days, the cells were trypsinized, collected by centrifugation and transferred to new 100 mm cell-culture dishes in 10 ml/dish of the DMEM-10% FBS/HS medium supplemented with 400 µg/ml of G418 (a neomycin analog). The medium was changed every two days. After two weeks of selection, transfected cell pools were maintained in the DMEM-10% FBS/HS medium containing 100 µg/ml of G418.

HPLC and radioimmunoassay of prosomatostatin and its derivatives

Subconfluent transfected AtT20 cells were harvested in phosphate buffered saline (PBS) (5 ml per Petri dish) with a rubber policeman. A 0.5 volume of glacial acetic acid was added to the cell suspension. The cell suspension was then sonicated four times for 30 sec each time and cell debris were pelleted by centrifugation. The supernatant was passed through an ODS-silica cartridge (Sep-pak C18, Waters Inc.) equilibrated with 0.1 % trifluroacetic acid (TFA). The absorbed peptides were eluted with 80% acetonitrile in 0.1 % TFA. Peptide from the culture media were collected by adding 0.5 volume of glacial acetic acid to the medium prior to loading on Sep-Pak cartridges. TFA and acetonitrile were removed by lyophilization and the dry peptides resuspended in 5% acetic acid. Peptides from both the cell extracts and the culture media were fractionated by gel filtration high performance liquid chromatograpy (HPLC) using two columns (two Protein-Pak 60 in tamdem, Waters Inc.). The columns were equilibrated in 40% acetonitrile containing 0.1% TFA prior to loading samples. Fractions of 0.5 ml were collected and then analyzed using a specific RIA for somatostatin-14 (8, 14). The position of elution of

ProSOM was determined theoretically after calibration of the columns with bovine serum albumine, ovalbumine, S-28 and S-14. The positions of elution of S-28 and S-14 were determined using synthetic peptides.

Stimulation of transfected AtT20 cells with 8-BrcAMP

AtT20 cells (3-4 x 10^6 cells per dish) were plated in 100 mm tissue culture dishes, and incubated at 37 ^{0}C for 48 h in DMEM-10%FBS/HS. For stimulation of secretion, cells were rinsed twice with PBS and incubated in 3 ml of the serum-free synthetic media with or without 5 mM 8-BrcAMP for one hour. At the end of the incubation period, cells were collected for counting by incubation in PBS supplemented with 20 mM EDTA and centrifugation.

RESULTS AND DISCUSSION

To study processing of ProSOM in AtT20 cells, expression vector pN2-Som (15) was used to introduced in these cells a human ProSOM cDNA. Stable transfectants were obtained by selection in G418 and the peptides present in extracts and culture media of expressing cells were fractionated by gel permeation high performance liquid chromatography. In the cell extract and culture medium of AtT20 cells expressing ProSOM, somatostatin immunoreactive material was detected in the column fractions corresponding to the elution position of human ProSOM and also at elution positions of S-28 and S-14 (Fig.2A and 2B,

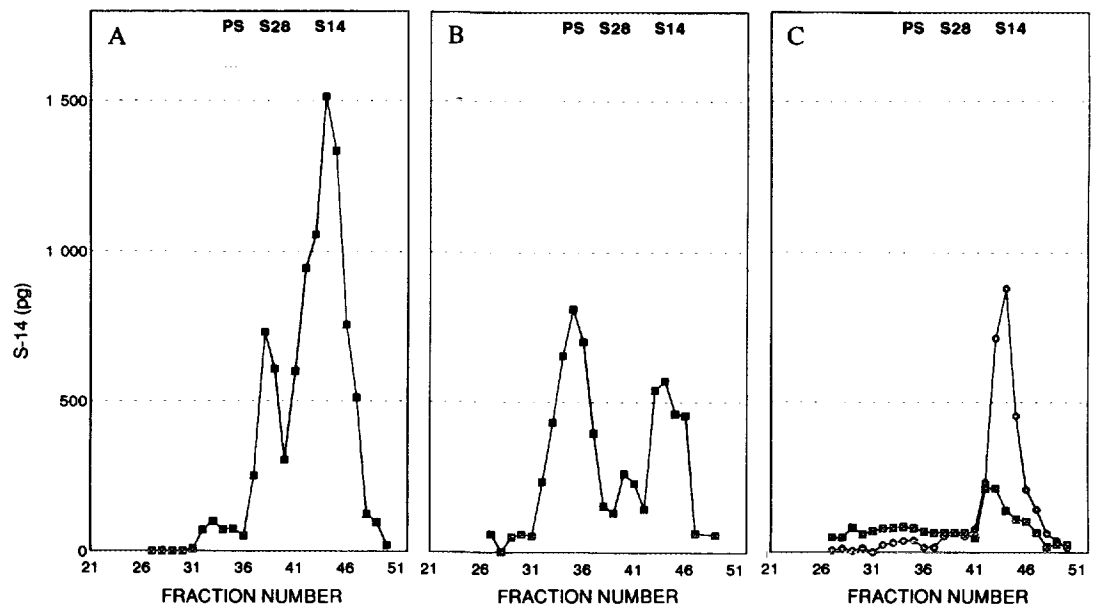

Fig. 2. HPLC analysis of ProSOM processing in transfected AtT20 cells. Peptides in cell extracts (A), and culture media in the absence (B) or the presence (C) of 5 mM 8-BrcAMP were separated by HPLC and detected by RIA (see Materials and Methods). The arrows indicate the elution positions of the standard peptides. PS: prosomatostatin, S-28: somatostatin-28, S-14: somatostatin-14.

respectively). The amounts of processed peptides were evaluated at 90% and 50% of total somatostatin immunoreactive material recovered in the cell extract and culture medium, respectively. It is interesting that the cell extract contained mostly processed peptides while the culture medium showed the presence of a large peak of ProSOM. This result suggests that ProSOM is released through the constitutive secretory pathway. In order to determine the pathway of secretion of S-28 and S-14, we fractionated the products released in the culture medium with or without stimulation of the cells for 1 h with 8-BrcAMP. In the presence of the secretagogue, the amount of S-14 in the medium increased 5 fold (Fig. 2C). No ProSOM or S-28 was released upon stimulation of the cells with 8-BrcAMP. These results suggest that only S-14 is generated in the regulated secretory pathway. However, our experiments do not rule out the possibility that some ProSOM is processed into S-28 which is then rapidly transformed into S-14.

To study independently the processing of S-14 and S-28, mutations affecting selectively either the Arg^{-15} or the Arg^{-2}-Lys^{-1} processing site were created in the human ProSOM cDNA (Fig.1). Mutant ProSOM(N^{-15}) allows us to study the processing of S-14 independently of S-28. In the extract of AtT20 cells expressing ProSOM(N^{-15}) we found almost exclusively S-14 (Fig.3A). This result was expected from the mutation introduced and indicated that S-14 can be generated directly from the precursor without prior processing of S-28. This result is in agreement with our previous observations in Neuro2A cells (30). The culture medium of the same cells contained only the precursor (Fig. 3B) while the addition of 8-BrcAMP to the culture medium stimulated the release of S-14 (Fig. 3C). The lack of S-14 along the expected absence of S-28 in the culture medium of unstimulated cells is surprising. This observation suggests that S-14 found in the culture medium of cells expressing ProSOM, in the absence of stimulation of the cells with a secretagogue, is generated from S-28 in a pathway different from the regulated secretory pathway. It is surprising to note that ProSOM(N^{-15}) which cannot lead to the production of S-28 did not accumulate in cells. The reason for this observation is not clear but one explanation is that cleavage of the precursor may be a prerequisite for entry into this pathway. Alternatively, the uncleaved precursor may be rapidly recycled to the constitutive pathway.

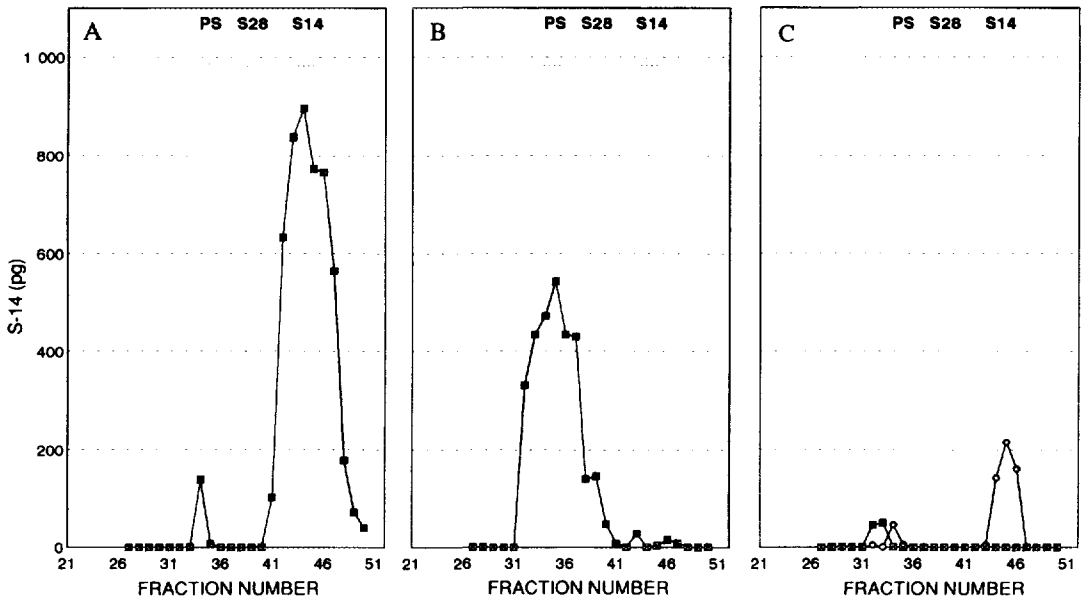

Fig. 3. HPLC analysis of ProSOM(N^{15}) processing in transfected AtT20 cells. Separation and detection of peptides were as described in Legend to Fig. 2.

With mutant ProSOM (N^{-2}, N^{-1}) we studied the production of S-28 without any further proteolytic processing into S-14. As observed for ProSOM, mostly peptides were observed in the extract of cells expressing ProSOM (N^{-2}, N^{-1}) while the culture medium showed approximately the same amounts of precursor and processed peptides (Fig. 4A and B, respectively). As expected, no S-14 was detected. More importantly, no somatostatin-immunoreactive material was released upon stimulation of the cells with 8-BrcAMP (Fig. 4C). Two important conclusions can be drawn from this last observation. First, as suggested by the analysis of mutant ProSOM(N^{15}), S-28 can be generated in a pathway different from the regulated secretory pathway. This pathway is also likely to be different from the constitutive secretory pathway since in cells expressing ProSOM, S-28 accumulated to a certain extent into the cells while the precursor was rapidly secreted (Fig. 2A and 2B). The exact nature of the pathway where processing of S-28 occurs is unknown. However, the existence of alternative secretory pathways in exocrine (31, 32, 33) and endocrine (34, 35, 36, 37) cells has recently been postulated. Furthermore, it was shown in Rin5F cells that a proregion mutant of ProSOM entered

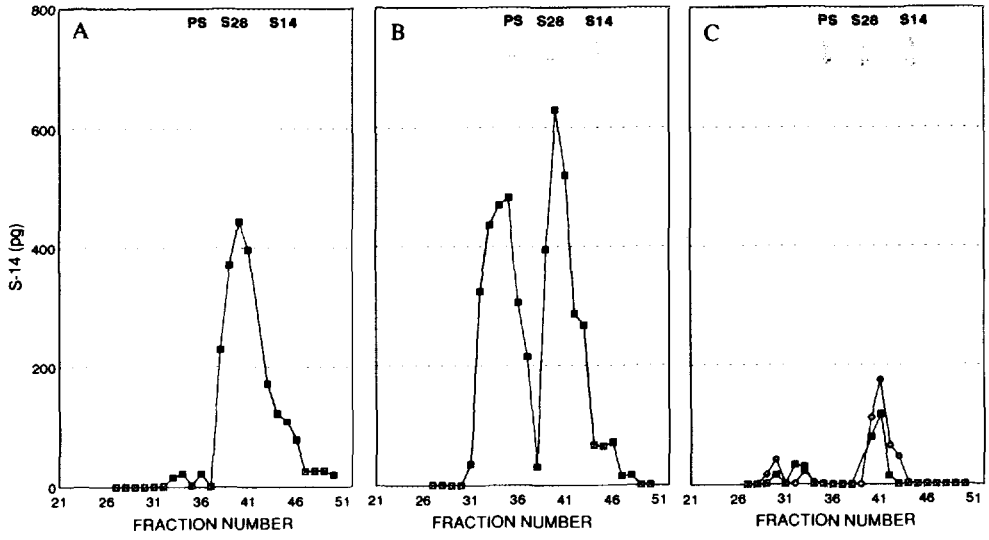

Fig. 4. HPLC analysis of ProSOM(N^{-2},N^{-1}) processing in transfected AtT20 cells. Separation and detection of peptides were as described in Legend to Fig. 2.

a cAMP resistant secretory pathway (38). The second conclusion concerns the targeting of ProSOM to the regulated secretory pathway: it appears that the R^{-2}-K^{-1} cleavage site is essential for this process to occur.

Thus our results suggest that in AtT20 cells ProSOM can enter at least three distinct pathways. First, the full length precursor can be rapidly secreted through the cellular constitutive secretory pathway (Fig. 5, pathway I). No significant amount of precursor can be

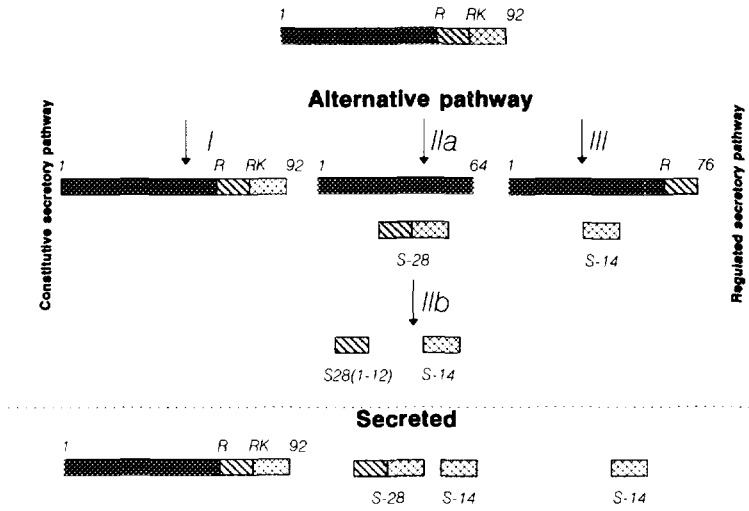

Fig. 5. Proposed pathways for the differential processing of prosomatostatin in AtT20 cells.

detected in cell extracts while large amounts are found in the culture medium of unstimulated cells. This is true for ProSOM and mutated ProSOM. Second, the precursor can enter an alternative pathway (Fig. 5, pathway II) where S-28 is first generated and then partially processed to S-14. The peptides accumulated to a certain extent in the cells but their release from the cells was not stimulated by 8-BrcAMP. They appeared to be released constitutively but more slowly than ProSOM. Finally, the precursor can be targeted to the regulated secretory pathway where exclusively S-14 is stored (Fig. 5, pathway II). ProSOM and ProSOM(N^{-15}) entered this pathway. ProSOM(N^{-2},N^{-1}) did not appear to enter the regulated pathway since no somatostatin immunoreactive material was released upon stimulation of the cells with 8-BrcAMP. This last provocative observation is presently under further investigation.

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