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Identification of prosomatostatin in pancreatic islets

(hormone biosynthesis/prohormone conversion/tryptic peptides)

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ABSTRACT **A 12.5-kilodalton protein not related to insulin or glucagon was detected in pulse-chase-labeled rat islets of Langerhans. Although this protein reacted poorly with various somatostatin antisera, analysis of two-dimensional peptide maps showed that it contains all of the tryptic fragments of somatostatin, which is located at its COOH terminus. Proteolytic conversion of the putative prosomatostatin, which took place parallel to the processing of proinsulin and proglucagon in pulse-chase experiments, coincided with the appearance of newly synthesized somatostatin and proceeded without the apparent involvement of major intermediate forms.**

The tetradecapeptide somatostatin was isolated from hypothalamic extracts in 1973 (1) and has since been found to be present in other parts of the central nervous system, as well as in the gastrointestinal tract and in the D-cells of pancreatic islets (2–6). Because the formation of such a small peptide hormone is likely to proceed via the biosynthesis of a prohormone, many attempts have been made to identify larger forms of immunoreactive somatostatin in tissue extracts and several "large" somatostatins have been reported (7–14).

However, most of these studies have not provided clear evidence for a somatostatin precursor that is processed on a time scale similar to that for the conversion of proinsulin (15), proglucagon (16), or other hormone precursors (17, 18). Only studies on isolated fish islets have demonstrated the probable formation of somatostatin via the processing of cysteine- and tryptophan-labeled precursor material slightly larger in size than proinsulin (13).

The data reported here demonstrate that a distinct rapidly synthesized protein of 12.5 kilodaltons (kDal) in rat pancreatic islets contains the tryptic peptides of somatostatin and that its cellular conversion is accompanied by the formation of tetradecapeptide somatostatin.

MATERIALS AND METHODS

Labeling of Islet Proteins. Isolation of pancreatic islets from Sprague–Dawley rats has been described (19, 20). Samples of 100 isolated islets were incubated at 37°C in 100 μl of Hank's buffer supplemented with 2.5 mM glucose and 50 μCi (1 Ci = 3.7×10^{10} becquerels) of the indicated radioactively labeled amino acid (New England Nuclear, Boston, MA, or the Radiochemical Centre, Amersham, England). For subsequent chase incubations, 5 ml of Hanks' buffer containing 1 mM unlabeled amino acid was added. Washing and lysis of islets and electrophoresis of lysates on $NaDodSO_4$/polyacrylamide gels followed by fluorography were performed as described (21).

Elution of Proteins from Electrophoresis Gels. Protein bands were excised from dried and fluorographed electrophoresis gels of lysates of 1000–1200 islets that had been incubated for 2 hr with the indicated radioactive amino acid as described above. Proteins were eluted by shaking the chopped gel bits twice for 48 hr at 37°C either in 2 ml of 0.1 M NH_4HCO_3 (proteins < 6 kDal) or in 2 ml of 25 mM Tris/200 mM glycine/0.1% $NaDodSO_4$ (proteins > 6 kDal). Bovine serum albumin (100 μg) was added to all samples as carrier. After removal of $NaDodSO_4$ by dialysis, samples were concentrated by precipitation in 10% trichloroacetic acid and the precipitates were dissolved finally in 0.1 M NH_4HCO_3 for further analyses.

Analysis of Tryptic Peptides. For tryptic digestion, eluted proteins were incubated in 2 mM $CaCl_2$ after addition of 2 μg of trypsin treated with tosylphenylalanine chloromethyl ketone (Worthington) for 12 hr at 37°C. Digestion was stopped by freezing and lyophilization. Dried samples were dissolved in 10 μl of 30% (wt/wt) formic acid, tryptic peptides from 50 μg of synthetic somatostatin (generously provided by J. Rivier, Salk Institute, La Jolla, CA) were added, and samples were electrophoresed on 20 × 20 cm cellulose thin-layer plates (Eastman) for 5 hr at 100 V in 30% formic acid followed by chromatography in 1-butanol/pyridine/acetic acid/water (60:40:12:48, vol/vol) in the second dimension. Plates were coated with 2,5-diphenyloxazole (22) and exposed on X-Omat R films (Kodak) at −80°C. Fluorographed plates were then stained with ninhydrin reagent.

Immunoprecipitations. Eluted proteins were incubated in 0.1 M Tris·HCl (pH 7.6)/0.05 M NaCl/0.01 M EDTA/0.1% bovine serum albumin, total volume 0.5 ml, after addition of 1 μl of somatostatin antiserum from sheep (no. 486, J. E. Gerich, Mayo Clinic, Rochester, MN) or from rabbit (no. 575, Y. C. Patel, Fraser Laboratories, McGill University, Montreal) for 24 hr at 4°C. Synthetic somatostatin (10 μg) was added to control samples. Immunoprecipitation was achieved by addition of the respective anti-IgG sera (Miles) and further incubation for 24 hr at 4°C. Precipitates were washed in excess buffer containing 1% Triton X-100, dissolved in 0.2 ml of 3 M acetic acid, and assayed for radioactivity in 10 ml of Aquasol (New England Nuclear). [Tyr^1]- and [Tyr^{11}]somatostatins (Beckman) were iodinated by a chloramine-T procedure (23). Electrophoretic analysis of immunoprecipitates was performed on a 12.5% polyacrylamide gel containing 0.1% $NaDodSO_4$ and 8 M urea (24).

Quantitation of Fluorographed Samples. Labeled protein bands were excised from fluorographed gels and dissolved in 0.2 ml of 30% (wt/wt) H_2O_2 by incubation at 60°C. Labeled tryptic peptides were collected by scraping from fluorographed thin-layer cellulose plates. All samples were assayed in 10 ml of Aquasol in a liquid scintillation counter.

RESULTS

Incorporation of Labeled Amino Acids into Precursor Proteins in Isolated Islets. During the course of our studies on preproinsulin biosynthesis in intact pancreatic islets (21) we observed an additional precursor-like protein of 12.5 kDal, which exhibited proinsulin-like turnover characteristics. This

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Abbreviation: kDal, kilodaltons.

protein, designated protein 4 in the previous study (21), was shown to be unrelated to insulin on the basis of the following criteria: its biosynthesis was not influenced by glucose; the protein was not present in two different types of insulin-producing tumors from rats; its pattern of tryptic peptides differed from that of proinsulin; and it was not reactive with (pro)insulin antisera. However, the kinetics of labeling of this component paralleled those of proinsulin, in that (*i*) it was detected directly after a short pulse-incubation with radioactive amino acids (1–2 min) and (*ii*) it disappeared after a chase period of 30–120 min. This kinetic behavior was later confirmed in separate experiments in which isolated rat islets were incubated with various labeled amino acids. Three proteins exhibiting prohormone-like kinetic behavior were distinguished: the predominant band of proinsulin, a less prominent protein of 18 kDal, and the rather faintly labeled band of the 12.5-kDal protein (16) (Fig. 1). After identifying the 18-kDal protein as proglucagon (16), we considered that the 12.5-kDal protein might represent a prohormonal form of somatostatin. Its relative abundance in these biosynthetic experiments also seemed to parallel the rather low level of D-cells in rat islets (16, 25).

Fluorograms of $NaDodSO_4$/polyacrylamide gels showing islet cell proteins pulse-labeled with various radioactive amino acids are compiled in Fig. 2*A*. The figure shows that the 12.5-kDal protein incorporated the less-abundant amino acids tryptophan and cysteine, which occur in the somatostatin tetradecapeptide sequence, and also methionine, as well as the amino acids leucine, proline, and phenylalanine, which occur more frequently in proteins.

Identification of the 12.5-kDal Protein as Prosomatostatin. The 12.5-kDal protein, labeled with either cysteine or phenylalanine, was eluted from polyacrylamide gels and subjected to further analysis. Two-dimensional tryptic maps of the 12.5-kDal protein are compiled along with the tryptic map of somatostatin in Fig. 3. The splitting of the NH_2-terminal and COOH-terminal tryptic peptides of somatostatin into vertical doublets, as shown in Fig. 3*A*, is likely to result from cysteine oxidation during the initial isolation procedures. As illustrated in Fig. 3, the 12.5-kDal protein contains all three tryptic peptides of somatostatin: the NH_2-terminal and COOH-terminal peptides were labeled with [^{35}S]cysteine (Fig. 3*B*), and the COOH-terminal and central peptides were labeled by [^{3}H]-phenylalanine (Fig. 3*C*). Quantitation of the phenylalanine-labeled peptides collected from the fluorographed plate gave 277 cpm for the central peptide and 144 cpm for the COOH-terminal peptide. These findings are consistent with the known presence of two phenylalanine residues in the former and one in the latter peptide. Taken as a whole, these results suggest that the entire primary structure of somatostatin occurs within that of the 12.5-kDal protein.

The identical migration of a cysteine-labeled peptide from this 12.5-kDal protein and the COOH-terminal tryptic fragment of somatostatin, as shown in Fig. 3, indicates that the labeled peptide does not carry a basic amino acid at its COOH terminus. Accordingly, treatment of the peptide with carboxypeptidase B did not influence its electrophoretic mobility, whereas the cluster of spots related to the NH_2-terminal peptide was shifted towards the anode (data not shown). These results suggest that the somatostatin sequence is located at the COOH terminus of the 12.5-kDal protein. Because the NH_2-terminal tryptic peptide of somatostatin is cleaved from the larger molecule by tryptic digestion, the somatostatin sequence is

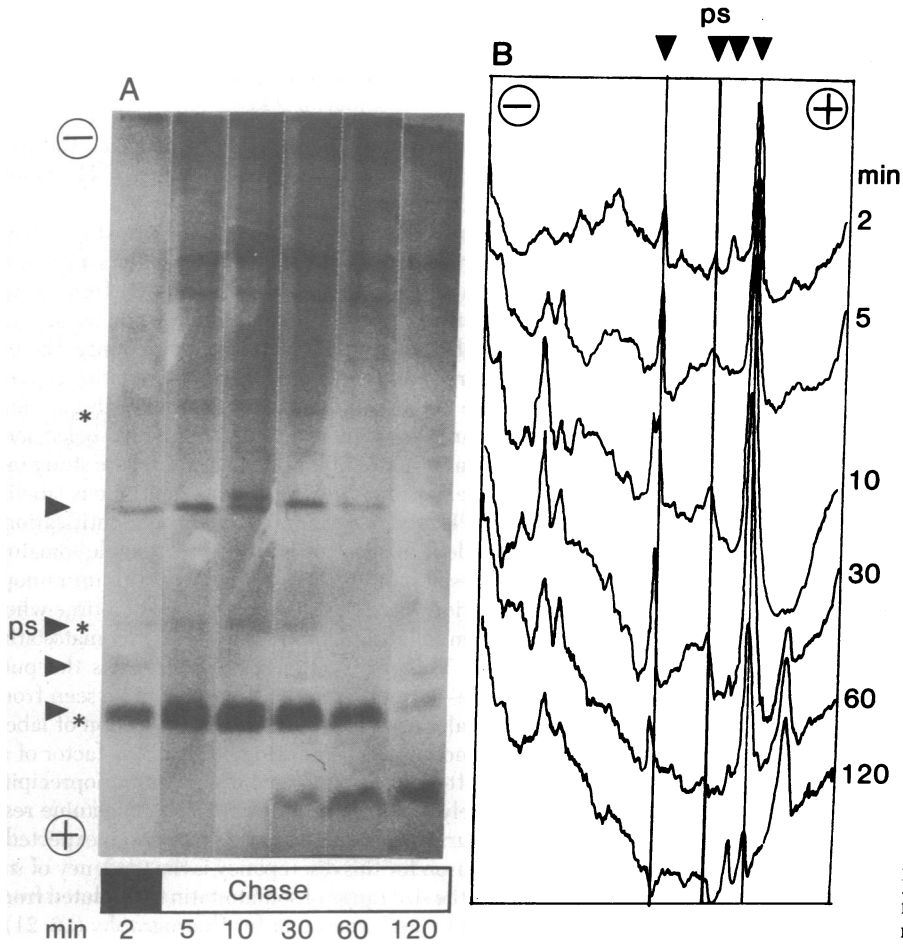

FIG. 1. Pulse-chase labeling of pancreatic islets with [^{3}H]phenylalanine. Total incubation periods are given, including the initial pulse period of 2 min. (*A*) Fluorograph; (*B*) densitometric scans. Asterisks indicate the position of marker proteins (26, 12.5, and 9 kDal) and arrowheads point to proglucagon, prosomatostatin (ps), preproinsulin, and proinsulin (all from top to bottom and from left to right, respectively).

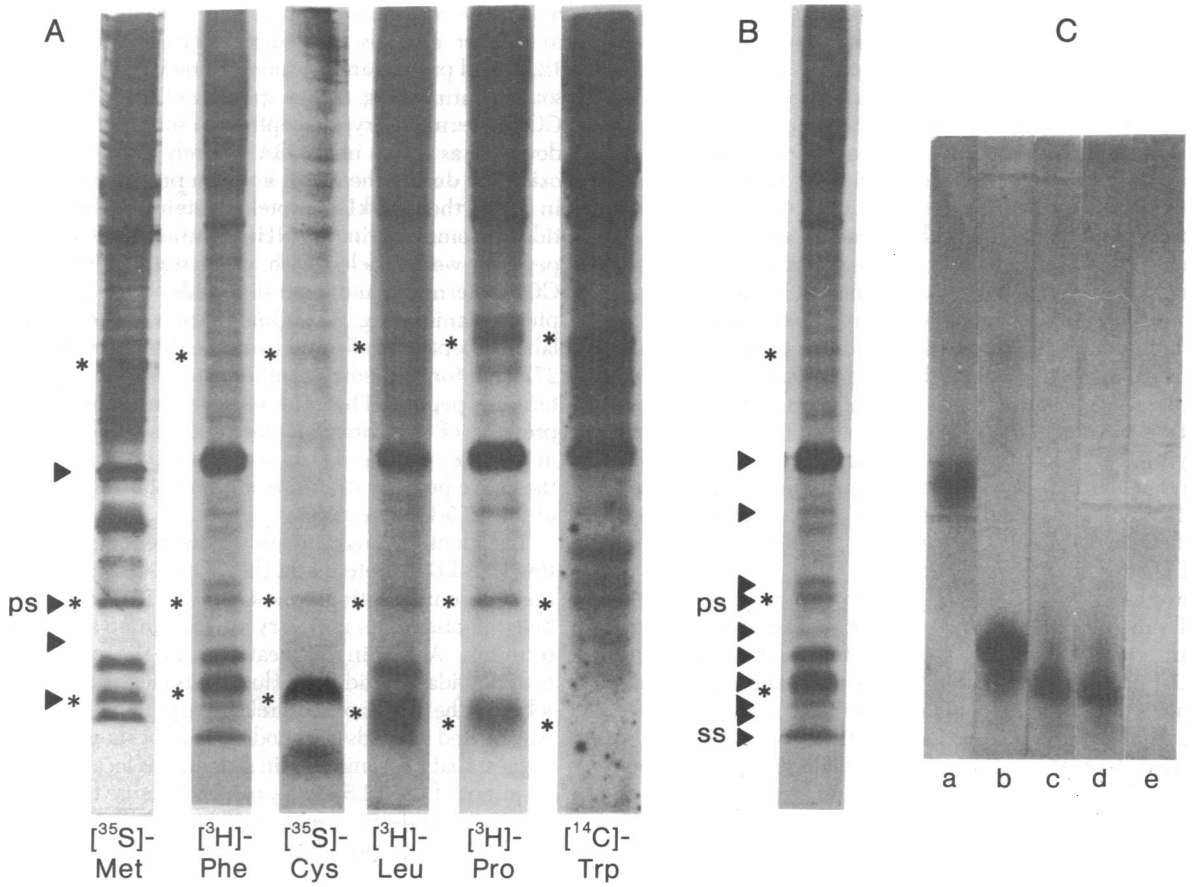

FIG. 2. Differential labeling of islet proteins and electrophoretic analysis of newly synthesized, immunoprecipitated somatostatin. (*A*) Fluorographs of electrophoresed islet lysates labeled for 2 hr by the indicated amino acids. Asterisks and arrowheads as in Fig. 1. (*B*) [^{3}H]Phenylalanine-labeled islet proteins. Arrowheads point to proteins examined in addition to prosomatostatin (ps) and somatostatin (ss) but found to be unrelated to somatostatin. (*C*) Electrophoresis of immunoprecipitated eluate from the somatostatin-containing band on a 0.1% $NaDodSO_4$/9 M urea/12.5% acrylamide gel. Lanes a–c: iodinated protein markers; a, proinsulin; b, glucagon; c, somatostatin; d, immunoprecipitate from eluate ss (antiserum 486); e, control precipitate.

likely to be preceded by basic amino acids within the precursor sequence. The leftmost two peptides in Fig. 3*B* probably represent partial digestion products that were formed by tryptic cleavage *between* a pair of basic residues rather than COOH-terminal to the second. This proposal is strengthened by our finding that the sum of the radioactivity in these peptides and the native NH_2-terminal fragments (479 cpm) equals that in the COOH-terminal tryptic fragment (475 cpm). It thus appears likely that cysteine occurs only within the somatostatin sequence in the 12.5-kDal precursor.

Further identification of this somatostatin-containing protein was attempted by immunoprecipitation with somatostatin antisera. The two antisera employed showed different specificities when tested with synthetic somatostatins containing iodotyrosine in position 1 or 11. However, the 12.5-kDal protein did not show consistent immunoreactivity with either antibody. Immunoprecipitation studies were carried out on all those protein bands that underwent prohormone-like processing during pulse-chase experiments, and on their apparent conversion products. The various protein candidates are indicated in Fig. 2*B* by arrowheads; their kinetics and characteristics were described in an earlier publication on proglucagon (16). Only the smallest of these proteins (Fig. 2*B*, band ss), which comigrated with iodinated somatostatin, was immunoprecipitated. Its apparently low immunoreactivity (only 1–2%) is probably due to the presence of other islet peptides, especially the insulin B-chain and glucagon, in this fraction. Analysis of the immunoprecipitate of this band on a urea/$NaDodSO_4$/acrylamide gel (Fig. 2*C*) allowed for better resolution in the low molecular weight range (Fig. 2 *B* versus *C*) and confirmed the specific immunoprecipitation of newly synthesized [^{3}H]phenylalanine-labeled somatostatin.

Conversion of Prosomatostatin to Somatostatin. Because labeled intermediates in the molecular weight range between somatostatin and its 12.5-kDal prohormonal form were not detectable either by immunoprecipitation or by analysis of [^{3}H]phenylalanine-labeled tryptic digests, only these two proteins were considered during studies on the conversion process. The kinetics of formation of prosomatostatin and somatostatin are shown in Fig. 4. Although no labeled somatostatin was immunoprecipitated in a pulse-chase study (analogous to the experiment of Fig. 1), the continuous labeling of islets with [^{3}H]phenylalanine allowed the identification of a time-dependent increase of immunoprecipitable somatostatin in the eluates of band ss. The formation of this immunoprecipitable peptide was delayed and occurred at a time when the incorporation of labeled amino acids into prosomatostatin had leveled off. This observation further supports the putative prohormone–hormone relationship. As can be seen from the different scales used in Fig. 4, the incorporation of label into the prohormone and the hormone differ by a factor of about 100. Even after considering incomplete immunoprecipitation and the likely presence of additional phenylalanine residues in the precursor, this difference is larger than expected. The probable reason for this discrepancy is the tendency of smaller peptides in the size range of somatostatin to be eluted from gels during fixation and treatment for fluorography (16, 21).

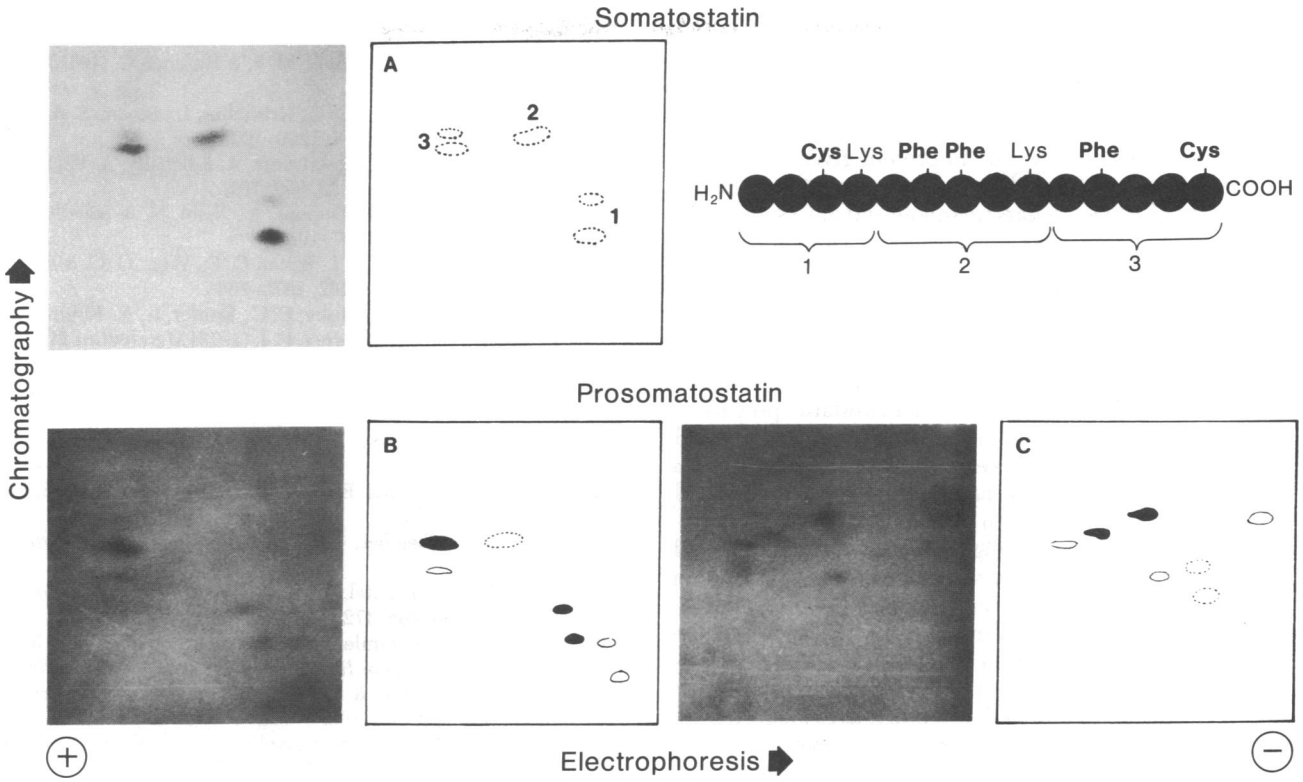

FIG. 3. Comparison of tryptic peptides of prosomatostatin to those of synthetic somatostatin (sequence in upper right). (*A*) Ninhydrin-stained tryptic peptides of synthetic somatostatin. (*B* and *C*) Fluorographed maps of [^{35}S]cysteine- or [^{3}H]phenylalanine-labeled tryptic peptides of prosomatostatin, respectively. Diagrams are on the right side of the respective maps: dotted spots show ninhydrin-stained peptides of synthetic somatostatin, solid black spots represent radioactive peptides at the position of ninhydrin-stained somatostatin peptides. Origins were at the lower left corners.

DISCUSSION

On the basis of several criteria, we have identified the 12.5-kDal protein of pancreatic islets as prosomatostatin. First, this protein contains all the tryptic peptides of somatostatin; second, the kinetics of its formation and conversion are similar to those of other islet prohormones; third, the conversion of this protein is accompanied by the formation of newly synthesized somatostatin. In further support of this conclusion, the NH_2 terminus of the somatostatin sequence appears to be linked to the precursor moiety by a pair of basic amino acids, as is the case for other hormone precursors (26). Moreover, as shown in the preceding report (16), the ratios of the biosynthetic rates for proinsulin, proglucagon, and the 12.5-kDal precursor are consistent with the relative proportions of B-, A-, and D-cells as the respective sites of synthesis of insulin, glucagon, and somatostatin in pancreatic islets.

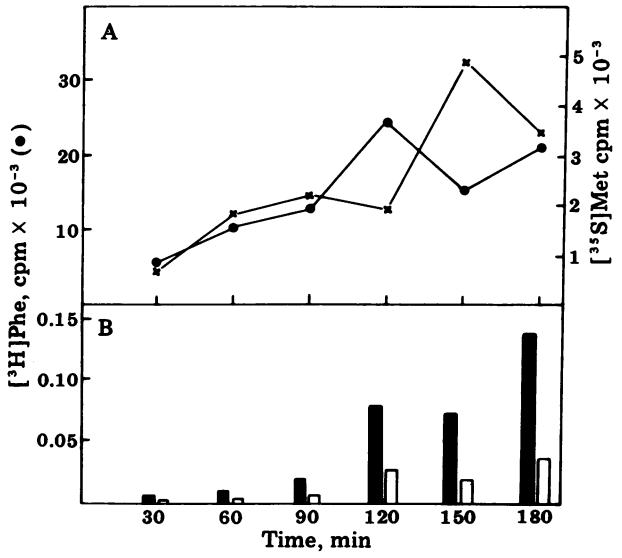

FIG. 4. Kinetics of formation and processing of prosomatostatin (*A*) and somatostatin (*B*). Samples of 100 islets were pulse-labeled for the indicated periods and then lysed and electrophoresed. Labeled prosomatostatin bands were excised from fluorographed gels, dissolved, and assayed. Somatostatin was eluted from the appropriate band for immunoprecipitation. ■, Immunoprecipitates; □, controls.

Thus, although higher molecular weight aggregates of somatostatin have been reported in pancreatic extracts (11, 12), our use of denaturing conditions for electrophoresis and of enzymatic digestion in the identification of prosomatostatin suggest that the 12.5-kDal protein represents a true biosynthetic precursor. The lack of immunoreactivity of this protein with somatostatin antisera may be explained by its modification or incomplete renaturation during preparation and elution from electrophoresis gels. Although we were unable to detect major intermediates in the conversion process, the existence of such transient forms cannot be excluded. Whether or not the 3.5-kDal immunoreactive peptide extracted from dog pancreas (12) and the 21-residue, NH_2-terminally extended form of somatostatin identified in intestinal extracts (27) represent biosynthetic intermediates or side-products of conversion requires further investigation.

Our knowledge of precursor processing in neural peptide formation is not extensive enough as yet to permit us to conclude that somatostatin is synthesized in all cells where it occurs or that the posttranslational processing of its precursor(s) follows the same pathway in all tissues. Nevertheless, the size of islet prosomatostatin is consistent with that of a large immunoreactive somatostatin that has been isolated from brain extracts

(11). However, in a more recent report (14), a still higher molecular weight of 25,000 has been estimated for the largest form of immunoreactive somatostatin in extracts of rat hypothalamus and neurohypophysis.

Discrepancies in molecular weight determinations by different approaches could be due to protein glycosylation. In biosynthetic studies with rat islets we observed a rather selective incorporation of labeled sugars (mannose and glucosamine) at the electrophoretic position of prosomatosatin. However, similar experiments performed in the presence of tunicamycin, an inhibitor of protein glycosylation, did not reveal apparent changes in the electrophoretic mobility of this protein.

In analogy to the multihormonal precursor of corticotropin and lipotropin in the pituitary gland (28) the presence of additional bioactive sequences in the somatostatin precursor, which is about eight times larger than its derivative hormonal product, is not unlikely. However, by histochemical studies no other known peptide hormone has thus far been demonstrated unambiguously to be present in pancreatic D-cells. The presence of gastrin in these cells is highly controversial (29–33), and the simultaneous production of somatostatin and calcitonin in a human tumor of the endocrine pancreas (34) has been attributed to the presence of different cell types in this tumor. On the other hand, the existence of the same prohormone in histologically different cell types and its conversion to different end products seems to be a real possibility, as evidenced from studies on the precursor of corticotropin and lipotropin in the anterior and intermediate lobes of the pituitary gland (18).

This confusing situation shows the need of further biosynthetic studies to correctly establish the origin and prohormonal nature of "large immunoreactive hormones" in various tissues. *In vitro* translation studies are also necessary in order to detect and characterize the primary translation products—i.e., the preprohormonal form. If prosomatostatin is synthesized with a leader sequence (35), the size of this hypothetical preprosomatostatin would be expected to be in the molecular range of 14–15 kDal. However, a short-lived protein of this size would not be readily distinguished in islet lysates subjected to one-dimensional electrophoresis; a more reliable means of identification would be based on cell-free translation experiments using mRNA extracted from pancreatic islets or other tissues synthesizing somatostatin.

Since these results were submitted for publication we have further substantiated the association of the 12.5-kDal component with islet D-cells by means of fluorescence-activated cell sorting. Suspensions of rat islet cells were partially resolved into subfractions enriched in A-, B-, or D-cells by using a Becton Dickinson FACS III instrument. After labeling of the D-cell-enriched fraction with [^{35}S]methionine for 1 hr followed by slab gel electrophoresis and autoradiography as described herein, the 12.5-kDal prosomatostatin band was selectively enhanced, whereas those bands associated with proglucagon and its proteolytic products (16) were all markedly diminished. Conversely, there was no indication of the 12.5-kDal labeled component in an A-cell-enriched fraction.

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