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Higher molecular weight forms of immunoreactive somatostatin in mouse hypothalamic extracts: Evidence of processing *in vitro*

(prohormones/radioimmunoassay/affinity chromatography/posttranslational cleavage/proteolytic enzymes)

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ABSTRACT **Extracts of mouse hypothalamus made in acid/urea containing protease inhibitors were analyzed for somatostatin immunoreactivity after molecular sieve filtration on Sephadex G-50. Higher molecular weight (higher-M_r) somatostatin-like forms with apparent molecular weights of 15,000, 10,000, and 6000 could be identified, besides the molecular weight 1600 somatostatin. Immunological identities with somatostatin were unambiguously demonstrated by the analysis of the displacement curves in the radioimmunoassay. The M_r 15,000, 6000, and 1600 species were purified by affinity chromatography on an anti-somatostatin immune serum covalent conjugate with Sepharose used as immunoadsorbant. After disulfide reduction by dithiothreitol, the size of the M_r 15,000 and 6000 somatostatin-like species was assessed either by molecular sieve filtration or by polyacrylamide gel electrophoresis. The results indicated that the higher-M_r somatostatin-like species isolated from the hypothalamus did not result from hormone polymerization by means of disulfide interchange. The processing *in vitro* of the 15,000 higher-M_r form of somatostatin was achieved by proteolytic enzymes coeluted with this species during the fractionation of hypothalamic extracts. Under neutral pH conditions the intermediary higher-M_r forms were generated together with the M_r 1600 somatostatin-like species. This processing activity could be either strongly inhibited at acidic pH or in acid/urea medium or else eliminated by selective immunoadsorption of the 15,000 higher-M_r form. Neither trypsin nor the γ subunit of 7S nerve growth factor was able to produce this processing, suggesting that enzymes with other kinds of specificity may be involved. It is concluded that somatostatin biosynthesis in the mouse hypothalamus may occur via a high-M_r precursor that is processed into intermediary forms leading to the tetradecapeptide hormone.**

It is now well recognized that the tetradecapeptide hormone somatostatin (SRIF; somatotropin-release inhibiting factor) elicits a broad spectrum of biological effects (1–3). This important hormone, which was identified in the mammalian hypothalamus (1), seems also to be localized both in other regions of the central nervous system and in epithelial cells of the digestive tract (4–7). The perikarya of some neurons belonging to the magnocellular nuclei of the hypothalamus appear also to react positively with both anti-somatostatin and anti-neurophysin antibodies (8).

There is increasing evidence that the synthesis of many secretory proteins and peptides, including hormones, occurs in the producing cells on ribosomal systems via higher molecular weight (higher-M_r) forms (9–12). These larger molecules have been identified as precursors of final compounds obtained by posttranslational cleavage. Their processing, in some cases, has been achieved *in vitro* with trypsin-like enzymes (12, 13) or cell extracts (14). In the particular case of pro-opiocortin, the larger plurifunctional precursor molecule (15–18) has been shown to contain within its sequence several peptide hormones (adrenocorticotropin, lipotropin, and melanotropin). This scheme may also apply to the synthesis of hormones of the neurosecretory tract in the hypothalamoneurohypophysis. In the course of our search for putative proneurophysins and proneurohypophyseal hormones we have analyzed mouse hypothalamic extracts for somatostatin immunoreactivity. Our preliminary observations (19,*) suggested that immunoreactive higher-M_r forms of somatostatin, distinct from the putative proneurophysins, could be detected. In this report we describe the biochemical properties of these possible intermediary biosynthetic forms of somatostatin and we show their conversion *in vitro* into the biologically important hormone.

EXPERIMENTAL PROCEDURE

Materials. Phenylmethylsulfonyl fluoride ($PhMeSO_2F$) and Trasylol (aprotinin) were purchased from Sigma. Triton X-100 was supplied by Calbiochem. Activated CH-Sepharose was provided by Pharmacia. The anti-somatostatin antisera 3638 and 3636 were provided by the Unité de RadioImmunologie Analytique (M. Dray, Institut Pasteur).

Fractionation of Hypothalamic Extracts. All operations were run at 4°C. Mice hypothalami (20 fragments) were removed under a binocular microscope (Nikon type 102) immediately after sacrifice. The glands were homogenized in a Potter–Elvehjem homogenizer (clearance 0.23 μm) in 3 ml of 0.1 M HCl containing 8 M urea, 5 mM $PhMeSO_2F$, and Trasylol at 130 kallikrein inhibitor units/ml. Centrifugation was performed for 1 hr at 100,000 × *g* in a SW 50 rotor on a Beckman L5-65 centrifuge. The supernatant solution was then submitted to molecular sieve filtration on a Sephadex G-50 column equilibrated and eluted in the same solution (0.1 M HCOOH containing 0.1% Triton X-100, 0.5 mM $PhMeSO_2F$, and Trasylol at 130 kallikrein inhibitor units/ml). The columns were calibrated with a series of ^{125}I-labeled markers: immunoglobulins (150,000), human growth hormone (22,000), bovine neurophysin (10,000), aprotinin (6000), and somatostatin (1600). M_r evaluations are given ±15%.

Radioimmunoassays. All the fractions from the molecular sieve filtration were tested for somatostatin immunoreactivity by a radioimmunoassay (RIA) procedure using anti-somatostatin antiserum 3636 or 3638 and [^{125}I-Tyr^1]somatostatin as tracer. The assay was conducted in 0.25 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl, Trasylol at 500 kallikrein inhibitor units/ml, and bovine serum albumin at 4 mg/ml, at 4°C for 24 hr. The antibody-bound iodinated tracer was separated from the free one by means of propanol precipitation, and the radioactivity was evaluated on an Intertechnique (CG-2000) gamma counter. Under these conditions,

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Abbreviations: SRIF, somatotropin-release inhibiting factor (somatostatin); $PhMeSO_2F$, phenylmethylsulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; RIA, radioimmunoassay.

* Camier, M., Lauber, M., Nicolas, P. & Cohen, P. (1978) *Abstracts of the Fourth EMBO Annual Symposium, Molecular Neurobiology*, Heidelberg, W. Germany.

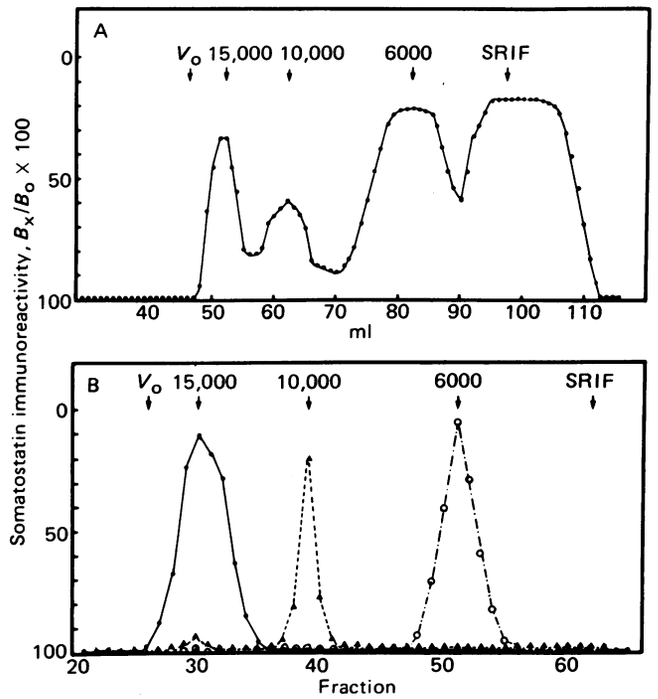

FIG. 1. Fractionation by molecular sieve filtration of higher-M_r forms of somatostatin from hypothalamic extracts. (*A*) The extracts were fractionated on a 95 × 1.2 cm Sephadex G-50 column in 0.1 M HCOOH containing 0.1% Triton X-100, 0.5 mM $PhMeSO_2F$, and Trasylol at 130 kallikrein inhibitor units/ml. Elution (7 ml/hr) of the somatostatin-like material was monitored by RIA on aliquots of each 1-ml fraction. ^{125}I-Labeled somatostatin was added as internal marker. V_o indicates the exclusion volume of species with $M_r \geq 30{,}000$. (*B*) Filtration, on a Sephadex G-50 column (45 × 1.2 cm in 0.1 M HCOOH containing 0.1% Triton X-100, 0.5 mM $PhMeSO_2F$, and Trasylol at 130 kallikrein inhibitor units/ml; flow rate = 3 ml/hr), of each higher-M_r form recovered from the fractionation of hypothalamic extracts after 48-hr treatment by 8 M urea. ●, M_r 15,000; ▲, M_r 10,000; ○, M_r 6000. Each 0.75-ml eluted fraction was tested by RIA, and iodinated somatostatin was added as a marker. The total recovery of somatostatin-like immunoreactivity was ≧ 90%. The $B_x/B_o \times 100$ ratio represents the percentage of the antibody-bound ^{125}I-labeled somatostatin tracer.

with a 1:30,000 final dilution of the serum (3638 or 3636), the minimal amount of somatostatin that could be detected was 15 pg, and a 50% displacement of the tracer was obtained with 62 pg of the hormone. $B_x/B_o \times 100$ represents the percentage of antibody-bound ^{125}I-labeled somatostatin tracer, with B_o being the total amount of tracer bound to antibodies in the absence of competitor.

Affinity Chromatography by Immunoadsorption. The immunoadsorbant was prepared by covalent crosslinking of ammonium sulfate-precipitated anti-somatostatin antiserum (90 mg of 3636) to CH-activated Sepharose 4B (5 g), using the procedure recommended by the manufacturer (Pharmacia, Uppsala). Adsorption of the samples on the column and the washing operations were done in 0.1 M sodium phosphate buffer, pH 7.8, containing 0.15 M NaCl and 1 mM $PhMeSO_2F$. The immunoreactive material was desorbed by 1 M acetic acid. In all experiments the iodinated somatostatin tracer was added as an internal marker. Under these conditions 90% of the total radioactivity was retained on the column. The recovery yield of the immunoadsorbed material was 85%. The ^{125}I iodination of the immunoreactive 6000 M_r somatostatin-like product was performed by reaction with 0.5 mCi (1 Ci = 3.7×10^{10} becquerels) of the Bolton–Hunter reagent (20) (2000 Ci/mmol from New England Nuclear) in 0.5 M sodium phosphate buffer, pH 8.5, for 1 hr at 0°C. The reaction was stopped by addition of 0.5 ml of the same buffer containing 0.2 M glycine. The iodinated 6000 M_r derivative was separated from the excess reagent by filtration on a Sephadex G-25 column (24 × 0.9 cm in 0.1 M HCOOH containing 0.25% gelatin). The ^{125}I-labeled compound was purified by affinity adsorption on the immunoadsorbant and then analyzed by polyacrylamide gel electrophoresis under denaturing conditions (21). The sample was denatured in the sample buffer (21) containing 2% sodium dodecyl sulfate ($NaDodSO_4$) and 0.1 M dithiothreitol for 5 min at 100°C and then run on 20% acrylamide gels.

RESULTS

The immunoreactivity profile in the eluate of the molecular sieve filtration of hypothalamic extracts shown in Fig. 1*A* indicates the presence of four distinct immunoreactive somatostatin-like components. Their apparent M_rs were 15,000, 10,000, 6000, and 1600. The lowest-M_r was found to coelute with the radioactive somatostatin derivative added as internal marker (Fig. 1*A*). The behavior of each of these individual somatostatin-like components, after a 48-hr exposure to 8 M urea in 0.1 M HCl, was found to be unchanged when the components were submitted to a subsequent molecular sieve filtration on the Sephadex G-50 column (Fig. 1*B*). This demonstrates that these forms of somatostatin-like material are neither somatostatin aggregates nor noncovalent complexes of somatostatin with polypeptides.

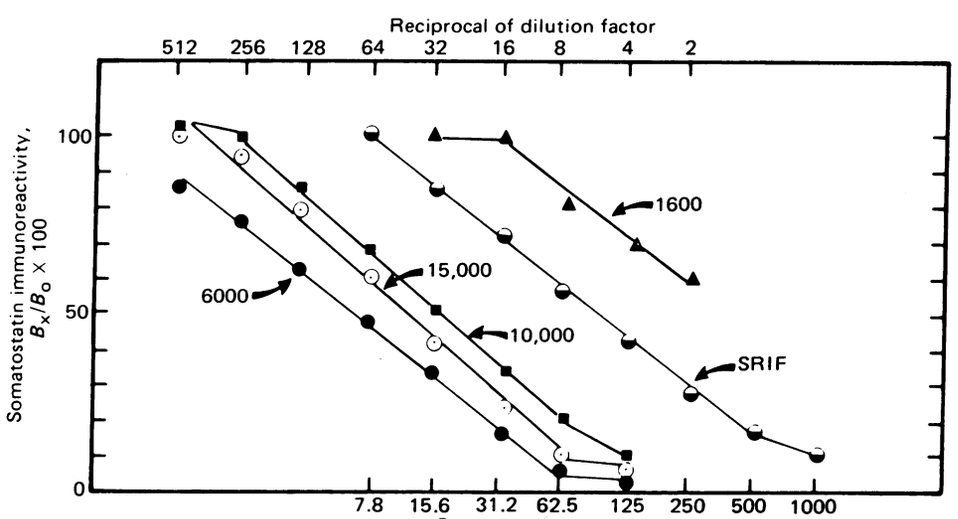

FIG. 2. Immunological characterization of the higher-M_r and M_r 1600 somatostatin-like material. Comparison of the crossreactivity of the reference somatostatin (◒) and the M_r 15,000 (⊙), 10,000 (■), 6000 (●), and 1600 (▲) species as studied by RIA. The latter was recovered after the *in vitro* processing of the M_r 15,000 species (see Figs. 4 and 5). Anti-somatostatin antiserum 3638 (final dilution 1:30,000) and tracer [^{125}I-Tyr^1]somatostatin were used.

The immunological similarities between the M_r 15,000, 10,000, and 6000 somatostatin-like species and the hormone somatostatin were established by a direct radioimmunological competition assay (Fig. 2). Serial dilutions of these compounds give displacement curves that were parallel to the one obtained when synthetic somatostatin was used as standard. Similar results where obtained with two different anti-somatostatin antisera (3636 and 3638).

The M_r 15,000 and 6000 species recovered as individual peaks from the molecular sieve fractionation of hypothalamic extracts were purified by means of affinity chromatography on a covalent conjugate of anti-somatostatin antiserum (3636) with CH-Sepharose 4B. After adsorption of the samples at 4°C, washing of the column at room temperature eluted significant amounts of proteins detected by UV absorption (Fig. 3). In contrast, whereas only 5% of the total immunoreactivity recovered was eluted with the washing buffer, 95% of the somatostatin-like immunoreactivity (15,000 or 6000) and of the radioactive somatostatin marker were eluted after irrigation of the sorbent by 1 M acetic acid (Fig. 3). The UV absorbance of this desorbed material was undetectable, suggesting a significant purification of the somatostatin-like material.

The molecular weights of these purified 15,000 and 6000 higher-M_r forms of somatostatin was determined after a treatment of the compound involving the complete reduction of disulfide linkages by dithiothreitol (22). In the case of the M_r 15,000 component recovered from the affinity chromatography process, followed by subsequent molecular sieve fractionation (see below and Fig. 4), a treatment by a 1000-fold molar excess of dithiothreitol in 8 M urea did not affect its elution profile on a Sephadex G-50 column (see Fig. 3*B inset*). The M_r 6000 fraction recovered after desorption from the immunoadsorbant was labeled with ^{125}I by derivatization with the Bolton–Hunter reagent (20). The labeled somatostatin-like derivative, after subsequent purification by passage on the same immunoadsorbant, was treated by dithiotheitol and detergent and then

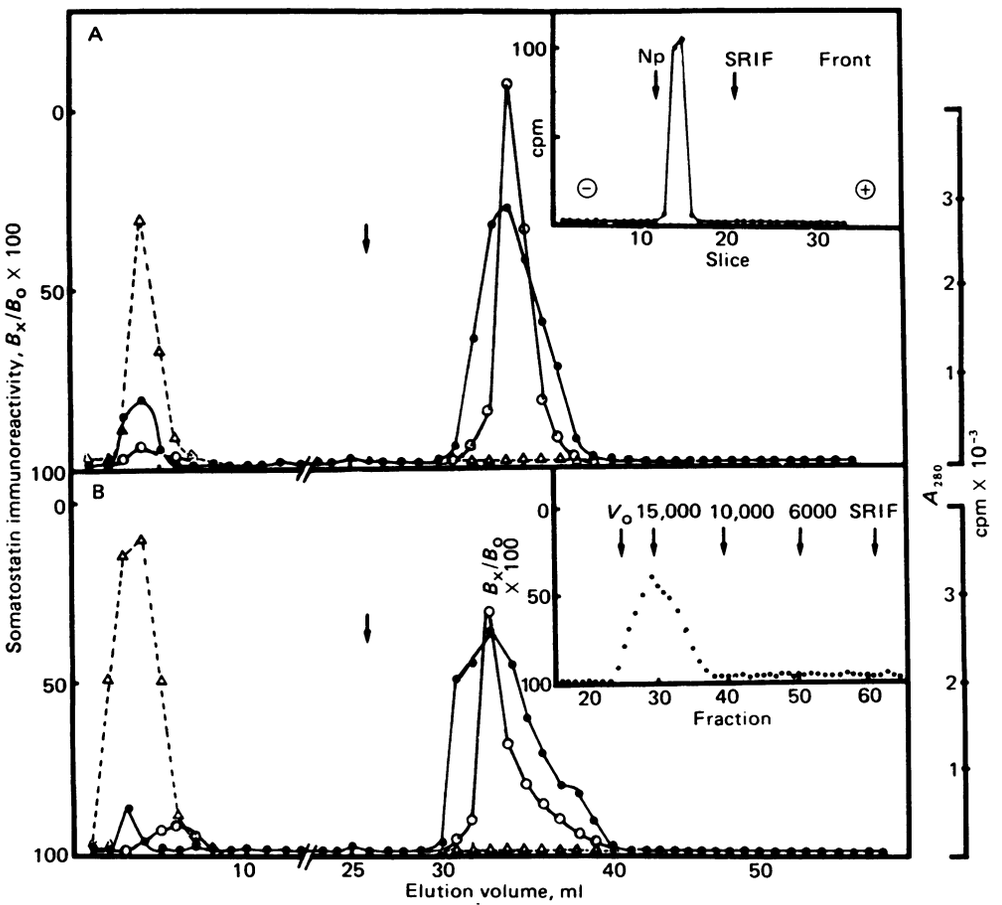

FIG. 3. Affinity chromatographic isolation of the M_r 6000 and 15,000 somatostatin-like materials. (*A*) The M_r 6000 peak recovered from the molecular sieve fractionation (Fig. 1*B*) and ^{125}I-labeled somatostatin (10^4 cpm) added as an internal marker were adsorbed for 1 hr at room temperature, then for 12 hr at 4°C on the affinity column (5 ml) in 0.1 M sodium phosphate buffer, pH 7.8, containing 0.15 M NaCl and 1 mM $PhMeSO_2F$. After washing by the same buffer solution (at room temperature), the immunoreactive material was desorbed (arrow) by 1 M acetic acid. Each eluted 1-ml fraction was tested for somatostatin immunoreactivity by the RIA procedure, with antisomatostatin antiserum 3638 (●). The ^{125}I radioactivity (○) and the absorbance monitored at 280 nm (△) were evaluated in each fraction. (*Inset*) Analysis of the M_r 6000 species after disulfide reduction. The M_r 6000 material recovered from the affinity immunoadsorbant was radioactively labeled by the Bolton–Hunter reagent. The labeled derivative was readsorbed on the immunoadsorbant and, after washing of the gel followed by desorption, analyzed by polyacrylamide slab gel electrophoresis under denaturing conditions (20% acrylamide gels containing 2% $NaDodSO_4$). The sample was previously boiled for 5 min in 0.1 M dithiothreitol and 2% $NaDodSO_4$ in 1 M Tris buffer, pH 8.8. After the migration (left to right) the gel was cut in 2-mm slices and assayed for ^{125}I radioactivity. ^{125}I-Labeled neurophysin (Np, M_r 10,000) and somatostatin were run on the same gel as internal markers (arrows).

(*B*) Same procedure as in *A* but on the M_r 15,000 peak recovered from the fractionation of the hypothalamic extracts (Fig. 1*B*). (*Inset*) Analysis of the purified M_r 15,000 species after disulfide reduction. The M_r 15,000 compound recovered after immunoadsorption followed by molecular sieve filtration was treated with a 1000-fold molar excess of dithiothreitol for 1 hr at 37°C under nitrogen atmosphere in 0.1 M phosphate buffer, pH 8.8, containing 8 M urea. After the reaction was stopped by 0.25 M HCl in 8 M urea, an aliquot of ^{125}I-labeled somatostatin (10^4 cpm) was added to the sample and the product was filtered on a calibrated Sephadex G-50 column (45 × 1.2 cm) in 0.1 M HCOOH containing 0.1% Triton X-100. Each 1-ml eluted fraction was tested by RIA. The total recovery of the somatostatin immunoreactivity was ≧90%.

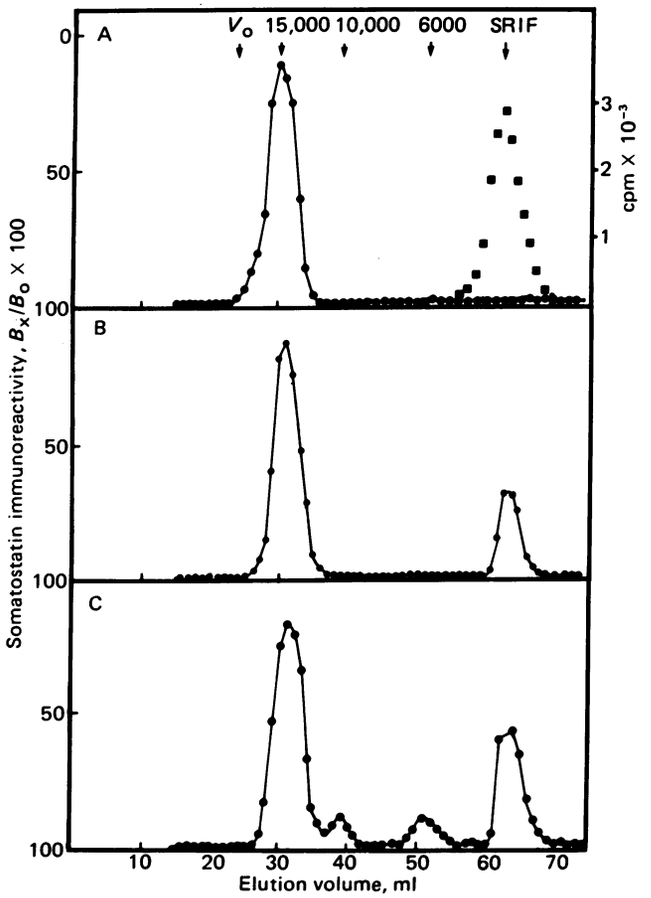

FIG. 4. Conversion of the M_r 15,000 somatostatin-like material. The M_r 15,000 somatostatin-like immunoreactive material recovered from the gel exclusion fractionation of hypothalamic extracts (Fig. 1*B*) was lyophylized in three identical aliquots. (*A*) One aliquot was solubilized in 1 ml of 0.1 M HCl containing 8 M urea and then filtered on a calibrated Sephadex G-50 column (45 × 1.2 cm) equilibrated and eluted in the same formic acid solution as for Fig. 1*B*. ^{125}I-Labeled somatostatin (■) was added to the sample as an internal standard. (*B*) The second aliquot was incubated at 37°C for 90 min in 1 ml of 100 mM phosphate buffer, pH 7.5, containing Trasylol at 130 kallikrein inhibitor units/ml. Reaction was stopped by addition of 0.5 ml of 0.25 M HCl containing 10 M urea. After 30 min at 4°C, the sample was filtered on the same column. (*C*) The last aliquot was treated as for *B* but for 6 hr at pH 7.5 (37°C). Each fraction in *A–C* was tested for somatostatin immunoreactivity (●) by RIA. The total recovery was in each case ≧85%.

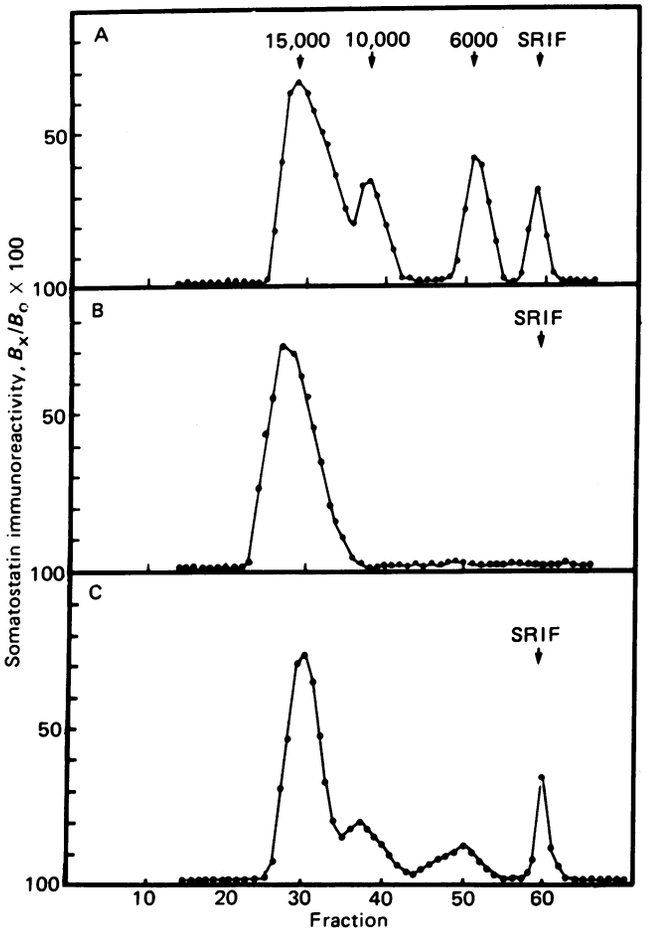

FIG. 5. Processing of the 15,000 higher-M_r somatostatin-like form. (*A*) Molecular sieve filtration, on the same Sephadex G-50 column as in Fig. 1*B*, of the immunoreactive material desorbed by the 1 M acetic acid treatment of the affinity column (Fig. 3*B*). (*B*) The M_r 15,000 species recovered in *A* was incubated for 90 min at 37°C in 1 ml of 100 mM phosphate buffer, pH 7.5, and then filtered on the column as in *A*. (*C*) A similar aliquot of the M_r 15,000 species was incubated in the same neutral buffer as in *B*, but in the presence of an aliquot of the proteins recovered from the exclusion volume of the affinity chromatography step (Fig. 3*B*).

analyzed by electrophoresis on polyacrylamide gels in 2% NaDodSO$_4$. The radioactivity pattern on the gel indicated (Fig. 3*A inset*) that the ^{125}I-labeled higher-M_r form of somatostatin migrated as an apparent 6000 species. These observations exclude the possibility that the 15,000 and 6000 higher-M_r somatostatin-like compounds result from somatostatin polymerization via disulfide interchange.

That the 15,000 higher-M_r form contains in its structure the sequence of the 10,000 or 6000 forms and of somatostatin was demonstrated by a series of experiments. First, it was observed that whereas the 6000 higher-M_r form recovered from the molecular sieve fractionation of extracts remained stable when incubated for several hours at 37°C at neutral pH, the M_r 15,000 species (from Fig. 1*B*) under these conditions generated the somatostatin-like M_r 1600 species together with various amounts of the intermediary forms (Fig. 4). Also under the conditions of the immunoadsorption of the crude M_r 15,000 preparation to the sorbent (Fig. 3*B*), the incubation, successively for 1 hr at 20°C and 12 hr at 4°C at neutral pH, was sufficient to generate all the lower M_r somatostatin-like species detected in the hypothalamic extract. This was clearly shown when the immunoreactive material recovered after desorption from the immunoadsorbant was submitted to further molecular sieve fractionation (Fig. 5*A*). The pattern obtained in a series of five successive experiments clearly indicated the appearance, in approximately equal amounts, of these three immunoreactive species (Fig. 5*A*). After this treatment the recovered M_r 15,000 component could be easily shown to be stable (Fig. 5*B*). This suggested that the processing of the higher-M_r 15,000 somatostatin-like species into the lower-M_r intermediary forms was achieved by proteolytic enzymes coeluting with the M_r 15,000 material in the molecular sieve fractionation of the hypothalamic extracts. Furthermore a control experiment indicated that the source of proteolytic activity was not the agarose-attached immunoserum proteins (not shown). The affinity chromatography step was obviously efficient in removing essentially all the contaminating proteases.

The stable M_r 15,000 immunoreactive form (Fig. 5*B*) was converted into the M_r 10,000, 6000, and 1600 somatostatin by means of exposure of this form to the enzymatic activities recovered in the void volume of the affinity chromatography step (Fig. 5*C*). Under neutral pH conditions, in the presence of serine protease inhibitors, some M_r 10,000 and 6000 species and significant amounts of M_r 1600 somatostatin-like material were

generated by this treatment (Fig. 5C). Neither trypsin (for different times of reaction or various doses of enzyme) nor the 7S nerve growth factor γ subunit (12) was able to produce this selective interconversion. Only unspecific degradation, producing fragments of smaller size than the M_r 1600 somatostatin, was observed (results not shown). The M_r 1600 somatostatin-like material generated during the processing (Fig. 4C or Fig. 5C) was identified by its ability both to bind to the immunoadsorbant and to compete with reference somatostatin in the competition RIA (Fig. 2).

DISCUSSION

Our observations provide evidence for the existence of higher-M_r forms of somatostatin in the hypothalamus. The demonstration that the largest form characterized can be processed into the final M_r 1600 somatostatin indicates that the M_r 15,000 species can be considered as a possible biosynthetic precursor of this hormonal peptide. Immunoreactive somatostatin-like material with higher M_r was observed during the molecular sieve fractionation of various organ extracts (23–26). These studies led neither to the characterization and isolation of these forms nor to their conversion into the hormone. In this respect it is interesting to compare our data with the preliminary observations from others (27) on the chemical nature of higher-M_r forms of somatostatin observed in extracts of canine pancreas. At variance with their conclusions that some higher-M_r forms can be shown to generate somatostatin by disulfide reduction, our experiments on the isolated M_r 15,000 and 6000 forms demonstrate that these molecules are not the result of somatostatin polymerization by disulfide interchange. This marked difference might possibly reflect different mechanisms of synthesis between the two glands. More likely they result from differences of extraction conditions, the strong alkaline treatment of pancreatic extracts (27) being a source of disulfide scrambling.

The analogy between the pattern of distribution of the immunoreactive forms extracted and those obtained by processing of the M_r 15,000 species suggest a high selectivity for the enzymes involved in the conversion of these precursors into somatostatin. Because this transformation could not be mimicked by trypsin or a trypsin-like proteolytic enzyme, it may be hypothetized that other kinds of cleavage specificity are involved. The considerable difference of size between the putative M_r 15,000 precursor and the final M_r 1600 somatostatin, together with the observation of forms with intermediary size, suggest that this molecule is precursor to several other peptides. The release of these as-yet-unidentified polypeptides (and corresponding biological activities) might follow a coordinated sequential process from the M_r 15,000 to the M_r 1600 hormone. The available data at this stage do not permit us to rule out different mechanisms for the posttranslational processing of the M_r 15,000 precursor. Of interest in this respect are the observations by two groups (28, 29) of a higher-M_r form of somatostatin, possibly an analog to the M_r 6000 form identified here, in the hypophysial portal blood. These data might be taken as suggestive that some of the somatostatin precursor is secreted and then converted in the blood. This mechanism remains speculative at this stage, but its possible physiological significance is worth elucidating.

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