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Biochemistry

Translation of mRNA from calf thymus in the wheat germ system: Evidence for a precursor of thymosin α_1

(thymosin fraction 5/cell-mediated immunity)

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ABSTRACT **When translated in the wheat germ system, mRNA from fresh calf thymus stimulates incorporation of radioactive amino acids into an acid-insoluble product, and 10–20% of the total radioactivity incorporated is precipitated with antisera to active thymosin fractions. In sodium dodecyl sulfate disc gel electrophoresis, radioactivity was recovered mainly in two peptides, corresponding to 16,000 and 11,000 daltons; the latter probably represents incomplete chains. Tryptic digests of each of these peptides yielded fragments corresponding to the sequence of residues 15–19 of thymosin α_1; these peptides were characterized by cochromatography with digests of synthetic thymosin α_1 and by Edman degradation. Thus, the 16,000-dalton peptide synthesized in the cell-free system appears to be a precursor of thymosin α_1 and possibly of other peptides in the fractions isolated from calf thymus. The results support the conclusion that this peptide is synthesized in the thymus gland.**

In recent years a number of laboratories have reported the isolation, from the thymus, of biologically active fractions that affect cellular immunity by controlling the development and function of thymus-dependent lymphocytes (for reviews, see refs. 1–3). Several fractions containing active thymic factors have been prepared and peptides from these fractions have been isolated and characterized (4). From one of these preparations, designated fraction 5 (4), Goldstein and his collaborators (5) have isolated a biologically active octaeicosapeptide, named thymosin α_1, whose structure has been determined.

The present experiments were designed to determine whether thymic hormones might be synthesized as a longer peptide chain that would then be processed (or degraded) to form the peptides detected in preparations isolated from the thymus. For this purpose we carried out the translation of mRNA isolated from calf thymus in the cell-free wheat germ system. The radioactive products were analyzed for material that was immunoprecipitable with antisera against thymosin fractions and contained sequences identical to those expected for tryptic peptides from thymosin α_1. The identification of three such peptides—a tripeptide, dipeptide, and an overlapping pentapeptide—are reported here.

MATERIALS AND METHODS

Materials. [^{3}H]Leucine (113 Ci/mmol), [^{3}H]lysine (60 Ci/mmol), [^{3}H]glutamic acid (51 Ci/mmol), and [^{3}H]aspartic acid (112 Ci/mmol) were purchased from New England Nuclear. ATP (sodium salt), phospho*enol*pyruvate, creatine phosphate, and creatine kinase were purchased from Sigma. Synthetic thymosin α_1 was provided by S. Wang (Hoffmann-La Roche).

Antibodies against calf thymosin fractions 5 and 6, prepared in the rabbit, were generously provided by John E. McClure (George Washington University School of Medicine and Health Sciences, Washington, DC).‡ Goat anti-rabbit IgG was obtained from Miles. Trypsin (TPCK-treated) and DNase (RNase-free) were purchased from Worthington.

Preparation of Thymus mRNA. Fresh thymus glands were obtained at a slaughterhouse and chilled in ice. Total nucleic acids were isolated by a modification of the method described by Green *et al.* (6). The thymus glands were dissected from the membranes, cut into small pieces, and homogenized directly for 2 min in a Waring blender with a mixture of 5 vol of water-saturated phenol, 5 vol of $CHCl_3$, and 10 vol of buffer I [50 mM Na acetate, pH 5.0/10 mM EDTA/0.5% sodium dodecyl sulfate ($NaDodSO_4$)]. The proportions were 100 g of thymus tissue and 2 liters of the phenol/$CHCl_3$/buffer mixture. The suspension was kept for 20 min at room temperature with frequent shaking, chilled in the cold room, and centrifuged. The phenol layer plus interface were removed and again extracted with an equal volume of buffer I. The combined aqueous extracts were extracted for 20 min with frequent shaking at room temperature with phenol/$CHCl_3$/isoamyl alcohol, 50:50:1 (vol/vol). The suspension was chilled and centrifuged, and the aqueous phase was adjusted to 2% K acetate, pH 5.5. DNA was precipitated with an equal volume of ethanol and spun on a glass rod. RNA was precipitated by the addition of 1 vol of ethanol followed by overnight stirring at −20°C. The precipitate was collected and dissolved in 300 ml of 2% K acetate, and the precipitations of DNA and RNA were repeated as above. This was necessary because of the high content of DNA in the thymus. The RNA precipitate was dissolved in water to a concentration of 100 A_{260} units/ml, digested with DNase, and purified as described by Green *et al.* (6).

Poly(A)-containing mRNA was isolated by chromatography on oligo(dT)-cellulose (type T-2, Collaborative Research, Inc., Waltham, MA) essentially as described by Green *et al.* (6). A solution of total RNA (100 ml; 30–40 A_{260} units/ml) in 10 mM Tris buffer, pH 7.5/0.5 M KCl was applied to an oligo(dT)-cellulose column (1 × 16 cm) previously equilibrated with the same buffer. The column was washed with the same buffer and then with 10 mM Tris buffer, pH 7.5/0.2 M KCl each time until the A_{260} was <0.002. The mRNA was eluted with 10 mM Tris buffer, pH 7.5. The fractions absorbing at 260 nm were pooled, made 2% with K acetate (pH 5.5), and precipitated at −20°C with 2.5 vol of ethanol. The precipitate was dissolved in water to a concentration of 20–40 A_{260} units/ml and stored at −70°C.

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Abbreviations: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; $NaDodSO_4$, sodium dodecyl sulfate.

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‡ Similar results were obtained with antibodies prepared against fraction 5 or fraction 6 (for the preparation of these fractions, see ref. 4), but the antiserum against fraction 6 had a higher titer.

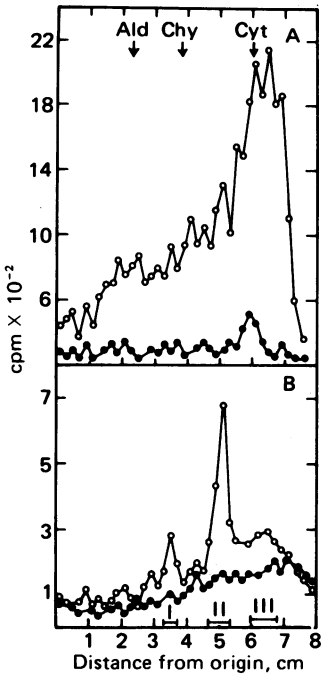

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of cell-free translation products directed by whole thymus RNA. (*A*) Total translation of thymus mRNA: products found in the presence (○) and absence (●) of thymus mRNA. An aliquot of the reaction mixture (50 μl) was treated with CCl_3COOH (10% final concentration), and the precipitate was collected, dissolved in 0.1 M NaOH, and again precipitated with CCl_3COOH. The precipitate was washed with 50% acetone, dissolved in 50 μl of 10 mM phosphate buffer, pH 7/1% $NaDodSO_4$/1% 2-mercaptoethanol, and heated for 5 min at 80°C. Glycerol and bromophenol were added to final concentrations of 10% and 0.002%, respectively, and the entire sample (50,000 cpm) was subjected to disc gel electrophoresis. The gels were stained, destained, and sliced into 2-mm sections for counting. Gels containing rabbit muscle aldolase (Ald), bovine chymotrypsin (Chy), and horse heart cytochrome *c* (Cyt) were run in parallel. (*B*) Immunoprecipitated peptides. Aliquots of the translation mixture containing 110,000 cpm were immunoprecipitated by using the IgG fraction from immune serum (○) or nonimmune serum (●). The immunoprecipitates were dissolved and fractionated on $NaDodSO_4$ as in *A*.

Cell-Free Protein Synthesis. The S-30 extract of wheat germ (Niblack Raw, Niblack Foods, Rochester, NY) was prepared as described by Roberts and Paterson (7), without preincubation and with K acetate (8) instead of KCl. The reaction mixtures (50 μl) contained: 28 mM Hepes buffer, pH 7.6; 0.1 M K acetate; 3 mM Mg acetate; 2 mM 2-mercaptoethanol; 1 mM ATP; 20 μM GTP; 8 mM creatine phosphate; 3 μg of creatine kinase; a mixture of 19 unlabeled amino acids, 24 μM each; 10 μCi of [^{3}H]leucine (113 Ci/mmol) or [^{3}H]lysine (72 Ci/mmol) or 100 μCi of [^{3}H]glutamic acid (51 Ci/mmol); 1.5 A_{260} units of wheat germ S-30 extract; and 0.04–0.08 A_{260} unit of poly(A)-containing RNA. The reaction mixtures were incubated at 23°C for 90 min and aliquots (10 μl) were removed and treated with 200 μl of 0.1 M NaOH containing 10 mM unlabeled amino acid corresponding to the radioactive amino acid used in the experiment. After 15 min at 37°C, 2 ml of 10% CCl_3COOH was added and the precipitate was collected on Millipore (HA 0.45 μm) filters. The filters were washed with 5% CCl_3COOH, dried, and assayed for radioactivity in 10 ml of Aquasol (New England Nuclear). Radioactivity measurements were carried out with a Beckman model LS250 liquid scintillation counter.

Sodium Dodecyl Sulfate Disc Gel Electrophoresis. For analysis of the cell-free translation products, the procedure of Weber and Osborn (9) was followed, except that the concentration of acrylamide was 12.5% and that of methylene-bis-acrylamide was 0.3%. After staining and destaining, the gels were sliced into 2-mm segments; these were transferred to scintillation vials and dissolved by incubation at 60°C for 3 hr in 0.5 ml of 30% H_2O_2. Aquasol (10 ml) was added to each vial and the radioactivity was determined as above. For the isolation of peptides from the gels, the 2-mm slices were extracted overnight with 0.5 ml of 0.2 M NH_4HCO_3, pH 8.5. Aliquots were taken for measurement of radioactivity. The extracts containing radioactive peptides were pooled, dialyzed against the same buffer, and lyophilized.

Immunoprecipitation. For this, the reaction mixture for cell-free synthesis was scaled up 5-fold. Usually, six such reaction mixtures were combined and diluted with 10 mM Tris buffer, pH 7.5, containing 10 mM unlabeled amino acid to a final volume of 2 ml. Ribosomes were removed by centrifugation at 200,000 × *g* for 1 hr and the supernatant solution was dialyzed overnight against 10 mM Tris buffer, pH 7.5. The dialyzed solution was adjusted to 150 mM NaCl, 1% Triton X-100, and 1% sodium deoxycholate. Rabbit anti-fraction 6 was added in a ratio of 100 μg for each 100,000 cpm of acid-precipitable product. After incubation for 1 hr at 37°C, goat anti-rabbit IgG (300 μg for each 100,000 cpm of radioactivity) was added and incubation was continued overnight at 4°C. The precipitate was collected by centrifugation, washed three times with the immunoprecipitation buffer, and dissolved in 10 mM

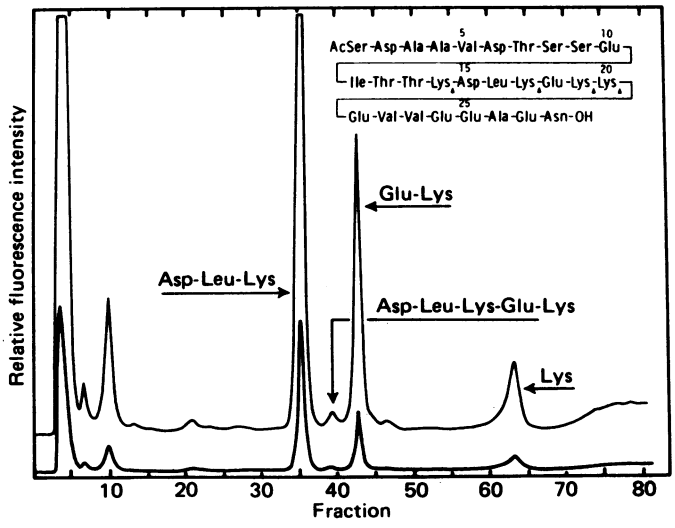

FIG. 2. Separation of thymosin α_1 tryptic peptides by ion exchange chromatography. Synthetic thymosin α_1 (300 μg) was dissolved in 200 μl of 0.2 M NH_4HCO_3, pH 8.5, and digested with 6 μg of TPCK-trypsin for 8 hr at 37°C. The reaction mixture was lyophilized, and the peptides were dissolved and analyzed by high-pressure ion exchange chromatography on HCB-X8. The tryptic peptides Asp-Leu-Lys and Glu-Lys were identified by amino acid composition and the results of Edman degradation. The pentapeptide Asp-Leu-Lys-Glu-Lys was identified by Edman degradation, using samples pooled from three such experiments. The upper curve represents the same analysis at 5 times higher sensitivity. (*Inset*) Sequence of thymosin α_1 (5).

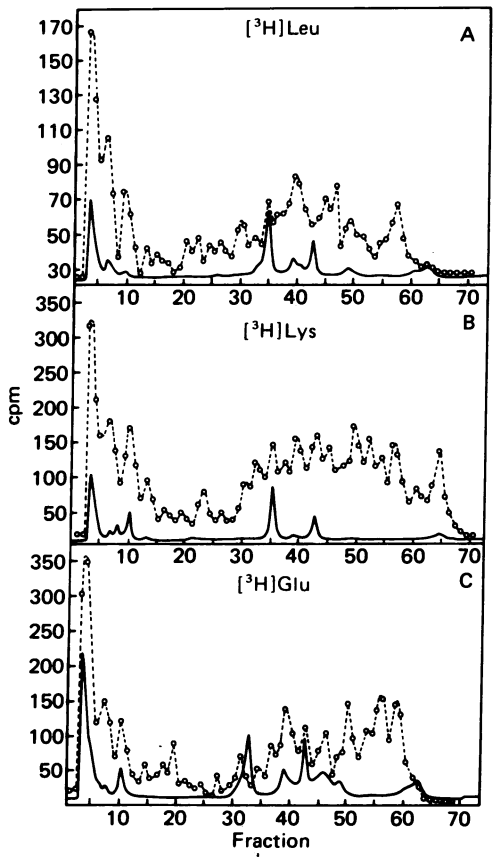

FIG. 3. Ion exchange chromatography of labeled peptides after tryptic digestion of fraction II isolated from the immunoprecipitated translation products. The radioactive peptides corresponding to fraction II were eluted from the gels and, after lyophilization, were dissolved in 200 μl of 0.2 M NH_4HCO_3, pH 8.5, containing carrier thymosin α_1 as indicated below and treated with 10 μg of TPCK-trypsin. After digestion for 18 hr at 37°C, the digests were analyzed as described in the legend to Fig. 2. o, Radioactivity; —, relative fluorescence intensity of the peptides derived from the carrier thymosin α_1. (*A*) Products labeled with [^{3}H]leucine (50,000 cpm). The amount of carrier thymosin α_1 was 150 μg. Aliquots (70 μl) of each fraction were taken for radioactivity assay. (*B*) Products labeled with [^{3}H]lysine (50,000 cpm). The amount of carrier thymosin α_1 was 120 μg. Aliquots (100 μl) of each fraction were taken for assay. (*C*) Products labeled with [^{3}H]glutamic acid (6000 cpm). The amount of carrier thymosin α_1 was 150 μg. The total volume in each fraction (≈350 μl) was taken for assay.

Na phosphate buffer, pH 7/1% $NaDodSO_4$/1% 2-mercaptoethanol for disc gel electrophoresis (see above).

Analysis of Tryptic Peptides. After digestion as indicated in the figure legends, the peptide mixtures were dissolved in 0.5 ml of 1 mM HCl/0.01% pentachlorophenol/0.001% thiodiglycol and applied to columns (0.4 × 25 cm) containing Hamilton ion exchange resin HCB-X8. The columns were eluted stepwise at 55°C with (*i*) 0.1 M pyridine/0.2 M acetic acid for 20 min, (*ii*) 0.25 M pyridine/0.5 M acetic acid for 20 min; (*iii*) 0.5 M pyridine/0.5 M acetic acid for 20 min; (*iv*) 2 M pyridine/1 M acetic acid for 30 min; (*v*) 6 M pyridine/2 M acetic acid for 20 min; and (*vi*) same as (*i*) for 90 min. The flow rate was 10 ml/hr and fractions were collected every 2.5 min. One-quarter of each sample was automatically taken for analysis with fluorescamine (see solid lines in figures) with the instrument described by Bohlen *et al.* (10).

Sequence Analysis. Manual Edman degradation was carried out as described by Tarr (11). The phenylthiohydantoin derivatives were identified by thin-layer chromatography on silica gel 60F-254 (Merck, Darmstadt, Germany) with the solvent system described by Bridgen *et al.* (12). Standard phenylthiohydantoin derivatives of amino acids were run in parallel sections on each plate.

RESULTS

Immunoprecipitation of Labeled Peptides Synthesized in the Wheat Germ System from Thymus mRNA. In the presence of 3 mM Mg^{2+}, the immunoprecipitable peptides accounted for approximately 18% of the total [^{3}H]leucine incorporated into acid-insoluble material. In $NaDodSO_4$ disc gel electrophoresis the total radioactivity incorporated (Fig. 1*A*) was found to be associated mainly with the small peptides, but the immunoprecipitable material was concentrated in three bands, corresponding to peptides of approximately 25,000, 16,000, and 11,000 daltons and designated bands I, II and III (Fig. 1*B*), respectively. The studies described below were carried out with bands II and III, which contained the bulk of the radioactivity. These yielded similar peptides after digestion with trypsin, and the smaller peptide may represent synthesis of incomplete chains. The ratios of radioactivity in peaks II and III varied depending on the particular wheat germ extract used.

Comparison of Tryptic Peptides from Peptide Fraction III with Those Derived from Thymosin α_1. Tryptic digests of thymosin α_1 were analyzed by high-pressure ion exchange chromatography and compared with the patterns obtained on digestion of the radioactive peptide II from disc gel electrophoresis. Thymosin α_1 would be expected to yield a tripeptide, Asp-Leu-Lys, and a dipeptide, Glu-Lys, together with free lysine, and all of these were identified in the fractionated tryptic digests (Fig. 2). In addition, we detected small quantities of the overlap peptide, Asp-Leu-Lys-Glu-Lys, which emerged between the tripeptide and the dipeptide. The larger peptides (see Fig. 2) derived from the NH_2-terminal and COOH-terminal portions of thymosin α_1 were not considered because these would not be identical with those formed from a larger precursor molecule containing the thymosin α_1 sequence.

Tryptic digestions of fraction II from the cell-free synthesis carried out with [^{3}H]leucine yielded a number of labeled peaks (Fig. 3*A*), two of which on further purification (see below) coincided with Asp-Leu-Lys and Asp-Leu-Lys-Glu-Lys from the carrier thymosin α_1. Both peaks were also labeled when the cell-free synthesis was carried out with [^{3}H]lysine (Fig. 3*B*) and, in addition, radioactivity emerged at the positions corresponding to those of Glu-Lys and free lysine. When [^{3}H]glutamic acid was the radioactive precursor, the label emerged with the peptide Glu-Lys and the overlap peptide Asp-Leu-Lys-Glu-Lys (Fig. 3*C*). Identical results were obtained with tryptic digests of fraction III.

The quantitative aspects are of interest. The amount of radioactivity recovered in the overlap peptide was always greater than one would have predicted from the amount of that peptide present in digests of the carrier thymosin α_1 (Fig. 3). This will be discussed later.

Purification of the Tryptic Peptides and Edman Degradation. The identity of the three radioactive peptides was confirmed by rechromatography and Edman degradation. The fractions labeled with [^{3}H]leucine (Fig. 3*A*) and corresponding to the tripeptide Asp-Leu-Lys and the pentapeptide Asp-Leu-Lys-Glu-Lys were purified by rechromatography on the same ion exchange columns. In each case the radioactivity emerged in a single symmetrical peak that coincided with the fluorescamine peak of the unlabeled carrier (Fig. 4 *A* and *B*). Similarly, rechromatography of the lysine-labeled peaks corresponding to Asp-Leu-Lys, Asp-Leu-Lys-Glu-Lys, and Glu-

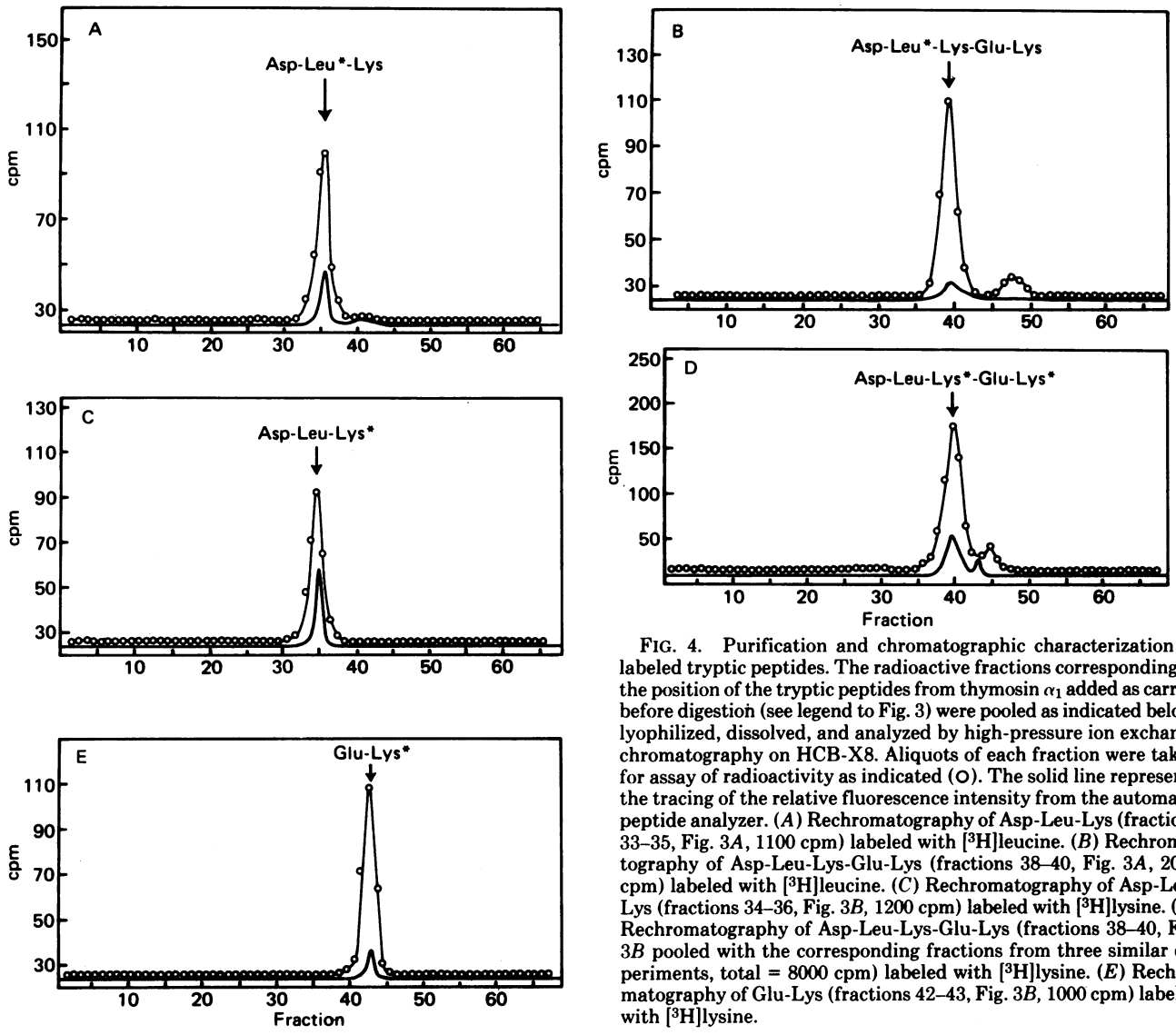

FIG. 4. Purification and chromatographic characterization of labeled tryptic peptides. The radioactive fractions corresponding to the position of the tryptic peptides from thymosin α_1 added as carrier before digestion (see legend to Fig. 3) were pooled as indicated below, lyophilized, dissolved, and analyzed by high-pressure ion exchange chromatography on HCB-X8. Aliquots of each fraction were taken for assay of radioactivity as indicated (○). The solid line represents the tracing of the relative fluorescence intensity from the automatic peptide analyzer. (*A*) Rechromatography of Asp-Leu-Lys (fractions 33–35, Fig. 3*A*, 1100 cpm) labeled with [^{3}H]leucine. (*B*) Rechromatography of Asp-Leu-Lys-Glu-Lys (fractions 38–40, Fig. 3*A*, 2000 cpm) labeled with [^{3}H]leucine. (*C*) Rechromatography of Asp-Leu-Lys (fractions 34–36, Fig. 3*B*, 1200 cpm) labeled with [^{3}H]lysine. (*D*) Rechromatography of Asp-Leu-Lys-Glu-Lys (fractions 38–40, Fig. 3*B* pooled with the corresponding fractions from three similar experiments, total = 8000 cpm) labeled with [^{3}H]lysine. (*E*) Rechromatography of Glu-Lys (fractions 42–43, Fig. 3*B*, 1000 cpm) labeled with [^{3}H]lysine.

Lys yielded radioactive peaks coinciding with the unlabeled carriers (Fig. 4 *C–E*).

Edman degradation was carried out with the peptides labeled with leucine and lysine, and in each case the radioactivity emerged at the expected cycle (Fig. 5). In all of these experiments the identity of phenylthiohydantoin derivative from the carrier peptide was confirmed by thin-layer chromatography.

Edman degradation of the glutamic acid-labeled peptides could not be carried out because insufficient radioactivity was incorporated. Even less radioactivity was incorporated in cell-free translation experiments with [^{3}H]aspartic acid despite the use of high concentrations of the highly labeled amino acid. This may be due to the presence of these amino acids in the reaction mixtures containing the wheat germ extracts or their formation from unlabeled precursors. However, both amino acids were detected on the thin-layer plates as the phenylthiohydantoin derivatives at the appropriate cycles, confirming the identity of the marker peptides.

Similar results were obtained when fractions corresponding to fraction II in the gel electrophoresis patterns of the total translation products (see Fig. 1*A*) were purified and analyzed. This fraction accounted for 3–5% of the total cell-free synthesis products, compared with 1.5–2% when the immunoprecipitation step was included. The peptides in the fraction corresponding to fraction II in Fig. 1*A* were digested with trypsin and analyzed by chromatography and Edman degradation, with results identical to those reported for the peptides in the immunoprecipitated material.

The material recovered from fraction III (Fig. 1*B*) labeled with [^{3}H]leucine and [^{3}H]lysine was also digested with trypsin and the radioactive peptides Asp-Leu-Lys and Asp-Leu-Lys-Glu-Lys were identified and subjected to Edman degradation, with results similar to those shown in Figs. 4 and 5. This confirms that this fraction contains incomplete chains.

DISCUSSION

The results reported here establish that the sequence Asp-Leu-Lys-Glu-Lys, corresponding to positions 15–19 of thymosin α_1 (see Fig. 2), is present in peptides of approximately 16,000 and 11,000 daltons translated in the wheat germ system by mRNA from the thymus gland. The probability of occurrence of a unique pentapeptide sequence is only one in 3.2×10^6, and it may therefore be concluded that thymosin α_1 arises from a larger precursor that is processed to yield the peptide found in purified fractions from calf thymus extracts (5). Alternatively, the smaller peptides in the thymosin preparations, including thymosin α_1, may arise as a result of proteolytic activity during the isolation procedure.

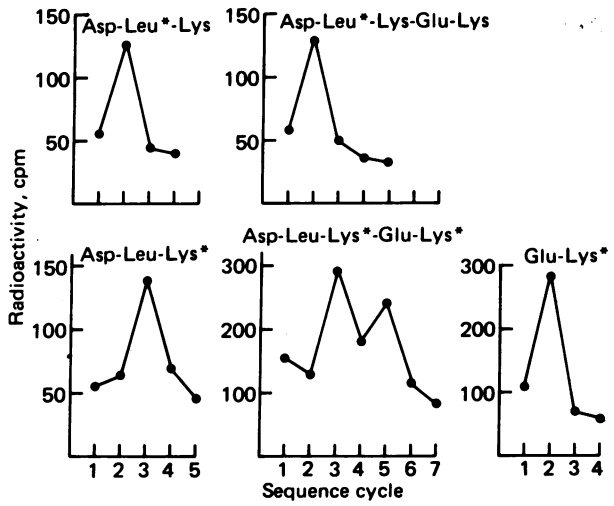

FIG. 5. Sequence analysis of radiolabeled tryptic peptides purified by ion exchange chromatography. The peptides recovered from the experiments in Fig. 4 were subjected to manual Edman degradation. After each step in the degradation, the phenylthiohydantoin derivatives were identified by chromatography on silica gel thin-layer plates. The fluorescent spots, representing derivatives from the carrier peptides, were scraped from the plates and assayed for radioactivity. The values are not corrected for quenching or for losses incurred during Edman degradation. The ^{3}H-labeled amino acids used are indicated by the asterisks.

Of particular interest is the fact that a relatively high proportion (≈10–20%) of the total products synthesized from unfractionated thymus mRNA is precipitated by antibody prepared against the mixture of thymic peptides. These results establish that the thymus gland contains mRNA for the synthesis of the peptide isolated from this organ by Goldstein *et al.* (5). Whether the mRNA that codes for this product is contained in epithelial cells or thymocytes remains to be determined, but it should be pointed out that the source of the mRNA was the whole thymus gland, in which thymocytes represent the major cell type.

Tryptic digestion of the 16,000-dalton peptide found in the cell-free translation system yielded a much larger proportion of radioactivity in the overlapping pentapeptide than would have been predicted from the analyses of the tryptic digests of thymosin α_1. For the purposes of this study, this was a fortunate circumstance, because identification of the pentapeptide sequence greatly increases the probability that the 16,000-dalton translation product is the precursor of thymosin α_1. It also suggests that the precursor peptide may possess some secondary structure that renders the Lys-Glu bond more resistant to trypsin than it is in thymosin α_1.

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