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Complete amino acid sequence of bovine thymosin β_4: A thymic hormone that induces terminal deoxynucleotidyl transferase activity in thymocyte populations

(differentiation of thymus-dependent lymphocyte/DNA nucleotidylexotransferase/acetylserine NH_2-terminal polypeptide)

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ABSTRACT **The amino acid sequence of thymosin β_4, a polypeptide isolated from calf thymus, was determined. Thymosin β_4 is composed of 43 amino acid residues and has a molecular weight of 4982 and an isoelectric point of 5.1. The NH_2 terminus of the peptide is blocked by an acetyl group. This molecule induces expression of terminal deoxynucleotidyl transferase (DNA nucleotidylexotransferase, EC 2.7.7.31) in transferase-negative murine thymocytes *in vivo* and *in vitro*. Thus, it appears that thymosin β_4 acts on lymphoid stem cells and may control the early stages of the maturation process of thymus-dependent lymphocytes. This peptide is one of several present in thymosin fraction 5 that participates in the regulation, differentiation, and function of thymus-dependent thymocytes.**

An important advance in medical research in the past decade has been the development of our understanding of the role played by the thymus-dependent immune system in various diseases, including the primary immunodeficiency diseases, cancer, and autoimmune diseases. Of particular importance in this area has been the elucidation of the endocrine role of the thymus gland (cf. ref. 1). These basic studies have resulted in the isolation and partial characterization of a family of acidic heat-stable polypeptides from the thymus that have been collectively termed "thymosin" (2).

A partially purified thymosin preparation termed "thymosin fraction 5" (3) has been studied most extensively for biological activity (4–10), as well as in clinical trials (11–15). Thymosin fraction 5 is a potent immunopotentiating preparation and can act in lieu of the thymus gland to reconstitute some immune functions in thymus-deprived or immuno-deprived individuals.

Analytical polyacrylamide gel electrophoresis and isoelectric focusing have demonstrated that fraction 5 consists of 10–15 major components and 20 or more minor components with molecular weights ranging between 1000 and 15,000.

A goal of our thymosin research program is to understand the molecular events by which the thymus gland exerts control over thymus-dependent lymphocyte (T cell) development. In order to understand the detailed mechanism(s) by which each thymosin polypeptide exerts control over the development and senescence of immunological responses, it has been necessary to isolate and characterize the components of thymosin fraction 5 and to determine their individual biological functions.

We have reported (16, 17) the isolation and complete amino acid sequences of two polypeptides purified from thymosin fraction 5, thymosin α_1* and polypeptide β_1*. In this paper, we report the structural determination of another biologically active component, namely thymosin β_4*. Thymosin β_4 was found to be a potent inducer of terminal deoxynucleotidyl transferase (terminal transferase; DNA nucleotidylexotransferase, EC 2.7.7.31) *in vivo* and *in vitro*. Thymosin fraction 5 (18, 19) and thymosin β_4 (19) were found to induce terminal transferase in bovine serum albumin gradient-separated bone marrow cells from NIH Swiss *nu/nu* mice *in vitro*. In this paper, we also demonstrate that thymosin fraction 5 and thymosin β_4 can greatly accelerate the reappearance of terminal transferase-positive cells in the thymus after steroid-induced immunosuppression.

EXPERIMENTAL PROCEDURES

Materials. Thymosin fraction 5 and spleen fraction 5 were isolated from calf thymus and spleen as described (16). Ion-exchange chromatographic medium carboxymethylcellulose CM-52 was obtained from Whatman. Gel filtration media Sephadex G-50, G-25, and G-10 were purchased from Pharmacia Chemicals, Inc. Trypsin, treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, and carboxypeptidase A were purchased from Worthington Biochemicals. Thermolysin was purchased from Calbiochem. All reagents used for Edman degradation were purchased from Pierce Chemical Co. Fluorescamine (Fluram) was obtained from Hoffmann–La Roche. All other chemicals were of reagent grade and were used without further purification. Sterile pyrogen-free water used in the preparation of all buffers was purchased from Travenol Laboratories or Abbott Laboratories.

Methods. Thymosin β_4 was isolated from fraction 5 by a combination of ion-exchange chromatography and gel filtration. The procedure used for the preparation of this polypeptide is illustrated in Fig. 1.

Lyophilized thymosin fraction 5 (9.58 g) was chromatographed on a column (2.5 × 128 cm) of CM-52 in 10 mM NaOAc/1.0 mM 2-mercaptoethanol, pH 5.0. The column was first washed with 3.2 liters of starting buffer, then with a linear gradient of 4 liters of 0–0.5 M NaCl in starting buffer. Final elution was by a second gradient of 4 liters of 0.5–1.0 M NaCl in starting buffer. The fractions were pooled as indicated in Fig. 1 (pools A–N). The second retained peak (pool G) was desalted on a Sephadex G-25 column (5 × 95 cm) in sterile water. The first protein peak (pool G1) was further fractionated by gel filtration on a Sephadex G-50 column (1.8 × 150 cm) in 6 M guanidinium·HCl. The third peak (pool III) was desalted on a Sephadex G-10 column and lyophilized. The sample so obtained is designated thymosin β_4 (43.5 mg).

For amino acid analysis, samples were hydrolyzed in doubly distilled 6 M HCl in evacuated sealed tubes for 24–120 hr at

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* See *Discussion* for a description of the nomenclature used to identify this peptide.

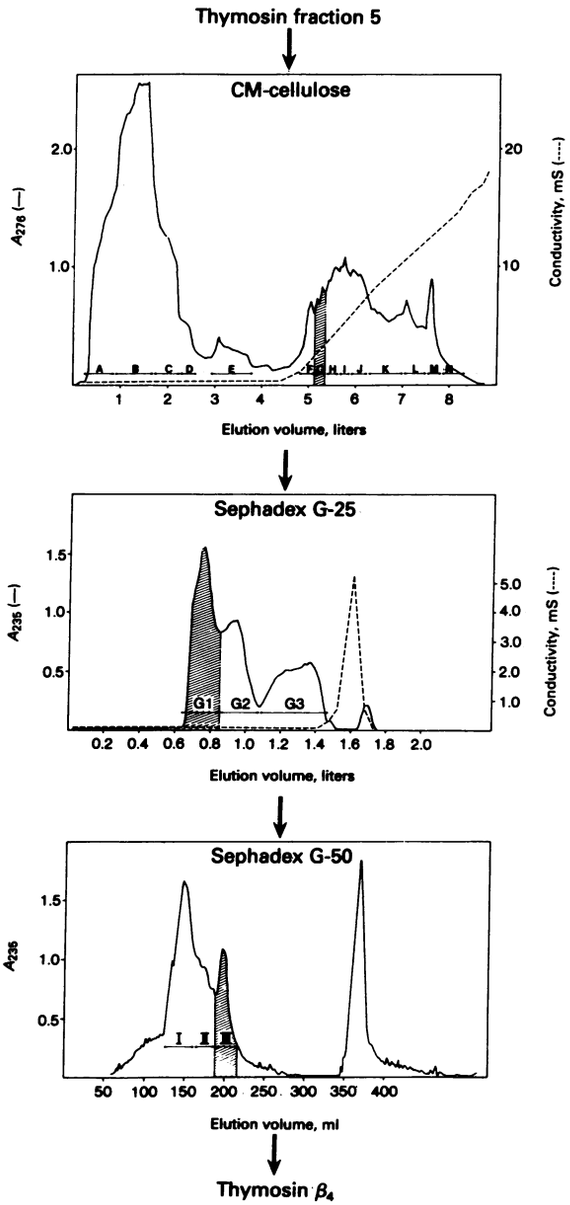

FIG. 1. Flow diagram of the fractionation of thymosin β_4 from bovine thymosin fraction 5. Shaded areas in elution profiles indicate fractions pooled for purifying thymosin β_4. Thymosin fraction 5 (9.58 g) was first chromatographed on a column packed with carboxymethylcellulose (CM-52). Fraction G was desalted on a Sephadex G-25 column. The first protein peak (G-1) was further fractionated on Sephadex G-50. Peak III was desalted on Sephadex G-10, lyophilized, and designated thymosin β_4 (43.5 mg).

110°C. A Beckman/Spinco Model 119CL amino acid analyzer was used in analysis based on the method of Spackman *et al.* (20). Thymosin β_4 was also hydrolyzed with 3 M mercaptoethansulfonic acid (21) and 6 M HCl containing 0.21 M dimethyl sulfoxide (22) to determine content of tryptophan, cysteine, or cystine.

Gels for isoelectric focusing were purchased from LKB (PAG plate, pH 3.5–9.5). As described previously (16), isoelectric focusing was carried out for 90 min at a constant power of 25 W. The gels were fixed in 20% trichloroacetic acid (wt/vol). The staining solution contained 0.1% Coomassie brilliant blue (G-250) in 20% trichloroacetic acid. Gels were destained in 10% trichloroacetic acid (wt/vol).

Enzymatic digestion was performed in 1% ammonium bicarbonate at pH 8.3 for 2–3 hr at 37°C. Trypsin or thermolysin was added to the protein solution for a final enzyme-to-substrate ratio of 1:50 (wt/wt). Cyanogen bromide cleavage was performed in 70% formic acid (vol/vol) at room temperature for 4 hr. The ratio of cyanogen bromide to protein was 5:1 (wt/wt). Cyanogen bromide was added in equal portions to the protein solution with stirring at intervals of 1 hr. At the end of 4 hr, the reaction product was diluted with 5 vol of distilled water and lyophilized.

Partial acid hydrolysis was achieved in 0.03 M HCl at 110°C for 4–16 hr in sealed evacuated tubes.

Enzymatic digests of partial acid hydrolysis products of thymosin β_4 were separated by paper electrophoresis, chromatography, or both. In a two-dimensional separation, paper chromatography was carried out first with 1-butanol/glacial acetic acid/water (4:1:5, vol/vol). This was followed by high-voltage electrophoresis at pH 1.9 for 30–50 min at 60 V/cm. Peptides were detected with cadmium/ninhydrin reagent (23) or with fluorescamine in acetone (24).

Cyanogen bromide cleavage products were separated by gel filtration on Sephadex G-50 in 0.1 M NH_4OH. Effluents were monitored by absorbance at 235 nm, as well as by fluorescamine assay after alkaline hydrolysis, as described by Nakai *et al.* (25). Peak 2 (F1 in Fig. 2) was further purified by high-voltage paper electrophoresis at pH 1.9.

The amino acid sequences of the peptides were determined by the dansyl-Edman (26) or subtractive-Edman (27) procedures and by automated sequence analysis on a Beckman 890C sequencer (dimethylallylamine program). Acids and amides were determined from the mobility on high-voltage paper electrophoresis at pH 6.5 according to Offord (28). Fluorescence measurements were made with excitation set at 390 nm and emission at 475 nm, using an Aminco–Bowman spectrophotofluorometer (American Instrument Co.).

In the method used for *in vivo* terminal transferase assay of thymosin, 11 daily injections of thymosin or other agents were given to 6-week-old C57BL/6J mice that were treated with 1.25 mg of hydrocortisone acetate the day before the first thymosin injection. The animals were sacrificed 24 hr after the last injection, and thymuses were removed and lymphocytes were prepared. The terminal transferase was extracted (29) and specific activity was determined (30). One unit of enzyme activity was defined as the amount catalyzing the incorporation of 1 nmol of dGTP into trichloroacetic acid-insoluble material per hr. The specific activity was calculated on the basis of the total enzyme activity recovered from phosphocellulose per 10^8 nucleated viable cells.

RESULTS

Thymosin β_4, isolated according to the procedure outlined in Fig. 1, has a molecular weight of 4982, as calculated from its structure. This correlates with the value 5000 estimated from a calibrated Sephadex G-50 column in 6 M guanidinium·HCl by the method of Fish *et al.* (31). The yield of thymosin β_4 from fraction 5 is about 0.45%.

The purity of the preparation was examined by analytical isoelectric focusing on an LKB PAG plate (pH 3.5–9.5). When the gels were fixed in 20% trichloroacetic acid, the thymosin β_4 preparation showed an intense protein precipitate band with a pI of 5.1. A few faint bands with pI between 5.1 and 7 were also observed. Subsequent staining of the fixed gels with 0.1% Coomassie brilliant blue in 20% trichloroacetic acid revealed a single sharp band with a pI of 5.1.

Amino acid analyses of thymosin β_4 showed, in terms of res-

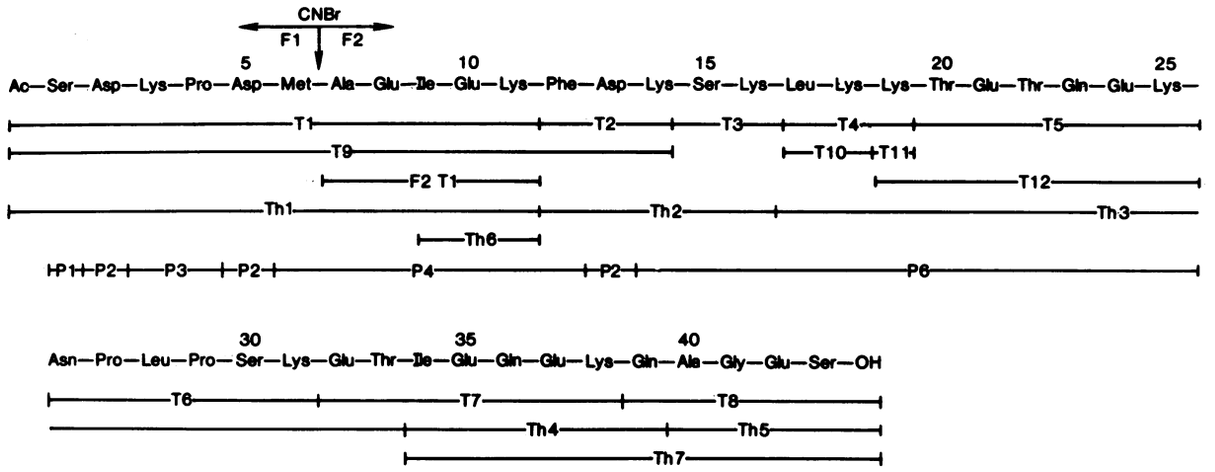

FIG. 2. Diagram of the sequence analyses that led to the elucidation of the primary structure of thymosin β_4. Line segments denote the peptides isolated after digestion of thymosin β_4 with trypsin (T1, etc.), thermolysin (Th1, etc.), and partial acid hydrolysis (P1, etc.). F1 and F2 are the fragments obtained from cyanogen bromide cleavage of thymosin β_4. F2 T1 was derived from tryptic digestion of F2. Acids and amides were assigned according to the charges determined by high-voltage paper electrophoresis at pH 6.5 of the original enzymatic peptides or the peptides derived from stepwise manual Edman degradation.

idues per molecule: Lys, 8.71 (9); His, 0.00 (0); Arg, 0.00 (0); Asp, 4.73 (4); Thr, 2.62 (3); Ser, 3.73 (4); Glu, 12.10 (11); Pro, 4.85 (3); Gly, 1.35 (1); Ala, 2.30 (2); Cys, 0.00 (0); Val, 0.37 (0); Met, 0.51 (1); Ile, 1.85 (2); Leu, 2.05 (2); Tyr, 0.00 (0); Phe, 0.85 (1); and Trp, 0.00 (0) [after hydroloysis with mercaptoethanesulfonic acid (21)]. The numbers in parentheses are the values from the established sequence, as shown in Fig. 2. The small discrepancies between the amino acid analysis results and the values obtained from the established sequence are attributed to minor contaminants in the preparation, which did not interfere with sequence analysis.

The failure of thymosin β_4 to give α-amino derivatives upon reaction with dansyl chloride or phenylisothiocyanate showed that its NH_2 terminus was blocked.

The complete amino acid sequence of thymosin β_4 is shown in Fig. 2, along with the peptide fragments isolated from proteolytic digests of trypsin and thermolysin and cyanogen bromide cleavage products that provided information toward the sequence elucidation.

Tryptic peptides T1 and T9 are assigned to the NH_2-terminal region of thymosin β_4 because they lack a free α-amino group. Peptide T8 is derived from the COOH terminus because of its lack of a lysine residue. Cyanogen bromide cleavage of thymosin β_4 produces two fragments, F1 and F2. F1 has a blocked NH_2 terminus. Partial acid hydrolysis of F1 gave rise to serine, aspartic acid, lysylproline, and homoserine. Tryptic digestion of F2 gave a new peptide Ala-Glu-Ile-Glu-Lys.

The NH_2-terminal blocking group of thymosin β_4 was identified as an acetyl group by the method of Schmer and Kreil (32). The sample was allowed to react first with anhydrous hydrazine and subsequently with dansyl chloride. The reaction product, 1-acetyl-2-dansylhydrazine, was extracted with chloroform and identified by thin-layer chromatography.

Table 1. *In vivo* induction of terminal transferase by thymosin in hydrocortisone acetate-treated C57BL/6J mice

Treatment*	Dose, μg	Terminal transferase: Specific activity†	Terminal transferase: % increase
Experiment 1			
Control saline	—	17.99	—
Spleen fraction 5	100	15.88	0
Thymosin fraction 5	100	28.54	58.6
Thymosin β_4	1	28.07	56.0
Experiment 2			
Control saline	—	13.0	—
Spleen fraction 5	100	14.4	10.8
Thymosin fraction 5	100	21.2	63.1
Thymosin β_4	1	19.8	52.3
Experiment 3			
Control saline	—	6.6	—
Spleen fraction 5	100	6.4	0
Thymosin fraction 5	100	9.9	50.1
Thymosin β_4	1	9.2	40.0

* Mice were treated with 1.25 mg of hydrocortisone acetate followed by daily injection of the indicated agents for 11 days. Amount used per injection is given in the dose column.

† Specific activity is expressed as nmol of dGTP transferred per hr per 10^8 cells.

Table 1 shows the terminal transferase assay results. It was found that the adminstration of thymosin β_4 at a dose of 1 μg/day (40 μg/kg) is effective in inducing terminal transferase activity in immunosuppressed mice.

DISCUSSION

During the last few years, several studies have indicated that the enzyme terminal transferase is uniquely associated with early T-cell differentiation (33–36). *In vitro*, terminal transferase polymerizes deoxynucleotides and, although it requires a primer, it does not require a template. The function of the enzyme *in vivo*, however, is not known. Terminal transferase is found in the cortisone-sensitive cortical thymocytes but not in the medullary thymocytes or more mature T cells in the periphery. Small numbers of terminal transferase-positive thymocytes (<5%) are also found in bone marrow cells, and this activity has been shown to be localized in a minor cell population separable by bovine serum albumin gradient fractionation. This population of prothymocytes is Thy-1-negative, but can be induced *in vitro* by thymic hormones to express Thy-1 antigen. Previously, we have reported that the expression of terminal

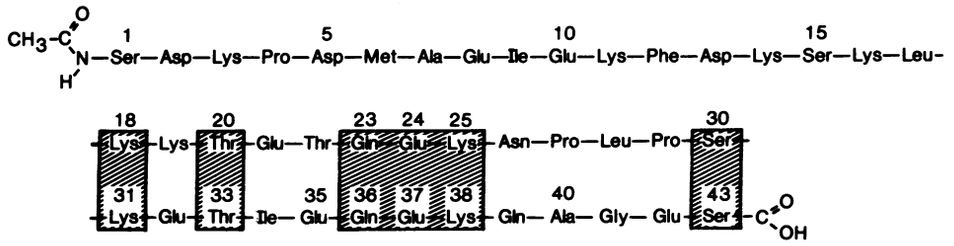

FIG. 3. Regions of internal duplication in thymosin β_4. Residues 31–43 are aligned with 18–30, and six identities are identified by shaded areas.

transferase in the bone marrow of athymic or normal mice can be induced both *in vivo* and *in vitro* by thymosin fraction 5 (18, 19) and thymosin β_4 (19). The results reported herein (Table 1) also indicated that these preparations are active in induction *in vivo* of terminal transferase in the thymus of steroid-suppressed mice. These results suggest that thymosin β_4 can influence the early stages in T-cell differentiation.

To facilitate the identification and comparison of all thymic peptides from one laboratory to another, we have previously proposed a nomenclature (37) based on the isoelectric focusing pattern of thymosin fraction 5 in the pH range 3.5–9.5. The separated polypeptides are divided into three regions: The α region consists of polypeptides with isoelectric points below 5.0; the β region 5.0–7.0; and the γ region above 7.0. Subscript numbers—α_1, α_2, β_1, β_2, etc.—are used to identify the polypeptides from that region as they are isolated, thus, the designation of thymosin β_4 for the peptide described in this paper.

Our previous studies have shown that, although thymosin fraction 5 possesses a wide spectrum of biological activities (4–10), the individual components purified from fraction 5 (3, 16, 17) exhibit only a portion of the total fraction 5 activity. This suggested to us that more than one peptide component may be necessary to elicit full immunological responsiveness (3). Consequently, the thymosin polypeptides are generally first purified by biochemical procedures to obtain homogenous products. The purified components are then tested in various assay systems for immunological efficacy. Thus, in the case of thymosin β_4 as described in this paper (Fig. 1), analytical isoelectric focusing gels were used to monitor the purification scheme rather than any particular assay system.

The complete amino acid sequence of thymosin β_4 is shown in Fig. 2. It is composed of 43 amino acid residues with an acetyl group at the NH_2 terminus. This polypeptide is rich in glutamic acid (eight residues) and lysine (nine residues) but devoid of arginine, histidine, half-cystine, valine, tyrosine, and tryptophan. A computer search of the sequence of thymosin β_4 against other protein sequences published to date (38) for possible sequence homology has been conducted at the National Biomedical Research Foundation, Washington, DC. The results do not indicate a statistically significant relationship of either segment A (residues 1–25) or segment B (residues 19–43) of thymosin β_4 to anything that is currently stored in the data base (38). A total of 138,972 segments of length 25 residues were compared to segments A and B. However, a possible duplicated region in the sequence of thymosin β_4 was noted. As shown in Fig. 3, when residues 31–43 are aligned with 18–30, there are six identities, as well as some other similarities.

Our studies on the endocrine role of the thymus gland strongly suggest the existence of a family of polypeptide hormones from the thymus gland. Thymosin β_4 reported here appears to act on stem cells to form prothymocytes. Thymosin α_1 (16, 17) and another peptide, α_7 (39), may act on prothymocytes to form more mature T cells.

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