Biochemistry. In the article "Isolation and complete amino acid sequence of human thymopoietin and splenin" by Tapan Audhya, David H. Schlesinger, and Gideon Goldstein, which appeared in number 11, June 1987, of *Proc. Natl. Acad. Sci. USA* (**84,** 3545–3549), the authors request that the following be noted. "Because much of the data are no longer available, it is necessary to retract this paper. Correct sequences for human thymopoietin(s) have been determined from cDNAs (1)."

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Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3545–3549, June 1987
Biochemistry

Isolation and complete amino acid sequence of human thymopoietin and splenin

(thymopentin/thymic hormone/polypeptides/radioimmunoassay)

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Communicated by Edward A. Boyse, December 29, 1986

ABSTRACT **Human thymopoietin and splenin were isolated from human thymus and spleen, respectively, by monitoring tissue fractionation with a bovine thymopoietin RIA cross-reactive with human thymopoietin and splenin. Bovine thymopoietin and splenin are 49-amino acid polypeptides that differ by only 2 amino acids at positions 34 and 43; the change at position 34 in the active-site region changes the receptor specificities and biological activities. The complete amino acid sequences of purified human thymopoietin and splenin were determined and shown to be 48-amino acid polypeptides differing at four positions. Ten amino acids, constant within each species for thymopoietin and splenin, differ between the human and bovine polypeptides. The pentapeptide active site of thymopoietin (residues 32–36) is constant between the human and bovine thymopoietins, but position 34 in the active site of splenin has changed from glutamic acid in bovine splenin to alanine in human splenin, accounting for the biological activity of the human but not the bovine splenin on the human T-cell line MOLT-4.**

Thymopoietin (TP) is a polypeptide hormone of the thymus with pleiotropic actions on prothymocytes, mature T cells, the nicotinic acetylcholine receptor, and pituitary corticotrophs (1–8). The complete 49-amino acid sequence of bovine TP (bTP) has been determined (9), and the biological activity has been shown to reside in the pentapeptide Arg-Lys-Asp-Val-Tyr corresponding to residues 32–36 [TP-(32–36) pentapeptide, named thymopentin (TP-5)] (10). Antisera to bTP were developed and used to demonstrate TP in epithelial cells of the thymus and also in basal keratinocytes of skin (11, 12). A RIA for bTP was developed (13, 14), and this detected immunoreactive material in extracts of bovine spleen or lymph node in addition to thymus (9). The immunoreactive substance in bovine spleen [bovine splenin (bSP)] was isolated using the bTP RIA to monitor purification; the complete amino acid sequence of bSP was determined (9). The amino acid sequence of bSP differed from that of bTP by only two residues, this similarity accounting for their both reacting with anti-bTP antibodies. Like bTP, the active site of bSP was shown to reside in residues 32–36 (Arg-Lys-Glu-Val-Tyr, named splenopentin), the change in residue 34 accounting for the changes in biological activities between the two molecules (15).

The RIA for bTP also detects immunoreactive material in extracts of human thymus and spleen. Therefore, we used this RIA to monitor the purification of the immunoreactive material in extracts of human thymus and spleen. We now report the isolation of these polypeptides and the determination of their complete amino acid sequences.

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MATERIALS AND METHODS

Materials. Human thymus obtained from pediatric surgical specimens and human spleen from surgical or autopsy specimens were trimmed and stored at −35°C. Separation materials and apparatus were purchased commercially: Sephadex (Pharmacia), hydroxyapatite (Bio-Gel HTP; Bio-Rad), and preparative large-pore anion-exchange column (2.5 × 25 cm; DE 500, particle size 40–60 μm) for HPLC (Separation Industries, Metuchen, NJ).

RIA of hTP and Human SP (hSP). The purification of hTP and hSP was monitored by using a RIA for bTP that was cross-reactive with hTP and hSP (13, 14). Briefly, bTP was labeled with ^{125}I by the Bolton–Hunter method (16), and the radiolabeled bTP was purified as described (17). Anti-bTP antibody (diluted 1:10,000) and ^{125}I-labeled bTP (10,000 cpm), in the presence or absence of various peptide fractions in phosphate buffer, were incubated for 8 hr at 22°C. Separation of bound and free bTP was with 24% polyethylene glycol at room temperature, followed by centrifugation at 700 × *g* for 15 min. The bound bTP was assayed in a LKB γ spectrometer.

Isolation of hTP and hSP from Tissue Extracts. A 714-g batch of human thymus or 9000-g batch of human spleen was extracted in ice-cold 100 mM ammonium bicarbonate [25% (wet weight) containing 50 ng of 2-mercaptoethanol, 175 μg of phenylmethylsulfonyl fluoride, and 375 μg of EDTA per ml) by homogenization in a Waring Blendor (Dynamics, New Hartford, CT). After centrifugation at 14,000 × *g* for 10 min at 4°C, the supernatant was filtered through cheese cloth and stored at 4°C after addition of 0.1% thimerosal and 0.1% sodium azide. For thymus, the soluble extract was processed by ultrafiltration at 4°C using Amicon Diaflo membrane XM100 (molecular exclusion limit ≈ M_r 100,000) and concentrated with Diaflo UM2 (molecular exclusion limit ≈ M_r 1000), reducing the volume to 1/10th to 1/50th. For spleen, the extract was processed through an Amicon hollow fiber cartridge H10×100, followed by concentration on a Diaflo YM2 membrane in a thin-channel ultrafiltration system.

Scheme 1 summarizes the isolation procedures. The retentate of the UM2 or YM2 filtration was chromatographed on bead-type Sephadex G-75 (particle size, 40–120 μm) at room temperature on a 5 × 150 cm column in 50 mM ammonium bicarbonate. Immunoreactive fractions were lyophilized. Two major peaks containing immunoreactive material were found for both thymus and spleen (Fig. 1). The lower molecular weight peak (M_r 5000–8000) was processed further by hydroxyapatite column chromatography using Bio-Gel

Abbreviations: TP, thymopoietin; SP, splenin; hTP, human TP; hSP, human SP; bTP, bovine TP; bSP, bovine SP; FPLC, fast protein liquid chromatography; TP-5, thymopentin [synthetic TP-(32–36) pentapeptide].

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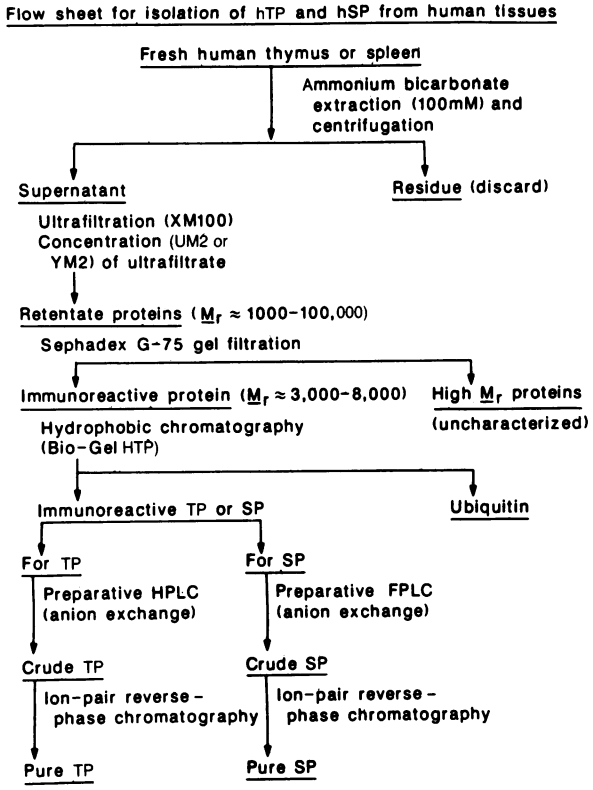

Scheme 1

HTP (2.5 × 60 cm column; 5 mM sodium phosphate buffer, pH 6.8), the sample being applied in the same buffer. Elution was with 30 ml of the same buffer and then with 35 ml of 50 mM phosphate buffer. Immunoreactive fractions were pooled, concentrated on an Amicon Diaflo UM2 membrane, desalted on an 0.6 × 30 cm Sephadex G-25 (fine) column in 10 mM ammonium bicarbonate (pH 8.0), and lyophilized.

hTP was subjected to HPLC using a 2.5 × 25 cm preparative DE 500 anion-exchange column. A linear solvent gradient started with 20 mM Tris acetate buffer (pH 6.0) and finished with 20 mM Tris acetate 500 mM sodium acetate, pH 6.0. Immunoreactive fractions were pooled, dialyzed using Spectrapor-6 (molecular exclusion limit ≈ M_r 1000), and lyophilized. Further chromatography was on a Vydac C_{18} reverse-phase column (218TP54) with a linear gradient starting with 50 mM ammonium formate (pH 6.5) and finishing with 60% dilution with acetonitrile. The fractions eluted by HPLC were partly evaporated, lyophilized, and reconstituted in 500 μl of 50 mM ammonium bicarbonate (pH 8.0). Volumes of 25 μl were tested in duplicate by RIA with pooling and lyophilization of immunoreactive fractions.

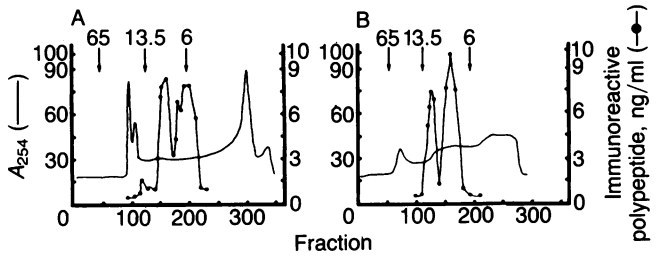

FIG. 1. Sephadex G-75 molecular sieve chromatography of extracts of human thymus (*A*) and human spleen (*B*), with measurement of immunoreactive material by RIA for bTP. The lower molecular weight peaks were further purified to yield hTP or hSP.

For hSP, after hydroxyapatite column chromatography, fast protein liquid chromatography (FPLC) was performed with a Mono Q HR 5/5 column (Pharmacia). Elution was with 20 mM monoethanolamine (pH 9.5) and a linear gradient finishing with 0.3 M NaCl in the same solvent. The eluted fractions were partially evaporated, lyophilized, and reconstituted in 500 μl of ammonium bicarbonate. Volumes of 10 μl were tested in duplicate by RIA, and the immunoreactive fractions were pooled and lyophilized.

Yields from the purification steps are seen in Table 1.

Amino Acid Analysis. Amino acid analysis was performed with a Liquimat III amino acid analyzer following acid hydrolyses in 5.7 M HCl containing 0.5% 2-mercaptoethanol for 22 hr at 100°C (18).

Preparation of Maleated hTP and hSP. Maleic anhydride in 1,4-dioxane (100 μl) was added at a 20-fold excess over the free amino groups of the polypeptides (500 μg), which were dissolved in 100 mM sodium borate (pH 9.3). Maleic anhydride was added stepwise over a 4-hr period, pH 9.3 being maintained with 6 M NaOH. The maleated protein was then desalted on Bio-Gel P-2 in 100 mM NH_4HCO_3 (pH 8.2) and lyophilized.

Enzymatic Digestion. Tryptic digestions of maleated hTP and hSP were carried out in 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.1) at 37°C for 6 hr. Bovine pancreatic trypsin (diphenylcarbamyl chloride-treated; 8400 Nα-benzoyl-L-arginine ethyl ester units per mg of protein, Sigma type XI) was added to a final enzyme/substrate ratio of 1:100 (on a weight basis). The pH was maintained at 8.5 during the digestion and then lowered to 2.0 by addition of 10 mM HCl to terminate the reaction. The mixture was then lyophilized.

CNBr Cleavages. hTP and hSP (200 pmol) were cleaved with CNBr in 150 μl of 70% formic acid for 23 hr at room temperature in the dark; a 200-fold molar excess of CNBr over the methionine content in the sample was used. The volume was then reduced to 60 μl under N_2 and applied directly on to the glass filter of the gas-phase sequencer (19).

Purification of Trypsin-Digested and CNBr-Cleaved Peptides. The tryptic peptides were dissolved in 0.1% orthophosphoric acid (pH 2.2) and were centrifuged at 15,600 × *g* for 5 min; the supernatant was applied to the C_{18} reverse-phase column [Vydac 218TP54, 46 mm × 25 cm, 5-μm particle size (The Separation Group, Hesperia, CA)] for HPLC (LDC gradient module with spectromonitor II and CI-10 computing integrator attached to a LKB Redirac fraction collector 2112). A linear elution gradient was used starting with 0.1% phosphoric acid and finishing with 80% acetonitrile. High-purity-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI) and distilled in glass. Phosphoric acid (1%) was filtered through a Millipore type HA 0.45-μm filter (Millipore). All solvents were degassed for 20 min under vacuum with stirring.

Automated N-Terminal Protein Sequencing. Automated sequence analyses on intact hTP, hSP, and tryptic and cyanogen bromide fragments of hTP and hSP were performed by gas-phase sequencing that used a model 470A applied biosystems gas-phase sequencer with Polybrene as carrier and a standard single-coupling single-cleavage program. The resulting phenylthiohydantoin-derivatized amino acids were identified by HPLC with a 1084B Hewlett Packard high-pressure liquid chromatograph (20).

C-Terminal Analysis with Carboxypeptidase Y. C-terminal analysis of hTP and hSP (150 μg each) was performed with yeast carboxypeptidase Y (2 nmol) dissolved in 100 mM sodium acetate (pH 6.0) at 37°C. At various time periods, aliquots were removed from the digest and added to tubes containing 10 μl of glacial acetic acid, and the mixture was then lyophilized. The sample was dissolved in 0.2 M sodium citrate (pH 2.2) and applied to the amino acid analyzer. Enzyme and peptide blanks were also run.

Table 1. Summary of purification hTP and hSP

Purification step	Total protein, mg	Immunoreactive protein mg	Immunoreactive protein %	Fold purification	Recovery, %
		hTP			
Ultrafiltered protein	68,800	309.5	0.45	1	100
Gel filtration of G-75	2,950	76.9	2.6	6	25*
Hydrophobic chromatography	181.2	32.6	18	40	11
Preparative HPLC	3.4	2.8	82	182	0.9
Reverse-phase chromatography	1.4	1.3	93	207	0.42
		hSP			
Ultrafiltered protein	230.9	134.7	0.05	1	100
Gel filtration of G-75	305.8	97.3	3.2	650	72*
Hydrophobic chromatography	148.5	66.3	45	900	49
Preparative FPLC	22.5	17.4	77	1540	12.9
Reverse-phase chromatography	6.8	6.1	90	1800	4.5

*Lower molecular weight peak.

RESULTS

Isolation of hTP. Approximately 1.4 kg of freshly frozen human thymus yielded 1.3 mg of immunoreactive hTP (recovery = 0.4%; Table 1). This product had one major band and few minor bands on isoelectric gel focusing. The isoelectric point of the major band was 6.1 ± 0.2.

Amino Acid Composition. The amino acid composition is shown in Table 2.

Cyanogen Bromide Cleavage and Separation of Fragments. Cyanogen bromide cleavage of hTP produced three distinct fractions (Fig. 2*A*), and the amino acid sequence results for each are summarized in Table 3.

Structural Determination of hTP. Automated N-terminal sequencing of 240 pmol of hTP through 46 cycles of the Edman degradation gave a repetitive yield (average yield per cycle) of 93.4% and identified each of the 46 N-terminal residues (Fig. 3*A*). The timed release of amino acids from the C terminus of intact hTP with carboxypeptidase Y (Table 4) provided an unequivocal C-terminal tetrapeptide sequence with a two-amino acid overlap (residues 45 and 46) with the N-terminal sequence. Partial sequence of hTP was confirmed by the structure determination of CNBr fragment I (Fig. 2*A*), which encompassed residues 32–40 and which includes the active site. In addition, the amino acid composition of intact hTP (Table 2) accounted for all of the amino acids found by sequence analysis.

Table 2. Amino acid composition of hTP and hSP (molar ratios)

Amino acid	hTP Found	hTP Mean ± SD*	hSP Found	hSP Mean ± SD*
Cysteic acid	0	ND	0	ND
Aspartic acid	2	2.29 ± 0.12	2	1.78 ± 0.13
Threonine	3	3.11 ± 0.23	3	2.86 ± 0.18
Serine	1	0.81 ± 0.19	2	1.84 ± 0.13
Glutamic acid	6	5.55 ± 0.22	7	7.36 ± 0.42
Proline	3	2.80 ± 0.30	3	2.56 ± 0.18
Glycine	3	2.28 ± 0.27	2	2.22 ± 0.14
Alanine	4	4.25 ± 0.10	5	4.84 ± 0.17
Valine	6	6.23 ± 0.30	6	5.62 ± 0.27
Methionine	1	0.87 ± 0.13	1	0.89 ± 0.14
Isoleucine	0	0.1 ± 0.11	0	0.21 ± 0.12
Leucine	9	8.72 ± 0.36	8	8.41 ± 0.41
Tyrosine	2	1.90 ± 0.12	2	1.69 ± 0.19
Phenylalanine	0	0.16 ± 0.11	0	0.22 ± 0.10
Lysine	5	4.92 ± 0.31	5	5.36 ± 0.34
Histidine	2	1.76 ± 0.12	1	0.96 ± 0.13
Arginine	1	1.07 ± 0.13	1	1.16 ± 0.12

ND, not detected.
*Average of two determinations (24 and 76 hr). Serine was increased by 10% and threonine by 5% to compensate for destruction by acid. Hydrolysis was performed with constant-boiling HCl at 110°C *in vacuo*.

Isolation of hSP. Approximately 9 kg of freshly frozen spleen yielded 6 mg of homogenous protein (recovery 4.5%; Table 1). Analytical disc gel electrophoresis at pH 8.9 and 4.3 gave a single sharp band with an isoelectric point of 6.0 ± 0.15.

Amino Acid Composition. The amino acid composition is shown in Table 2.

Cyanogen Bromide Cleavage and Separation of Fragments. CNBr cleavage of the hSP produced three peaks (Fig. 2*B*). The amino acid sequence and results for each are summarized in Table 3.

Tryptic Digestion of Maleated hSP and Separation of Fragments. Maleated tryptic digestion of hSP produced five distinct peaks on HPLC (Fig. 4). The sequence regions of peptide in each peak are summarized in Table 3.

Structural Determination of hSP. hSP (110 pmol) was subjected to automated gas-phase sequence analysis by Edman degradation, and continuous identifications were made through the N-terminal 34 residues. The average yield per cycle was 90.2%, and the measured recoveries of selected residues are shown in Fig. 3*B*. The C-terminal sequence (Table 4) of the intact protein was determined with carboxypeptidase Y giving time-dependent release of amino acids. The C-terminal sequence of -Ala-Glu-His-COOH- provided a two-amino acid sequence overlap with the sequence of peak II obtained from a tryptic digest of maleated hSP (Fig. 4). Amino acid compositions of hSP and the fragments obtained from both CNBr cleavage and enzymatic digestion were

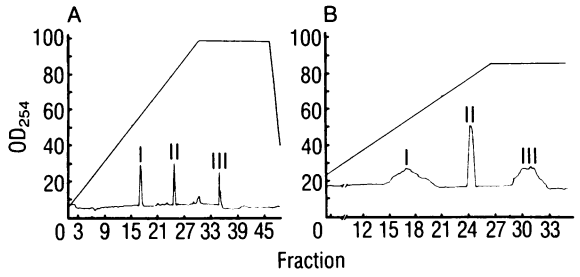

FIG. 2. Separation of CNBr digest of hTP (*A*) and hSP (*B*) dissolved in 0.1% orthophosphoric acid on a C_{18} reverse-phase column (Vydac 218TP54, 46 mm × 25 cm) at a flow rate of 2 ml/min. The peaks are designated I, II, and III. The gradient line represents the presence of organic solvent.

Table 3. Methods of sequence determination for the various regions of hTP and hSP

Method of sequencing	Residues identified
hTP	
N-terminal degradation	
Native TP	1–46
CNBr cleavage	
Peak I	1–11
Peak II	32–46
C-terminal carboxypeptidase method	44–48
hSP	
N-terminal degradation	
Native SP	1–34
CNBr cleavage	
Peak I	1–16
Peak II	32–39
Tryptic digest of maleated SP	
Peak I	1–15
Peak II	8–20
Peak III	37–47
Peak IV	No sequence obtained
Peak V	18–30
C-terminal carboxypeptidase method	45–48

consistent with the N-terminal sequence determinations and the sequence completion by carboxypeptidase analysis.

DISCUSSION

We had previously used the cross-reactivity of anti-bTP antibodies to detect and isolate the closely related polypeptide bSP from bovine spleen (9). Similarly, antibodies to bTP proved cross-reactive with hTP and hSP, and the bTP RIA was used to monitor the isolation of hTP and hSP from extracts of human thymus and spleen, respectively. Both isolations involved tissue extraction in aqueous solvent, ultrafiltration for approximate sizing and concentration, gel filtration for more accurate sizing, hydrophobic chromatography, and reverse-phase HPLC (for hTP) or anion-exchange FPLC (for hSP). Approximately 50% of the immunoreactive material in each extract was present in a higher molecular weight form that probably represents a biologically inactive precursor form (21). This was separated during molecular sieving on Sephadex G-75.

Despite simplification of the isolation procedure, the recovery of hTP was very low (0.42%), the purified material representing a 207-fold purification (Table 1). Large losses of hTP occurred during HPLC, but for splenin, preparative FPLC was substituted for HPLC, and the yield of 4.5% (1800-fold purification) (Table 4) was higher and comparable

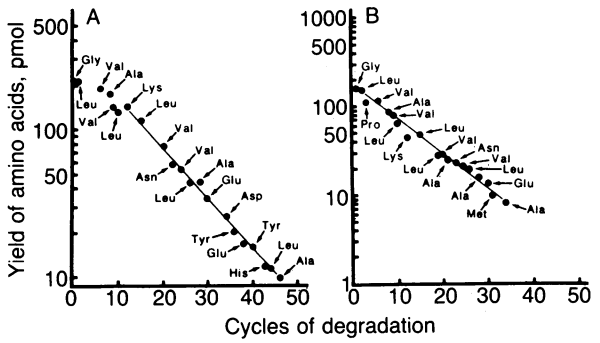

FIG. 3. Repetitive yields of phenylthiohydantoin-derivatized amino acids during automated Edman degradation of hTP through 46 cycles (*A*) and hSP through 34 cycles (*B*).

Table 4. Release of free amino acids with time from the C terminus with carboxypeptidase Y

Amino acid	Amino acid yield, nmol					
	100 s	120 s	150 s	200 s	250 s	300 s
			hTP			
Thr	—	—	—	75	210	263
Ala	—	—	88	258	276	288
Leu	—	148	275	283	418	503
His	—	289	310	300	308	299
			hSP			
Ala	—	—	—	0.32	—	0.80
Glu	0.27	—	—	0.80	—	0.80
His	0.91	—	—	0.80	—	0.90

to the yields for bSP (9). Purity of hTP and hSP was established by polyacrylamide gel electrophoresis, HPLC, and amino acid sequence (see below).

For hTP, residues 1–46 were determined by automated Edman degradation with a gas-phase sequencer, and residues 1–11 and 32–46 were confirmed by Edman degradation of two CNBr-cleavage fragments. This provided independent confirmation of the important active-site region, residues 32–36. The C-terminal sequence 44–48 was determined by time-release carboxypeptidase digestion.

For hSP, residues 1–34 were determined by automated Edman degradation with the gas-phase sequencer, with confirmation of residues 1–16 from a CNBr-cleavage fragment, and extension of sequence 32–39 was determined from the other CNBr-cleavage fragment. This provided independent confirmation of the active-site region of hSP, which is different from that of bSP and from those of both bTP and hTP. The remaining sequence was determined from Edman degradation of fragments produced by tryptic digestion of maleated hSP (residues 1–15, 8–20, 18–30, and 37–47) and by time-release carboxypeptidase digestion (residues 45–48). Tryptic cleavage of maleated hSP yielded fragments that represented cleavages between residues 7–8 (-Pro-Ala-), 17–18 (-Ser-Glu-), and 36–37 (-Tyr-Val-); there was no cleavage at the protected site 31–32 (-Met-Arg-). We are at a loss to explain these anomalous cleavages other than that

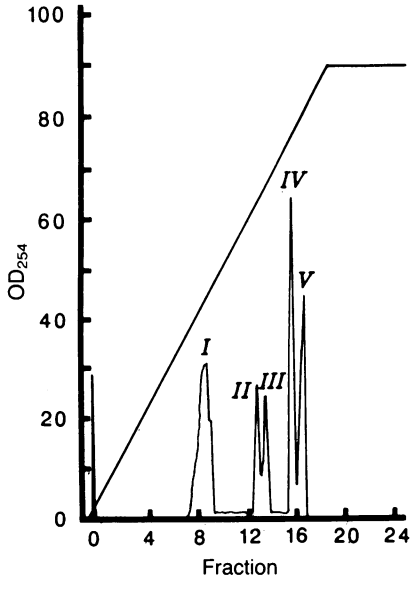

FIG. 4. Elution profile of the tryptic digest of maleated hSP on Mono Q HR 5/5 (Pharmacia) column used in HPLC system. Five major peptide-containing peaks were detected by UV absorption at 254 nm. These are designated I–V.

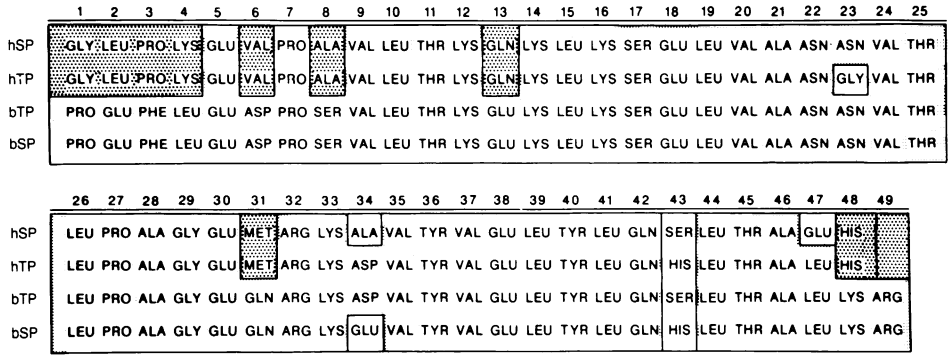

FIG. 5. Complete amino acid sequences of hSP, hTP, bTP, and bSP. The 10 species-specific residues shared by TP and SP within each species are shown with heavy stippling in the human sequences. Within the active site region (32–36), bTP and hTP are identical, but bSP and hSP differ at position 34 (Glu and Ala, respectively).

they are attributable to contaminating enzymes in the trypsin preparation and not to trypsin itself.

hTP and hSP were very similar in amino acid sequence, each being 48-amino acid linear polypeptides differing in only 4 residues, positions 23, 34, 43, and 47 (Fig. 5). They were also similar to bTP and bSP, with 10 residues being similar between hTP and hSP but different from the common residues in bTP and bSP at these positions (Fig. 5). These comparisons confirm that the amino acid sequences of hTP and hSP were indeed determined and indicate that gene conversion must have occurred during evolution to maintain the parallel sequence evolution of these two polypeptides within each species. Other important gene systems that utilize gene conversion include immunoglobulin (22) and the major histocompatibility complex (23) genes. Maintenance of parallel evolution of regions of TP and SP outside the active-site region (residues 32–36) implies that these C- and N-terminal regions of the molecule must also have an important function, with similar requirements for both TP and SP being maintained within each species.

Residues 32–36 of bTP are known to represent the active site, with the corresponding synthetic TP-(32–36) pentapeptide TP-5 having the biological activities of TP in animals and man (10, 15). The present studies establish that the active site of hTP (residues 32–36) is identical with that of bTP, so that synthetic TP-5 (Arg-Lys-Asp-Val-Tyr) also represents the human active site.

bSP differs from bTP by having a glutamic acid residue at position 34, in contrast to aspartic acid at this position in bTP, and this change confers contrasting biological activities on bSP and synthetic splenopentin or SP-5 (Arg-Lys-Glu-Val-Tyr) by comparison with bTP and TP-5. Interestingly, position 34 of hSP has an alanine at position 34, thus differing from bSP and TP. hSP differs in its biological activity from bSP in that hSP but not bSP induces elevation of intracellular cGMP in MOLT-4, a human T-cell line (B. Baker, G. I. Viamontes, T.A. and G.G., unpublished data). Therefore, it was predicted that the active site of hSP would have changed from that of bSP, and this is now confirmed directly from the amino acid sequence.

The isolation and amino acid sequence determination of hTP and hSP should contribute to the further understanding of these important immunoregulatory polypeptides in man.

The authors thank Ronald King, James Chen, and Gary Campbell for their technical expertise; Marilyn Sanders for editorial assistance; and Nancy Lawery for typing the manuscript.

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