Involvement of thymosin β_4 and endoproteinase Asp-N in the biosynthesis of the tetrapeptide AcSerAspLysPro a regulator of the hematopoietic system

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It is shown that AcSDKP a new regulator of the hematopoietic system can be generated from thymosin β_4 by a one-step enzymatic cleavage in vitro and in vivo. AcSDKP and Tβ_4 were both detected in bone marrow cells (BMC). Incubation of [^{3}H]Tβ_4 with either intact or lysed BMC led to the formation of [^{3}H]AcSDKP whereas the labelled tetrapeptide was not degraded under these conditions. Model enzymatic degradation of Tβ_4 carried out with bacterial enzymes suggests that a mammalian endoproteinase Asp-N might be involved in the formation of AcSDKP through the specific cleavage of the ^{4}Pro-^{5}Asp peptide bond of Tβ_4.

Thymosin β_4; Enzymatic maturation; Endoproteinase Asp-N; AcSDKP peptide; Hematopoietic regulator; Bone marrow cell

1. INTRODUCTION

Biologically active peptides are synthesized in cells as part of relatively high molecular weight precursors which are further processed by specific proteolysis before active peptides are generated. We recently isolated from fetal calf bone marrow a tetrapeptide Ac-N-Ser-Asp-Lys-Pro (AcSDKP). This peptide inhibits in vivo the entry into S-phase of cell populations which are normally blocked in G_o of the cell cycle. This property was demonstrated for murine hematopoietic pluripotent stem cells stimulated in vivo by cytosine arabinoside treatment [1], as well as for rat hepatocytes triggered into S-phase by caseine injection or partial hepatectomy [2].

It was suggested by us [1] that this tetrapeptide might derive from thymosin β_4 (Tβ_4). This polypeptide is presently the only sequenced mammalian protein which contains the complete sequence of the acetylated tetrapeptide at its N-terminus (Fig. 1) [3]. Tβ_4 was first isolated as a thymic hormone from calf thymus and described as an immunomodulatory factor [4].

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Abbreviations: AcSDKP, Ac-N-Ser-Asp-Lys-Pro; BMC, bone marrow cells; Tβ_4, thymosin β_4

In the present paper, we report evidence that (i) Tβ_4 is present as a constituent of bone marrow cells (BMC), (ii) BMC contain an enzymatic system which leads, starting from [^{3}H]Tβ_4, to the formation of [^{3}H]AcSDKP and (iii) that bacterial endoproteinase Asp-N mimics BMC maturation enzyme and forms AcSDKP through a one step enzymatic cleavage of Tβ_4.

2. MATERIALS AND METHODS

Flavobacterium meningosepticum α-prolyl-endopeptidase (EC 3.4.21.26) was purchased from Seikagaku Kogyo. Asp-N-endoproteinase was purchased from Boehringer (Mannheim). Tβ_4 was isolated from bovine spleen [5].

AcSDKP was a generous gift from IPSEN Beaufour. This peptide has been specifically tritiated on the lysine residue (Morgat et al., 1990, Proc. Eur. Pep. Symposium, in press).

2.1. *Isolation of Tβ_4 from murine BMC*

Tβ_4 extraction was carried out by a one step procedure as previously described [6]. An aliquot of the sample was analysed by reverse phase HPLC on a Licrospher 5 μm column (4 × 250 mm, Merck, Darmstadt, FRG) using a linear gradient of *n*-propanol (0–40% within 60 min) in 0.02 M pyridine/0.1 M formic acid/0.1 M lithium perchlorate at a flow rate of 0.75 ml/min. The elution was monitored by fluorescence measurement after post-column derivatization of the peptides with fluorescamine [7].

2.2. *^{3}H-labelling of Tβ_4 by tritium exchange*

Tβ_4 (1.35 mg, 270 nmol) was dissolved in 450 μl of water and then frozen. The catalyst (38.4 mg PdO) was added and the reacting vial was connected to the tritium supplying automatic device [8]. At 10^2

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AcSDKPDMAEIEKFDKSKLKKT
ETQEKNPLPSKETIEQEKQAGES

Fig. 1. Structure of Thymosin β_4.

Pa, 1110-1480 GBq (30-40 Ci) of pure tritium gas were introduced and compressed to 1.6×10^5 Pa. After thawing, the reaction mixture was kept at room temperature and magnetically stirred for 2 h. The absorption of tritium gas produced a pressure reduction of 0.85×10^5 Pa. The catalyst was removed from the reacting solution by filtration over a Schlecher and Schuell filter (0.2 μm, FPO30/3) and labile tritium atoms were eliminated by successive flash evaporations in dilute acetic acid (0.5%). Total radioactivity recovered was: 83.62 MBq (2.26 mCi). The crude labelled material was purified by HPLC on a Hypersil C18 column (4 × 250 mm) (SFCC, Neuilly, France) with a linear gradient of two solvents A and B (A: 0.1% TFA, B: 60% CH_3CN with 0.1% TFA, elution pattern: 0 to 1 min, 100% A, 0.5 ml/min; 1 min to 30 min: from 100% A to 100% B, 1 ml/min). Quantitative and comparative estimations indicated that the specific activity was found to be around 288.6 GBq/mmol (7.8 Ci/mmol).

2.3. *Degradation of [^{3}H]Tβ_4 and [^{3}H]AcSDKP by rabbit BMC*

Bone marrow from rabbit femur was dissociated in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were resuspended at 10^8 cells/ml in the same medium. Either radiolabelled [^{3}H]Tβ_4 (150 kBq) or [^{3}H]AcSDKP (75 kBq, 4440 GBq mmol) were incubated at 37°C for various periods of time with 300 μl of either the cell lysate or the intact cell suspension. After protein precipitation with trichloracetic acid, supernatants were analysed by reverse phase HPLC.

2.4. *Digestion of Tβ_4 by endoproteinase Asp-N*

1 mg Tβ_4 was dissolved in 450 μl of phosphate buffer (50 mM, pH 8.0), then 2 μg of endoproteinase Asp-N in 50 μl water were added. The digestion was carried out at room temperature for 18 h. At various periods of time, 50 μl aliquots were analysed by analytical reverse phase HPLC (Fig. 3). After an 18 h digestion, 200 μl of the sample were separated by a preparative reverse phase HPLC system equipped with a sampling valve having an internal chamber (2 μl). The sampling valve diverted aliquots of the column effluent into the fluram detection system at regular intervals [7].

3. RESULTS

3.1. *Detection of Tβ_4 in mouse BMC*

Murine BMC perchloric acid extracts (A) were analysed by reverse phase HPLC. Spectra exhibited a peak at 30.36 min characteristic of the elution time of Tβ_4 (Fig. 2a). This peak co-eluted with an authentic sample of the polypeptide (Fig. 2b). After an oxidative treatment by H_2O_2, the 30.36 min peak present in sample A was shifted to 28.18 min (Fig. 2c), which corresponded to the elution time of the sulfoxide derivative of Tβ_4 formed following this treatment.

3.2. *Degradation of Tβ_4 into AcSDKP by the rabbit BMC enzymatic system*

Random labelled [^{3}H]Tβ_4 was prepared from Tβ_4 by a catalytic exchange of H_2 with $^3H_2$ and incubated for various periods of time with rabbit BMC.

Incubation media were analysed by HPLC. Fractions corresponding to AcSDKP were collected between 19–23 min whereas larger peptidic fragments derived from Tβ_4 and intact Tβ_4 were recovered as a unique fraction collected between 30–40 min. After a 2 h incubation, the highest level of radioactivity associated with 19–23 min fraction was reached and corresponded to 5% of the total radioactivity initially present in Tβ_4

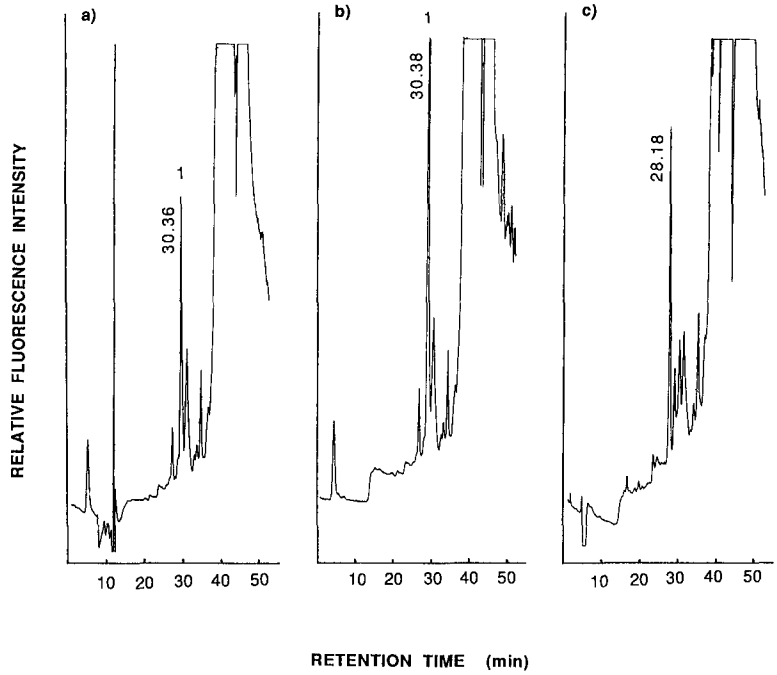

Fig. 2. HPLC analysis of murine BMC extract. BMC were extracted by perchloric acid (sample A). HPLC analysis was carried out with (a) sample A, (b) sample A spiked with 2 μg of Tβ_4, (c) sample A oxidized at 37°C with 5% H_2O_2 within 60 min. Peak 1 = Tβ_4.

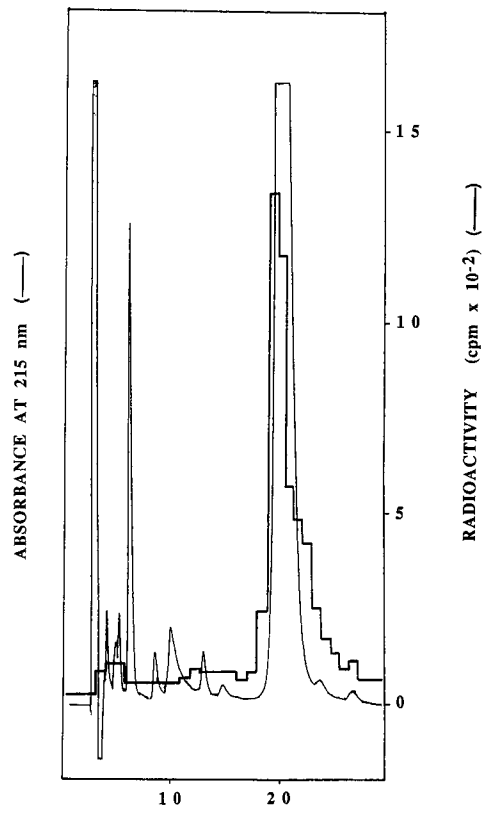

Fig. 3. Radioactive AcSDKP is formed by incubation of [^{3}H]Tβ_4 with BMC : HPLC analysis of the collected 19–23 min fraction. The collected 19–23 min fraction (Table I) was lyophilized and reinjected in the same conditions. Elution was monitored by UV detection at 215 nm and by radioactivity measurement of the collected fractions.

(Table I), i.e. to about 50% of the radioactivity theoretically associated with the N-terminal AcSDKP fragment of Tβ_4. This amount was unchanged after an 18 h incubation period. Comparative experiments carried out with rabbit BMC lysate led after a 2 h incubation period, to the recovery of a maximal 0.9% of the total radioactivity into the 19–23 min fraction, i.e. to about 10% of the radioactivity theoretically associated with the N-terminal AcSDKP fragment of Tβ_4. Longer incubation periods did not lead to the recovery of a larger amount of radioactivity in the 19–23 min fraction. For the two types of experiment, when the 19–23 min fraction was reanalysed by HPLC the radioactivity coeluted with added unlabelled AcSDKP (Fig. 3).

Table I

Degradation of [^{3}H]Tβ_4 by rabbit BMC enzymatic system

	Percentage of the total radioactivity associated with the corresponding fraction			
	Incubation time			
Collected fractions	0	1 h	2 h	18 h
19–23 min	0	2.5	5.0	4.0
30–40 min	100	91.5	77.1	78.6

[^{3}H]Tβ_4 was incubated for various periods of time with intact rabbit BMC. Soluble fraction was coinjected with 10 μg of AcSDKP and separated by reverse phase HPLC. Analysis was performed at a flow rate of 1 ml/min on a Hypersil C18 column (4 × 250 mm, SFCC) first with 4.5% CH_3CN, 0.1 TFA for 28 min and then with a gradient from 4.5% CH_3CN, 0.1% TFA to 0.1 TFA in CH_3CN for 5 min. Elution was monitored by UV detection at 215 nm and by radioactivity measurement of the collected fractions previously lyophilised and resuspended in 2.5 ml of a scintillation liquid (ACS, Amersham, Les Ulis, France). Fractions corresponding to AcSDKP were collected between 19–23 min whereas larger peptidic fragments derived from Tβ_4 and intact Tβ_4 were recovered between 30–40 min in a unique fraction.

3.3. *Degradation of [^{3}H]AcSDKP by rabbit BMC enzymatic system*

After a 4 h incubation of [^{3}H]AcSDKP with either intact rabbit BMC or their lysate, 97% of the radioactivity was recovered in the 19–23 min fraction, i.e. in the elution peak of the tetrapeptide.

3.4. *Degradation of Tβ_4 by bacterial endoproteinase Asp-N*

Treatment of Tβ_4 by endoproteinase Asp-N led to the formation of AcSDKP (Fig. 4a). In order to identify the various peptidic fragments generated from Tβ_4 through specific cleavages, analyses were carried out in modified HPLC conditions (Fig. 4b). The isolated fragments were subjected to acid hydrolysis and analyzed for amino acid composition. This proved the formation of 1–4, 13–23, 24–43, 21–31, 5–12 and 1–12 fragments through enzymatic cleavages of Tβ_4.

4. DISCUSSION

Previous studies have demonstrated that the tetrapeptide AcSDKP is a new regulator of mammalian cell proliferation active in vivo on the hepatocytes and hematopoietic stem cells [1,2]. Biosynthesis studies have shown that this factor is present in sera and in BMC, and biosynthesized in minute amounts [10]. The possibility that AcSDKP is formed through proteolytic maturation of a larger proteinous precursor appealed to us. According to a search in the Genomic Data Bank, rat phenylalanine hydroxylase, human and mouse Tumor Necrosis Factor α, human retinal S-antigen, human amphiregulin and rat, porc, human and mouse Tβ_4 are proteins claimed to contain the SDKP sequence in their structure. Except for this last protein, the SDKP sequence is included into the protein chains. A possible formation of AcSDKP from those hypothetical precursors would implicate three specific enzymatic reactions: two proteolytic cleavages followed by one N-terminal acetylation. Conversely, a unique proteolytic cleavage of the ^{4}Pro–^{5}Asp peptidic bond of Tβ_4 (Fig. 1) should be sufficient to form the active peptide from Tβ_4 as the AcSDKP sequence constitutes the N-terminal part of this polypeptide.

Tβ_4 has been detected in numerous species as a cell component of various tissues [11,12], but its presence in

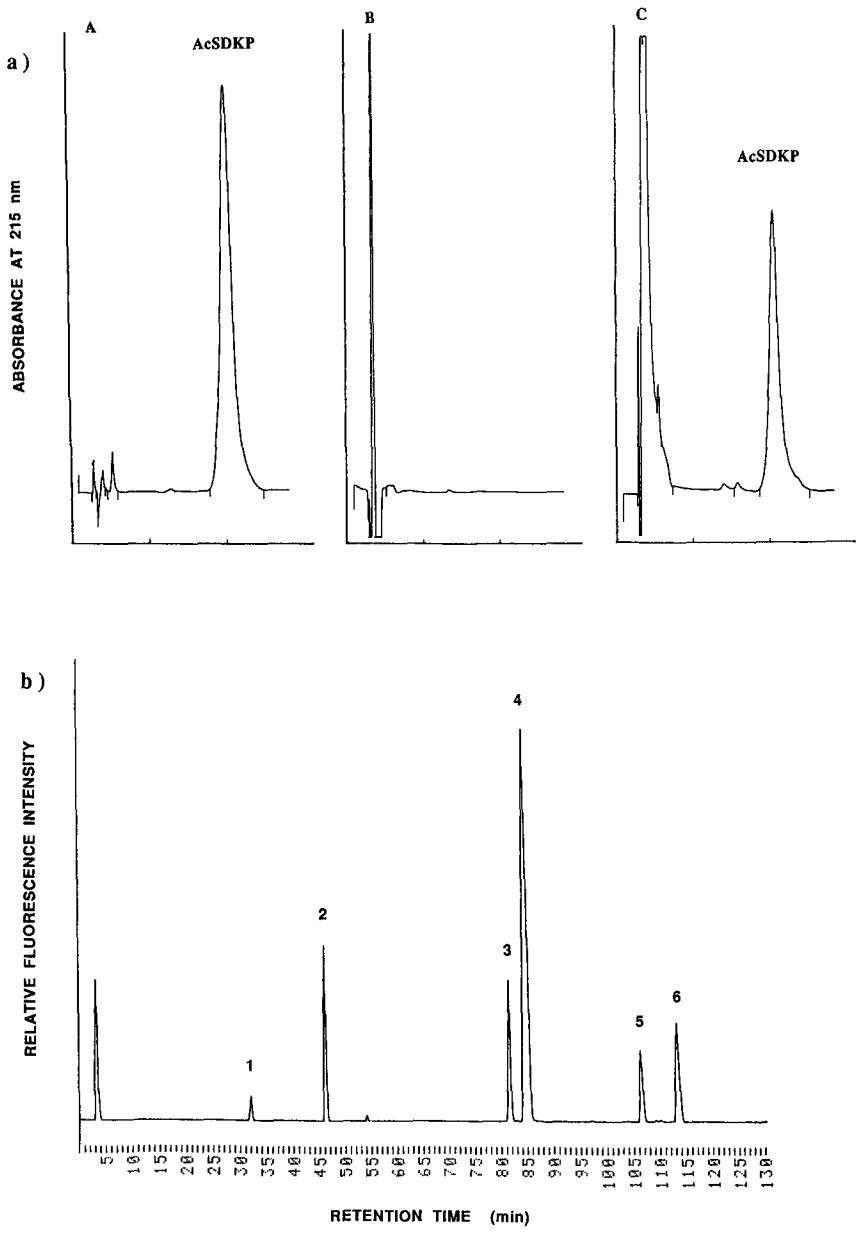

Fig. 4. HPLC analysis of degradative peptides formed through Tβ_4 digestion by endopeptidase Asp-N. Analysis was performed: (a) On a Hypersil C18 column in 4.5% CH_3CN, 0.1% TFA, (A) AcSDKP standard 10 μg, (B) control initial time, (C) after an 18 h incubation. (b) After an 18 h incubation, peptides formed were separated on a Lichrospher 5 μm (4 × 250 mm) column with a linear gradient formed from 0.1% aqueous TFA and acetonitrile up to 40% in 120 min. The isolated peptides were hydrolyzed in 6 M HCl for 60 min at 155°C. The amino acid composition of the generated fragments was determined by reverse phase HPLC after precolumn derivatization with *o*-phthalaldehyde/3-mercaptopropionic acid as described [9]. Identification of the following peptides is: 1, AcSDKP; 2, DKSKLKKTETQ; 3, EKNPLPSKETIEQEKQAQES; 4, ETQEKNPLPSK; 5, DMAEIEKF; 6, AcSDKPDMAEIEKF.

bone marrow has not been described until now. In a first set of experiments, mouse BMC extracts were analysed by HPLC. The presence in the HPLC spectra from mouse BMC extract (sample A, Fig. 2) of a peak identical to Tβ_4 which was shifted after oxidation to the elution time of Tβ_4 sulfoxide, demonstrated the presence of Tβ_4 in BMC.

In a second set of experiments the formation of radiolabelled tetrapeptide by intact or lysed BMC enzymatic systems through processing of a random tritiated [^{3}H]Tβ_4, in which about 10% of the radioactivity is located in the AcSDKP N-terminal fragment, was investigated. With the aim of limiting a possible inhibition of enzymatic degradative activity by AcSDKP already present in mouse BMC, [^{3}H]Tβ_4 was incubated with rabbit BMC preparations, which should be devoid of AcSDKP, as in this species, Tβ_4 is replaced by the corresponding T$\beta_{4\text{-Ala}}$ analogue, i.e. replacement of ^{1}Ser by ^{1}Ala [11]. Incubations using intact BMC were carried out up to 18 h. The evaluation of the radioac-

tivity associated with AcSDKP peptide demonstrated that BMC did contain an enzymatic system which was able to cleave Tβ_4 and generate the tetrapeptide (Table I, Fig. 3).

Several sets of experiments showed that a maximal degradation was observed with intact or lysed cells after a 2 h incubation period.

The stability of [^{3}H]AcSDKP observed under the same conditions of incubation suggests that the small amount of AcSDKP released by enzymatic cleavage of Tβ_4 by BMC lysate cannot be attributed to the further degradation of a neoformed tetrapeptide, and might suggest that the degradation products formed inhibit the enzymatic system implicated in their formation by a feedback control mechanism. This demonstrates that the ^{4}Pro–^{5}Asp peptidic bond of Tβ_4 could be cleaved specifically by enzymatic activities present in BMC and lead to AcSDKP formation.

Previous studies describe proteolytic degradations of Tβ_4 using trypsin and thermolysin [3], but none of those enzymes led to AcSDKP formation. In fact, two types of enzymes were characterized in mammalian tissues: the α-prolyl endopeptidase [13] and the endoproteinase Asp-N [14–15] were possible candidates to carry out the observed cleavage. To determine if those enzymatic systems were effective, processing assays of Tβ_4 were conducted with the respective bacterial enzymes. Whereas α-prolyl-endopeptidase from *Flavobacterium meningosepticum* was unable to carry out Tβ_4 processing, in spite of the fact that we could demonstrate the presence of such activity in BMC extracts (results not shown), endoproteinase Asp-N from *Pseudomonas fragi* cleaved specifically Tβ_4 to liberate AcSDKP (Fig. 4).

To our knowledge, until now the presence of such an endoproteinase Asp-N activity in mammalian system has been described only in monocyte membrane and was associated with the interleukine-1 maturation process [16–17]. The possibility that monocytes are involved in AcSDKP formation is appealing since those cells are known to participate in the regulation of the hematopoietic stem cell proliferation through cytokine secretion. Recently a new inhibitor of the hematopoietic stem cell has been characterized as one of the secreted monokines [18]. This possibility does not exclude however the endoproteinase Asp-N might be present in other cells such as stroma cells, T or B lymphocytes which are known to interfere with the regulation of stem cell proliferation [19]. Tβ_4 has been described as an immunoregulatory molecule. The possibility that a part of those activities is due to a liberation of AcSDKP might be questioned, as this molecule was shown to modulate T cell functions (unpublished results).

The present demonstration that AcSDKP can be generated in BMC from Tβ_4, through a one-step maturation process involving endoproteinase Asp-N enzymatic activity, does not exclude however the possibility that this tetrapeptide might be derived in mammalians through successive proteolytic cleavages, either from Tβ_4 or other SDKP containing proteins.

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