FEBS Letters 464 (1999) 14–20 FEBS 23099

Thymosin β_4 serves as a glutaminyl substrate of transglutaminase. Labeling with fluorescent dansylcadaverine does not abolish interaction with G-actin[1]

Thomas Huff[a,*], Edda Ballweber[b], Andreas Humeny[a], Thomas Bonk[a], Cord-Michael Becker[a], Christian S.G. Müller[a], Hans Georg Mannherz[b], Ewald Hannappel[a]

[a] *Institut für Biochemie, Medizinische Fakultät, Universität Erlangen-Nürnberg, Fahrstr. 17, D-91054 Erlangen, Germany*
[b] *Cytoskeletal Laboratory, Abteilung für Anatomie und Embryologie, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany*

Received 7 November 1999

Edited by Julio Celis

Abstract Thymosin β_4 possesses actin-sequestering activity and, like transglutaminases, is supposed to be involved in cellular events like angiogenesis, blood coagulation, apoptosis and wound healing. Thymosin β_4 serves as a specific glutaminyl substrate for transglutaminase and can be fluorescently labeled with dansylcadaverine. Two (Gln-23 and Gln-36) of the three glutamine residues were mainly involved in the transglutaminase reaction, while the third glutaminyl residue (Gln-39) was derivatized with a low efficiency. Labeled derivatives were able to inhibit polymerization of G-actin and could be cross-linked to G-actin by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. Fluorescently labeled thymosin β_4 may serve as a useful tool for further investigations in cell biology. Thymosin β_4 could provide a specific glutaminyl substrate for transglutaminase in vivo, because of the fast reaction observed in vitro occurring at thymosin β_4 concentrations which are found inside cells. Taking these data together, it is tempting to speculate that thymosin β_4 may serve as a glutaminyl substrate for transglutaminases in vivo and play an important role in transglutaminase-related processes.

Key words: Thymosin β_4; Transglutaminase; Actin; Dansylcadaverine

1. Introduction

Actin is present at high concentrations in virtually every eukaryotic cell. About half of the intracellular actin is stabilized in its monomeric form (G-actin) by interaction with sequestering factors [1]. This monomeric actin can be used for fast generation of new actin filaments after an appropriate intra- or extracellular signal [2]. The β-thymosins are a family of highly conserved polar 5 kDa peptides that are present in many tissues and cells of different vertebrates except human and chicken erythrocytes ([3], unpublished data). The most abundant species of β-thymosins in mammalian tissues is thymosin β_4, which was originally supposed to be a thymic hormone [4,5]. Safer and coworkers demonstrated in 1991 that thymosin β_4 is identical to the actin-sequestering factor Fx [6,7]. Thymosin β_4 is now regarded as the main intracellular G-actin-sequestering peptide in most mammalian cells [8–10] and it forms a 1:1 complex with G-actin, thereby inhibiting salt-induced polymerization to F-actin [11–15].

We had found that thymosin β_4 is present in very high concentrations in blood cells, with the exception of erythrocytes [3]. The concentration of thymosin β_4 in serum is normally low, but increases if the serum is not immediately removed from clotted blood. Cassimeris et al. [16] showed first that thymosin β_4 is the main G-actin sequestering peptide in resting human platelets and later the same group showed that it is involved in cellular events after activation of platelets [17].

In 1995 Grant et al. reported a 5-fold increase of thymosin β_4 mRNA during morphological differentiation of endothelial cells into capillary-like tubes [18]. The peptide itself increases the migration of human umbilical vein endothelial cells 4–6-fold in vitro and in vivo [19]. Frohm and coworkers reported high concentrations of thymosin β_4 in wound and blister fluids [20]. It was also postulated that β-thymosins may play a role in apoptosis [21].

Transglutaminases are Ca^{2+}-dependent enzymes, which catalyze the posttranslational modification of proteins through the exchange of primary amines for ammonia at the carboxamide group of glutamine residues. Peptide-bound lysine residues or naturally occurring polyamines serve quite unspecifically as primary amine substrates [22,23]. In the case of peptide-bound lysine residues covalent isopeptide bonds were formed, which are resistant against proteolysis. Transglutaminases have a broad specificity for primary amine substrates, whereas the number of proteins which serve as glutaminyl substrates is highly restricted. It is now well established that transglutaminases are implicated in a wide range of biological phenomena like blood coagulation (factor XIIIa), wound healing, terminal differentiation, and apoptosis [22,23]. Safer et al. described cross-linking of Lys-38 of thymosin β_4 to Gln-41 of actin by reaction with transglutaminase [24]. However, in this transglutamination thymosin β_4 served as an aminyl substrate.

When we further analyzed the possible role of thymosin β_4 as a substrate of transglutaminases we found that thymosin β_4 was labeled with dansylcadaverine by transglutaminase, hence reacting as a specific glutaminyl substrate. Here, we describe the molecular properties of the products formed. Additionally, we show that labeling of thymosin β_4 does not interfere with G-actin-sequestering activity and, therefore, may serve as a useful tool for further investigations.

*Corresponding author. Fax: (49)-9131-854605.
E-mail: t.huff@biochem.uni-erlangen.de

[1] This work is dedicated to Prof. B.L. Horecker on the occasion of his 85th birthday.

PII: S0014-5793(99)01670-1

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: LiChroprep RP-18 (40–63 μm) and trifluoroacetic acid (TFA, Uvasol) from Merck (Darmstadt, Germany); 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), monodansylcadaverine [*N*-(5-aminopentyl)-5-(dimethylamino)naphthalene-1-sulfonamide] (DNC), and guinea pig liver transglutaminase from Sigma; AsnC proteinase from PanVera Corporation (Madison, WI, USA) and trypsin from Boehringer Mannheim (Mannheim, Germany).

2.2. Proteins and peptides

Actin was prepared from bovine heart or rabbit skeletal muscle by the method of Pardee and Spudich [25] and further purified by gel filtration [26] on a Sephacryl S300 column (Pharmacia) equilibrated with G buffer (2 mM Tris–HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol, 0.05% NaN_3, pH 8.0). G-actin was stored in G buffer at 0°C. Rabbit skeletal muscle actin was pyrenylated following the procedure given in [27]. Thymosin β_4 was isolated from bovine spleen as described [28]. The purity of the preparations was demonstrated by reverse phase HPLC. The concentrations of thymosin β_4 and actin were determined by amino acid analysis after acid hydrolysis (6 M HCl, 155°C, 1 h) and precolumn derivatization with *o*-phthaldialdehyde/3-mercaptopropionic acid [29].

Proteolytic fragments of peptides (thymosin β_4 and modified thymosin β_4) were prepared by the following procedures: AsnC endoproteinase: 1 μg of peptide was incubated with 1 μg protease in 10 μl reaction buffer (50 mM sodium acetate, pH 5.0, 0.2 mM DTT, 0.2 mM EDTA); trypsin: 50 μg peptide TG51 was digested with 2 μg trypsin in 50 μl 1% NH_4HCO_3.

All digestions were incubated for 16 h at room temperature. Thereafter, reactions were stopped by adding an equal volume of 10% TFA. Prior to analysis by MALDI-TOF-MS the samples were concentrated in vacuo.

2.3. HPLC

Chromatographic conditions were controlled by a Merck-Hitachi L-6200 system supplemented with a diode array UV detector (L-7450A, Merck-Hitachi) and with a fluorometer (F-1050, Merck-Hitachi). The diode array detector signal was recorded on a computer using D-7000 HSM software (Merck) and the fluorescence signal on an integrator (D-2500, Merck-Hitachi). For high sensitivity detection of peptides, a postcolumn derivatization system was used [30].

Analytical separations. Flow rate: 0.75 ml/min; buffer: 0.1% TFA; gradient: linear from 0 to 40% acetonitrile in 60 min; column: Beckman ODS Ultrasphere (5 μm, 4.6×250 mm); detection: UV at 205 nm or fluorescence after postcolumn derivatization with fluorescamine.

Preparative separation of the peptides. Flow rate: 0.75 ml/min; buffer: 0.1% trifluoroacetic acid; gradient: linear from 0 to 40% acetonitrile in 120 min; column: Pharmacia SuperPac Pep-S (5 μm, 4×250 mm); detection: UV (215 nm) or dansyl fluorescence (λ_{ex} = 290 nm, λ_{em} = 540 nm).

2.4. Gel electrophoretic procedures

SDS polyacrylamide gel electrophoresis was performed either by the method of Laemmli [31] or using Pharmacia PhastGel Gradient 10–15. Native gel electrophoresis was performed as detailed previously [32].

2.5. Transglutamination of thymosin β_4 with dansylcadaverine

To determine the time course of transglutamination, thymosin β_4 (120 μM) was incubated with dansylcadaverine (5 mM) in 70 μl buffer consisting of 10 mM Tris–HCl, pH 7.4, 15 mM $CaCl_2$, 3 mM DTT. The reaction was started by addition of 0.1 U transglutaminase. Immediately after addition of the enzyme (t = 0) and at indicated times, 10 μl were taken from the mixture, diluted in 490 μl 0.1% TFA to stop the reaction and analyzed by HPLC.

For preparative separation of labeled peptides, 500 μg thymosin β_4 (200 μM) was incubated at room temperature with 500 μg dansylcadaverine (3 mM) and 0.5 U transglutaminase in 500 μl of the buffer described above. After 1 and 2 h, 10 μl was subjected to analysis by HPLC. The reaction was stopped after 4 h by addition of 5 μl TFA. Thereafter, the reaction mixture was subjected to preparative HPLC. Separated peptides were concentrated in vacuo and then characterized by amino acid analysis and MALDI-TOF mass spectrometry.

2.6. Matrix-assisted laser desorption mass spectrometry

Mass determinations were performed with a Biflex™ III MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The instrument is equipped with a nitrogen laser (λ = 337 nm) and a reflectron. Laser-desorbed positive ions were analyzed after being accelerated by 19 kV in the reflection mode. External calibration was performed by use of a standard peptide mixture. Usually, 30 individual spectra were averaged to produce a mass spectrum.

Dried peptide samples were dissolved in 0.1% TFA containing 33% acetonitrile to a final concentration of about 20 ng/μl. Each sample (1 μl) was mixed with 2 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, Germany) in 0.1% TFA, 33% acetonitrile and 1 μl of this mixture was spotted onto a stainless steel target.

2.7. Viscosimetry and EDC cross-linking

Viscosimetric measurements were done with a falling-ball viscometer according to Cooper and Pollard [33], at an angle of 40° relative to horizontal, distance of fall: 45 mm; ball diameter: 0.794 mm. G-actin solution (48 μl; 0.18 mg/ml in G buffer) was incubated with or without thymosin β_4 or dansylcadaverine-labeled peptides for 15 min at room temperature and thereafter 2 μl 50 mM $MgCl_2$ was added. The mixture was filled into a glass capillary (diameter 0.92 mm, 50 μl micropipettes, Brand No. 708733), sealed at one end and incubated for 4 h before measuring time of fall. Thereafter, mixtures were removed by centrifugation from the capillaries and incubated with 5 μl of an EDC solution (36 mg/ml). After 2 h, an additional 5 μl EDC solution was added for another 2 h incubation period. The reaction mixtures were dialyzed overnight in microcollodion bags (Sartorius) against G buffer. Prior to SDS gel electrophoresis, the solutions were concentrated in vacuo.

2.8. Transglutamination of thymosin β_4 in the presence of G-actin

G-actin and thymosin β_4 were incubated with transglutaminase in the presence or absence of dansylcadaverine in a buffer consisting of 10 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$, 5 mM DTE, 0.5 mM ATP, 0.2 mM NaN_3 overnight at room temperature.

3. Results

3.1. Derivatization of thymosin β_4 with dansylcadaverine by transglutaminase

To elucidate whether thymosin β_4 could serve as a gluta-

Table 1
Characterization of isolated peptides by MALDI-TOF-MS

Peptide	*m/z* (observed)	Composition	*m/z* (calculated)	Deviation in ‰
Thymosin β_4	4966.7	[$T\beta_4$-H^+]	4964.4	0.46
TG 47	5285.6	[$T\beta_4$*$(DNC)_1$-H^+]	5282.8	0.53
TG 48	5286.1	[$T\beta_4$*$(DNC)_1$-H^+]	5282.8	0.62
TG 49	5619.3	[$T\beta_4^{(o)}$*$(DNC)_2$-H^+]	5617.2	0.37
TG 51	5601.9	[$T\beta_4$*$(DNC)_2$-H^+]	5601.2	0.12
TG 55	5919.1	[$T\beta_4$*$(DNC)_3$-H^+]	5919.6	0.08

Peptides were isolated by preparative HPLC. Aliquots of the collected fractions were concentrated in vacuo and then applied to MALDI-TOF-MS.

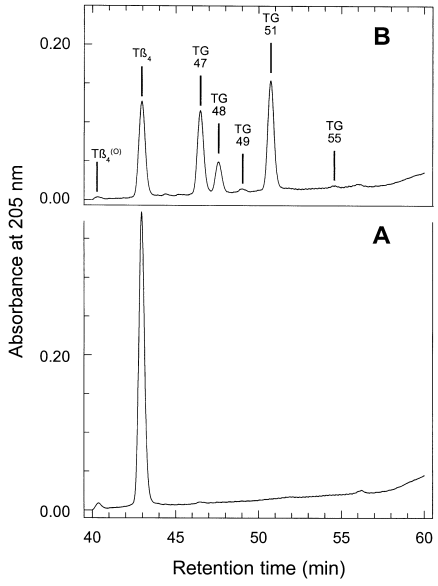

Fig. 1. HPLC analysis of the derivatization of thymosin β_4 with dansylcadaverine by transglutaminase. 10 μl was taken from the reaction mixture after incubation for 0 h (A) or 2 h (B). Retention time (min): thymosin β_4 sulfoxide ($T\beta_4^{(o)}$, 40.38); thymosin β_4 ($T\beta_4$, 42.95); TG47 (46.47); TG48 (47.57); TG49 (49.01); TG51 (50.72); TG55 (54.68).

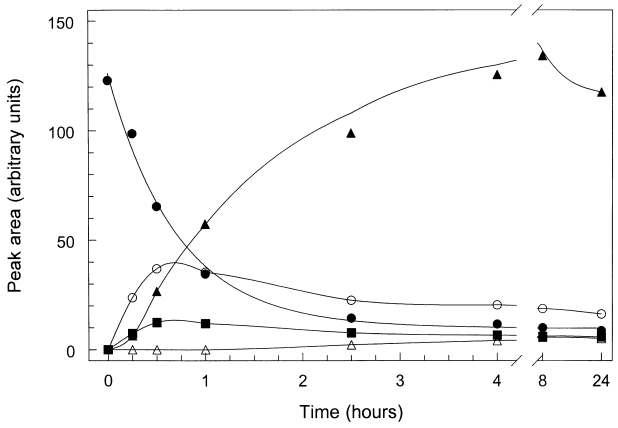

Fig. 2. Time course of the derivatization of thymosin β_4 with dansylcadaverine by transglutaminase. At indicated times 10 μl was taken from the reaction mixture and analyzed by HPLC as described in Section 2. Symbols: ● thymosin β_4; ○ TG47; ■ TG48; ▲ TG51; △ TG55.

minyl substrate for guinea pig transglutaminase we incubated the peptide with dansylcadaverine, a well-known fluorescent aminyl substrate of transglutaminase. HPLC analyses taken directly after addition of the enzyme ($t = 0$) and after 2 h are shown in Fig. 1. At the beginning of the reaction (A), the chromatogram showed a large peak for thymosin β_4 and a very small signal for its sulfoxide ($T\beta_4^{(o)}$). After 2 h (B), the amounts of thymosin β_4 sulfoxide and thymosin β_4 were diminished while five new peaks arose. Because of the presence of several new peaks we were interested to elucidate which and whether more than one of the three possible Gln residues were derivatized. Therefore we measured the kinetics of the derivatization reaction by HPLC analysis as described in Section 2. Fig. 2 shows the time course of the derivatization reaction, where the amount of thymosin β_4 (●) decreased in a logarithmic manner. The products TG47 (○) and TG48 (■) increased during the first 30 min and decreased thereafter. The third product TG49 (not shown in Fig. 2) increased to the same extent as thymosin β_4 sulfoxide decreased (Fig. 1). Product TG51 (▲) first developed nearly parallel to TG47 and TG48, but increased further and constituted the main product. After about 2 h another product (TG55, △) was detected which was only formed to a small extent during the incubation period.

3.2. Isolation and characterization of products formed

For isolation and characterization of the products, we carried out the reaction in a preparative manner as described in Section 2. After preparative HPLC, we isolated six products. Amino acid analysis of the isolated peptides showed identical amino acid compositions for all isolated products (data not shown). The peptides were then characterized by mass spectroscopy. Table 1 summarizes the observed molecular masses, assigned molecular formulas, calculated masses and their deviations. These data showed that there was still unreacted thymosin β_4 ($T\beta_4$), two mono-DNC derivatives ($[T\beta_4{*}(DNC)_1]$; TG47 and TG48), a very low amount of bis-DNC thymosin β_4 sulfoxide ($[T\beta_4^{(o)}{*}(DNC)_2]$; TG49). In addition to a small amount of tris-DNC thymosin β_4 ($[T\beta_4{*}(DNC)_3]$; TG55), a bis-DNC thymosin β_4 ($[T\beta_4{*}(DNC)_2]$; TG51) was identified as the main product.

3.3. Localization of derivatized Gln residues

To further elaborate which of the three possible Gln resi-

Table 2
Characterization of peptide fragments after AsnC digestion by MALDI-TOF-MS

Peptide	*m/z* (observed)	Composition	*m/z* (calculated)	Deviation in ‰
Thymosin β_4	4960.6	$[T\beta_4\text{-}H^+]$	4964.4	0.77
	3109.7	$[T\beta_4^{1-26}\text{-}H^+]$	3111.5	0.58
	1871.7	$[T\beta_4^{27-43}\text{-}H^+]$	1871.9	0.11
TG 47	5285.1	$[T\beta_4{*}(DNC)_1\text{-}H^+]$	5282.8	0.44
	3111.7	$[T\beta_4^{1-26}\text{-}H^+]$	3111.5	0.06
	2189.6	$[T\beta_4^{27-43}{*}(DNC)_1\text{-}H^+]$	2190.3	0.32
TG 48	5283.7	$[T\beta_4{*}(DNC)_1\text{-}H^+]$	5282.8	0.17
	3430.8	$[T\beta_4^{1-26}{*}(DNC)_1\text{-}H^+]$	3429.9	0.26
	1870.8	$[T\beta_4^{27-43}\text{-}H^+]$	1871.9	0.59
TG 51	3426.3	$[T\beta_4^{1-26}{*}(DNC)_1\text{-}H^+]$	3429.9	1.05
	2188.6	$[T\beta_4^{27-43}{*}(DNC)_1\text{-}H^+]$	2190.3	0.78

Peptides (1 μg) were digested with 1 μg of AsnC proteinase for 16 h at room temperature. Digestion was stopped by adding an equal volume of 10% TFA. Prior to analysis by MALDI-TOF-MS, samples were dried in vacuo.

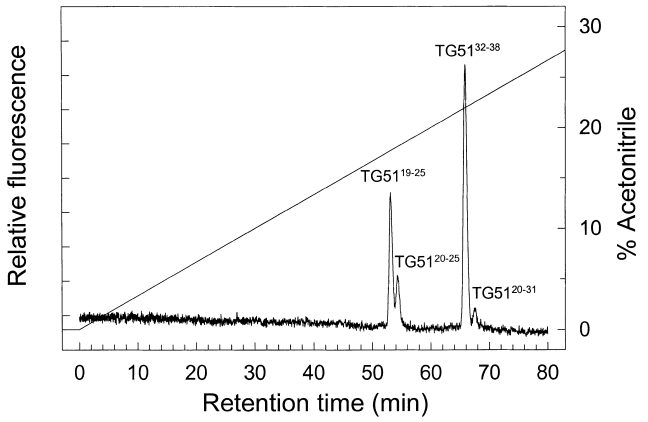

Fig. 3. HPLC analysis of tryptic fragments of TG51 detected by dansyl fluorescence.

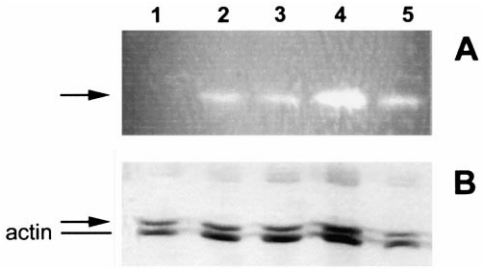

Fig. 4. Cross-linking of G-actin to thymosin β_4 or dansylcadaverine-labeled peptides by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. Detection was performed by fluorescence (A) or Coomassie blue staining (B). Lane 1, G-actin and thymosin β_4; lane 2, G-actin and TG47; lane 3, G-actin and TG48; lane 4, G-actin and TG51; lane 5, G-actin and TG55. The arrow indicates the position of actin cross-linked to thymosin β_4.

dues were modified, we digested thymosin β_4 as well as peptides TG47, TG48, and TG51 with AsnC proteinase and analyzed the samples by MALDI-TOF-MS. Because thymosin β_4 possesses only one Asn residue, at position 26, this digestion produces two fragments, $T\beta_4^{1-26}$ and $T\beta_4^{27-43}$, containing one and two of the Gln residues, respectively. In the case of thymosin β_4, the two expected fragments appeared at molecular masses of 3109.7 (*m/z*) and 1871.7 (*m/z*) (Table 2). In the MALDI mass spectra of digested TG47 the C-terminal fragment was shifted to 2189.6 (*m/z*), whereas in the case of TG48 the signal for the N-terminal fragment appeared at 3430.8 (*m/z*) corresponding to the addition of one DNC (335.5 Da). This indicated that Gln-23 of TG48 and either Gln-36 or 39 of TG47 were derivatized. In the case of digested TG51, both peaks were shifted. Thus, we concluded that Gln-23 and also one of the two Gln residues close to the C-terminus had been derivatized in this peptide. To elucidate which of these two residues was derivatized, we digested TG51 with trypsin. HPLC analysis of TG51 digested with trypsin yielded two major and two minor fluorescent peaks (Fig. 3). The two major peaks were identified by amino acid analysis and MALDI-TOF-MS as DNC-labeled tryptic fragments $TG51^{19-25}$ (*m/z* = 1181.4; expected 1182.4) and $TG51^{32-38}$ (*m/z* = 1194.6; expected 1195.3). The two minor fluorescent fragments represented amino acid residues 20–25 (*m/z* = 1053.3; expected 1054.3) and 20–31 (*m/z* = 1690.0; expected 1691.0) of TG51. No DNC-labeled fragment corresponding to $TG51^{39-43}$ was detected. Therefore, Gln-36 is the second residue which was readily modified by transglutaminase.

3.4. Interaction of modified thymosin β_4 with G-actin

After isolation and characterization of the labeled peptides, we asked how these modifications of thymosin β_4 influenced

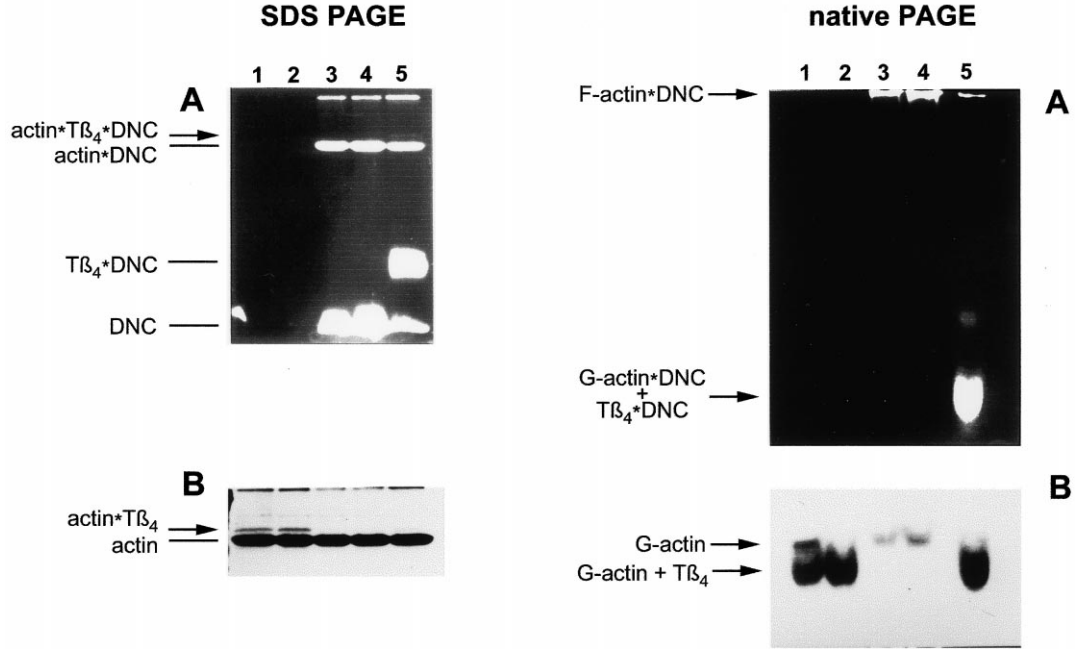

Fig. 5. Transglutamination of thymosin β_4 in the presence of G-actin. Detection was performed using either dansylcadaverine fluorescence (A) or Coomassie blue staining (B). All incubations were done in the presence of 2 μM transglutaminase. Additional conditions were as follows: Lane 1, G-actin (50 μM) and thymosin β_4 (50 μM); lane 2, G-actin (50 μM) and thymosin β_4 (100 μM); lane 3, G-actin (50 μM) and dansylcadaverine (100 μM); lane 4, G-actin (50 μM) and dansylcadaverine (250 μM); lane 5, G-actin (50 μM), thymosin β_4 (100 μM) and dansylcadaverine (100 μM).

Relative position	-6	-5	-4	-3	-2	-1	↓	+1	+2	+3	+4	+5	+6	+7	+8
β-Casein$^{161-175}$	***S***	V	L	***S***	L	***S***	**Gln**	S	**K**	V	L	P	V	P	E
Thymosin $\beta_4{}^{17-31}$	L	K	K	***T***	E	***T***	**Gln**	E	**K**	N	P	L	P	S	K
Thymosin $\beta_4{}^{30-43}$	***S***	K	E	***T***	I	E	**Gln**	E	**K**	Q	A	G	E	S	--
Thymosin $\beta_4{}^{33-43}$	***T***	I	E	Q	E	K	**Gln**	A	G	E	S	--	--	--	--

Fig. 6. Comparison of amino acid sequences of synthetic peptide substrate corresponding to β-casein$^{161-175}$, thymosin β_4^{17-31}, thymosin β_4^{30-43}, and thymosin β_4^{33-43}. Identical amino acids in bold, conservative substitutions in bold italics; –, end of sequence; ↓, Gln residue to be derivatized.

the interaction with G-actin. We first studied the polymerization-inhibiting capacity of the peptides using viscosimetry. The time of fall for polymerized actin was 55 ± 5 s (means ± S.D., $n = 6$). In the presence of an equal amount of thymosin β_4, this value decreased to 7 ± 1.5 s ($n = 6$), which equaled the value for the buffer solution and represented complete inhibition of polymerization. Using equimolar amounts of thymosin β_4 sulfoxide ($T\beta_4^{(O)}$), which is known to inhibit the polymerization only when present in a 20-fold excess to G-actin, but not at equimolar concentrations to actin [13], we determined a value of 40 ± 3 s ($n = 4$). For all labeled peptides, values in the range of 7–10 s were determined at equimolar ratios to G-actin. This indicates that the modification of neither a single nor all glutamyl residues of thymosin β_4 impaired the inhibition of the salt-induced polymerization. To verify these results, we further analyzed the samples by cross-linking with the zero-length cross-linker EDC and subsequent SDS-PAGE analysis using Pharmacia PhastGel Gradient 10–15. Following UV analysis (365 nm) of fluorescent bands (Fig. 4A), gel was stained with Coomassie blue (Fig. 4B). Indeed, the expected fluorescent bands appeared when actin was cross-linked with labeled peptides. Coomassie blue staining indicated that all peptides were cross-linked to G-actin to the same extent. Typically, about 50% of actin was cross-linked by EDC to β-thymosins.

3.5. *Transglutamination of thymosin β_4 in the presence of G-actin*

G-actin has been described as a glutaminyl substrate of transglutaminase [34] and the transglutaminase reaction has been used to cross-link G-actin and thymosin β_4 [24]. Therefore we studied transglutamination of G-actin in the presence of thymosin β_4 and dansylcadaverine (Fig. 5). Detection of dansylcadaverine fluorescence after SDS-PAGE (A) showed that the amount of actin–thymosin β_4 crosslink was very low in the presence of dansylcadaverine and transglutaminase (lane 5), while there were large amounts of dansylcadaverine-labeled thymosin β_4 (lane 5) and actin (lanes 3–5). Subsequent Coomassie blue staining (B) demonstrated that actin and thymosin β_4 were inefficiently cross-linked by transglutaminase (lanes 1 and 2). The amount of cross-link further decreased in the presence of dansylcadaverine competing for Lys residues of either G-actin or thymosin β_4 (100 μM, lane 5). In the absence of thymosin β_4, dansylcadaverine-labeled actin polymerized to F-actin as revealed by fluorescence detection after native PAGE (Fig. 5A, lanes 3 and 4). This was caused by the calcium concentration needed for activation of transglutaminase (1 mM). In contrast, actin polymerization was drastically inhibited in the presence of 100 μM thymosin β_4, due to complex formation with thymosin β_4 (lane 5). Coomassie blue staining of native gels (Fig. 5B) showed that both G-actin and its complex with thymosin β_4 were present regardless of whether dansylcadaverine was present or not (lanes 1, 2 and 5).

4. Discussion

In this work, we demonstrated that thymosin β_4 could serve as a glutaminyl substrate for guinea pig liver transglutaminase at concentrations that are present inside cells. Using proteolytic fragmentation and MALDI-TOF mass spectroscopy, we elucidated that Gln residues 23 and 36 are easily available for transglutamination, whereas Gln-39 reacts very slowly and to a very low extent. Moreover, with this reaction we were able

		5	10	15	20	25	30	35	40
$T\beta_4$	ac-SDKP	DMAEI	EKFDK	SK**LKK**	**TETQE**	**KNPLP**	**SK**ETI	EQEKQ	AGES
$T\beta_4{}^{Ala}$	ac-ADKP	DMAEI	EKFDK	SK**LKK**	**TETQE**	**KNPLP**	**SK**ETI	EQEKQ	AGES
$T\beta_9$	ac-ADKP	DLGEI	NSFDK	AK**LKK**	**TETQE**	**KNTLP**	***TK***ETI	EQEKQ	AK
$T\beta_9{}^{Met}$	ac-ADKP	DMGEI	NSFDK	AK**LKK**	**TETQE**	**KNTLP**	***TK***ETI	EQEKQ	AK
$T\beta_{10}$	ac-ADKP	DMGEI	ASFDK	AK**LKK**	**TETQE**	**KNTLP**	***TK***ETI	EQEKR	SEIS
$T\beta_{15}$	ac-SDKP	DLSEV	ETFDK	SK**LKK**	**TNTEE**	**KNTLP**	**SK**ETI	QQEKE	YNQRS

Fig. 7. Amino acid sequences of mammalian β-thymosins. Thymosin β_4 ($T\beta_4$ [4,8]), thymosin β_4^{Ala} ($T\beta_4^{Ala}$ [42]), thymosin β_9 ($T\beta_9$ [43]), thymosin β_9^{Met} ($T\beta_9^{Met}$ [28]), thymosin β_{10} ($T\beta_{10}$ [44]), thymosin β_{15} ($T\beta_{15}$ [37], deduced from cDNA sequence). Homologous residues are in bold letters and conservative substitutions around Gln-23 are in bold italics. Substitution of Gln-23 to Glu-23 in thymosin β_{15} is marked by double underlining.

to label thymosin β_4 with dansylcadaverine as a fluorescent marker without abolishing its interaction with G-actin.

The aim of this study was to elucidate whether thymosin β_4 could serve as a specific glutaminyl substrate for the transglutaminase reaction. In recent years it has been shown that thymosin β_4 may be involved in cellular events like angiogenesis [18,19], wound healing [20], apoptosis [21] and blood coagulation [3,16,17]. Because transglutaminases also participate in all these cellular reactions we investigated whether thymosin β_4 and transglutaminase may interact. Our results clearly show that thymosin β_4 could serve as a glutaminyl substrate for guinea pig transglutaminase at concentrations which are present inside cells (Fig. 1). Consequently it is likely that thymosin β_4, which is present in human platelets in a concentration of about 500 μM [3,35], serves as a glutaminyl substrate for factor XIIIa.

Our data also verify that not all Gln residues of thymosin β_4 are readily accessible for the transglutaminase reaction. Gorman and Folk have studied the glutaminyl substrate specificity of factor XIIIa and guinea pig liver transglutaminase [36]. They performed single and multiple amino acid substitutions of a synthetic peptide substrate corresponding to amino acid residues 161–175 of β-casein, a well-known substrate for factor XIIIa (Fig. 6). It was found that substitution of Lys by a Gly residue at position +2 results in a distinct decrease of its glutaminyl donor property with transglutaminase, which is even more pronounced with factor XIIIa. In the case of Gln-23 and Gln-36 of thymosin β_4 a Lys residue follows at the +2 position, while in the case of Gln-39 a Gly residue is at this position. This may partly explain why Gln-39 of thymosin β_4 is only labeled to a very low extent. Activity of both enzymes showed a distinct or drastic decrease by truncation of four C- or N-terminal residues. This may be an additional reason for the low derivatization of Gln-39. The environment of Gln-23 is highly conserved throughout all mammalian β-thymosins except thymosin β_{15} (Fig. 7), which has been described to be highly expressed in human cancer cells with high metastatic activity [37].

Safer et al. [6] proposed that the sequence ^{17}LKKTETQEK^{25} of thymosin β_4 may be involved in actin sequestering because of its high homology with the well-known actin-binding sequence of actobindin [38]. In accordance with their proposal, we have previously demonstrated that truncation of up to 13 N-terminal amino acid residues does not abolish chemical cross-linking to G-actin, while truncation of the first 23 amino acid residues completely destroys interaction [13]. Surprisingly, as demonstrated here, derivatization of Gln-23 with dansylcadaverine did not abolish G-actin binding, suggesting that this particular position may not be essential for actin binding. Indeed, Gln-23 is not conserved in the corresponding sequences of actobindin [38].

There are several reports dealing with G-actin as a glutaminyl substrate for the transglutaminase reaction. Takashi has shown that dansylcadaverine can be cross-linked to Gln-41 of G-actin and that this leads to a decrease of the critical concentration and faster polymerization of labeled G-actin [34]. It has also been reported that actin from human blood platelets and rabbit skeletal muscle actin serves as a substrate for factor XIIIa [39]. Moreover, the cross-linked matrix from platelets formed by the transglutaminase reaction contains actin [40]. Previously, Nemes et al. [41] have shown that actin seems to be the main endogenous substrate for tissue transglutaminase in HL60 and U937 cells undergoing apoptosis. For detection of substrate proteins, they used a synthetic hapten-labeled lysine derivative, which is able to cross the cell membrane. After inducing apoptosis, they detected actin as the major protein transglutaminated with hapten-labeled lysine by Western blot analysis. Our results prove that, at least under the conditions used in our assay, thymosin β_4 serves equally well as a substrate for guinea pig transglutaminase compared to actin (Fig. 5). Because of its low molecular weight and high solubility thymosin β_4 may have escaped their SDS-PAGE analysis. Therefore, it will be interesting to look for transglutamination of thymosin β_4 during apoptosis using HPLC techniques.

Taking these data together, it is tempting to speculate that thymosin β_4 may serve as a glutaminyl substrate for transglutaminases in vivo and thus may possibly play an important role in transglutaminase-related processes.

Acknowledgements: We express our appreciation to Doris Jaegers for her excellent technical assistance.

References

[1] Pollard, T.D. and Cooper, J.A. (1986) Annu. Rev. Biochem. 55, 987–1035.
[2] Carlier, M.F. and Pantaloni, D. (1994) Semin. Cell Biol. 5, 183–191.
[3] Hannappel, E. and van Kampen, M. (1987) J. Chromatogr. 397, 279–285.
[4] Low, T.L.K., Hu, S.-K. and Goldstein, A.L. (1981) Proc. Natl. Acad. Sci. USA 78, 1162–1166.
[5] Low, T.L.K. and Goldstein, A.L. (1982) J. Biol. Chem. 257, 1000–1006.
[6] Safer, D., Golla, R. and Nachmias, V.T. (1990) Proc. Natl. Acad. Sci. USA 87, 2536–2540.
[7] Safer, D., Elzinga, M. and Nachmias, V.T. (1991) J. Biol. Chem. 266, 4029–4032.
[8] Hannappel, E., Xu, G.-J., Morgan, J., Hempstead, J. and Horecker, B.L. (1982) Proc. Natl. Acad. Sci. USA 79, 2172–2175.
[9] Xu, G.J., Hannappel, E., Morgan, J., Hempstead, J. and Horecker, B.L. (1982) Proc. Natl. Acad. Sci. USA 79, 4006–4009.
[10] Hannappel, E. and Leibold, W. (1985) Arch. Biochem. Biophys. 240, 236–241.
[11] Safer, D. and Nachmias, V.T. (1994) BioEssays 16, 473–479.
[12] Hannappel, E. and Wartenberg, F. (1993) Biol. Chem. Hoppe-Seyler 374, 117–122.
[13] Huff, T., Zerzawy, D. and Hannappel, E. (1995) Eur. J. Biochem. 230, 650–657.
[14] Yu, F.-X., Lin, S.-C., Morrison-Bogorad, M., Atkinson, M.A.L. and Yin, H.L. (1993) J. Biol. Chem. 268, 502–509.
[15] Heintz, D., Reichert, A., Mihelic, M., Voelter, W. and Faulstich, H. (1993) FEBS Lett. 329, 9–12.
[16] Cassimeris, L., Safer, D., Nachmias, V.T. and Zigmond, S.H. (1992) J. Cell Biol. 119, 1261–1270.
[17] Nachmias, V.T., Cassimeris, L., Golla, R. and Safer, D. (1993) Eur. J. Cell Biol. 61, 314–320.
[18] Grant, D.S., Kinsella, J.L., Kibbey, M.C., Laflamme, S., Burbelo, P.D., Goldstein, A.L. and Kleinman, H.K. (1995) J. Cell Sci. 108, 3685–3694.
[19] Malinda, K.M., Goldstein, A.L. and Kleinman, H.K. (1997) FASEB J. 11, 474–481.
[20] Frohm, M., Gunne, H., Bergman, A.C., Agerberth, B., Bergman, T., Boman, A., Liden, S., Jornvall, H. and Boman, H.G. (1996) Eur. J. Biochem. 237, 86–92.
[21] Hall, A.K. (1994) Med. Hypotheses 43, 125–131.
[22] Folk, J.E. and Finlayson, J.S. (1977) Adv. Protein Chem. 31, 1–133.
[23] Greenberg, C.S., Birckbichler, P.J. and Rice, R.H. (1991) FASEB J. 5, 3071–3077.
[24] Safer, D., Sosnick, T.R. and Elzinga, M. (1997) Biochemistry 36, 5806–5816.

[25] Pardee, J.D. and Spudich, J.A. (1982) Methods Enzymol. 85, 164–181.
[26] MacLean-Fletcher, S. and Pollard, T.D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27.
[27] Kouyama, T. and Mihashi, K. (1981) Eur. J. Biochem. 114, 33–38.
[28] Hannappel, E., Wartenberg, F. and Bustelo, X.R. (1989) Arch. Biochem. Biophys. 273, 396–402.
[29] Hannappel, E., Kalbacher, H. and Voelter, W. (1988) Arch. Biochem. Biophys. 260, 546–551.
[30] Huff, T., Müller, C.S.G. and Hannappel, E. (1997) Anal. Chim. Acta 352, 239–248.
[31] Laemmli, U.K. (1970) Nature 227, 680–685.
[32] Ballweber, E., Hannappel, E., Huff, T. and Mannherz, H.G. (1997) Biochem. J. 327, 787–793.
[33] Cooper, J.A. and Pollard, T.D. (1982) Methods Enzymol. 85, 182–210.
[34] Takashi, R. (1988) Biochemistry 27, 938–943.
[35] Weber, A., Nachmias, V.T., Pring, M., Pennise, C.R. and Safer, D. (1992) Biochemistry 31, 6179–6185.
[36] Gorman, J.J. and Folk, J.E. (1981) J. Biol. Chem. 256, 2712–2715.
[37] Bao, L.R., Loda, M., Janmey, P.A., Stewart, R., Anandapte, B. and Zetter, B.R. (1996) Nature Med. 2, 1322–1328.
[38] Bubb, M.R., Vancompernolle, K., van Damme, J., Vandekerckhove, J. and Korn, E.D. (1991) Biophys. J. 59, 55a.
[39] Cohen, I., Blankenberg, T.A., Borden, D., Kahn, D.R. and Veis, A. (1980) Biochim. Biophys. Acta 628, 365–375.
[40] Harsfalvi, J., Fesus, L., Tarcsa, E., Laczko, J. and Loewy, A.G. (1991) Biochim. Biophys. Acta 1073, 268–274.
[41] Nemes, Z., Adany, R., Balazs, M., Boross, P. and Fesus, L. (1997) J. Biol. Chem. 272, 20577–20583.
[42] Ruggieri, S., Erickson-Viitanen, S. and Horecker, B.L. (1983) Arch. Biochem. Biophys. 226, 388–392.
[43] Hannappel, E., Davoust, S. and Horecker, B.L. (1982) Proc. Natl. Acad. Sci. USA 79, 1708–1711.
[44] Erickson-Viitanen, S., Ruggieri, S., Natalini, P. and Horecker, B.L. (1983) Arch. Biochem. Biophys. 225, 407–413.