Nuclear localisation of the G-actin sequestering peptide thymosin β_4

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Summary

Thymosin β_4 is regarded as the main G-actin sequestering peptide in the cytoplasm of mammalian cells. It is also thought to be involved in cellular events like cancerogenesis, apoptosis, angiogenesis, blood coagulation and wound healing. Thymosin β_4 has been previously reported to localise intracellularly to the cytoplasm as detected by immunofluorescence. It can be selectively labelled at two of its glutamine-residues with fluorescent Oregon Green cadaverine using transglutaminase; however, this labelling does not interfere with its interaction with G-actin. Here we show that after microinjection into intact cells, fluorescently labelled thymosin β_4 has a diffuse cytoplasmic and a pronounced nuclear staining. Enzymatic cleavage of fluorescently labelled thymosin β_4 with AsnC-endoproteinase yielded two mono-labelled fragments of the peptide. After microinjection of these fragments, only the larger N-terminal fragment, containing the proposed actin-binding sequence exhibited nuclear localisation, whereas the smaller C-terminal fragment remained confined to the cytoplasm. We further showed that in digitonin permeabilised and extracted cells, fluorescent thymosin β_4 was solely localised within the cytoplasm, whereas it was found concentrated within the cell nuclei after an additional Triton X100 extraction. Therefore, we conclude that thymosin β_4 is specifically translocated into the cell nucleus by an active transport mechanism, requiring an unidentified soluble cytoplasmic factor. Our data furthermore suggest that this peptide may also serve as a G-actin sequestering peptide in the nucleus, although additional nuclear functions cannot be excluded.

Key words: β-thymosins, Fluorescent labelling, Microinjection, Nuclear actin

Introduction

Actin is present at high concentrations in virtually every eukaryotic cell. About half of the intracellular actin is stabilised in its monomeric form (G-actin) by interaction with sequestering factors (Pollard and Cooper, 1986). This monomeric actin can be used for the fast generation of new actin filaments after an appropriate intra- or extracellular signal (Carlier and Pantaloni, 1994). The β-thymosins constitute a family of highly conserved water soluble 5-kDa polypeptides. Thymosin β_4 is the most abundant member of this family and is regarded as the main G-actin sequestering peptide in the cytoplasm of mammalian cells (Low et al., 1981; Hannappel et al., 1982; Hannappel and Leibold, 1985; Safer et al., 1991). This 43 amino acid oligopeptide forms a 1:1 complex with G-actin and thereby inhibits salt-induced polymerisation to F-actin (Hannappel and Wartenberg, 1993; Heintz et al., 1993; Yu et al., 1993; Huff et al., 1995; Huff et al., 2001). Additional members of the β-thymosin family have been identified and these peptides exhibit similar properties to thymosin β_4. Thymosin β_4 and other β-thymosins appear to be involved in a number of different processes like cancerogenesis (Hall, 1991; Bao et al., 1996; Verghese-Nikolakaki et al., 1996; Califano et al., 1998; Kobayashi et al., 2002; Otto et al., 2002) and apoptosis (Iguchi et al., 1999; Niu and Nachmias, 2000; Müller et al., 2003). In the extracellular space, thymosin β_4 participates in several physiological processes, e.g. angiogenesis (Grant et al., 1995; Malinda et al., 1997; Philp et al., 2003b), wound healing (Malinda et al., 1999; Sosne et al., 2001; Sosne et al., 2002; Philp et al., 2003a) and regulation of inflammation (Sosne et al., 2001; Sosne et al., 2002). We have recently shown that it also serves as a specific glutaminyl substrate of transglutaminases (Huff et al., 1999) which crosslink thymosin β_4 released from stimulated human platelets to fibrin and collagen (Huff et al., 2002).

Lately, there has been increasing evidence for the presence of cytoskeletal proteins in the nucleus, such as actin itself (Scheer et al., 1984; Gonsior et al., 1999), actin-related proteins (Arps) (Cairns et al., 1998; Harata et al., 1999; Harata et al., 2000) and a number of different actin binding proteins (Nowak et al., 1997; Giesemann et al., 1999; Pestic-Dragovich et al., 2000; Skare et al., 2003). Although the functions of these proteins in the nucleus are still under investigation, there is evidence that they are involved in activities ranging from nuclear assembly and shape changes to DNA replication and transcription (Rando et al., 2000; Olave et al., 2002; Pederson and Aebi, 2002; Shumaker et al., 2003). The intracellular localisation of thymosin β_4 has never been studied in detail. One study using immunofluorescence described that its

intracellular localisation in macrophages was most intense in the centre of the cell but was not nuclear (Yu et al., 1994). In another study, [^{125}I]-labelled thymosin β_4 was injected into the cytoplasm of *Xenopus laevis* oocytes and the nuclear and cytoplasmic radioactivity was monitored. In these cells thymosin β_4 was distributed roughly equally between cytoplasm and nucleus (Watts et al., 1990). Recently, McCormack and colleagues (McCormack et al., 1999) studied the influence of polyamine depletion on the relationship of F-actin to G-actin and thymosin β_4 in migrating IEC-6 cells. In untreated cells, they found only cytoplasmic staining for thymosin β_4, whereas they detected nuclear staining after polyamine depletion and speculated that the translocation of thymosin β_4 into the nucleus was a result of this particular treatment (McCormack et al., 1999). In the course of our studies on the possible role of thymosin β_4 in carcinogenesis, we studied the intracellular localisation of this peptide using a newly generated monospecific antibody against thymosin β_4. Using the human mammary carcinoma MCF-7 cell line, we found variable cytoplasmic staining but also additional nuclear staining.

As the previously published discrepancies on the intracellular distribution of thymosin β_4 and our own findings may have been caused by variations in the preservation of the original localisation of the highly soluble and diffusible thymosin β_4, we decided to re-investigate its intracellular localisation by microinjecting fluorescently labelled thymosin β_4 into cells of a number of different lines. Thymosin β_4 can be labelled at two of its three glutamine-residues by the enzymatic reaction of transglutaminase without influencing its G-actin sequestering activity (Huff et al., 1999). Here this technique was used to label thymosin β_4 with Oregon Green cadaverine as a fluorescent marker. Fluorescence microscopic inspection after microinjection of the labelled peptide into cells of a number of different lines revealed that a considerable amount of thymosin β_4 was located within their nuclei. The translocation of thymosin β_4 into the nucleus is not achieved by simple diffusion, as the labelled peptide could not be detected within nuclei when the cells were previously treated with digitonin under conditions that extract the soluble components of the cytoplasm by permeabilisation of the plasma membrane while leaving the nuclear envelope intact. Nuclear localisation was observed only after subsequent treatment and permeabilisation of the nuclear membranes with Triton X100. These data are further supported by results showing that after enzymatic cleavage of bis-labelled thymosin β_4 only the larger N-terminal fragment ($T\beta_4^{1-26}$), containing the proposed actin-binding site, was translocated to the nucleus. In contrast, the smaller C-terminal fragment ($T\beta_4^{27-43}$) and fluorescently labelled thymosin β_4 chemically crosslinked to ADP-ribosylated actin were retained in the cytoplasm.

Materials and Methods

Materials

Reagents were obtained from the following sources: LiChroprep RP18 (40-63 μm) and trifluoroacetic acid (Uvasol) from Merck (Darmstadt, Germany); guinea pig transglutaminase and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) from Sigma (Munich, Germany); AsnC-Proteinase from PanVera Corporation (Madison, WI); TRITC-phalloidin, Oregon Green-labelled deoxyribonuclease I and Oregon Green cadaverine were from Molecular Probes (Eugene, OR).

Protein purification

Actin was prepared from rabbit skeletal or bovine heart muscle by the method of Pardee and Spudich (Pardee and Spudich, 1982) and stored as G-actin in G-buffer (2 mM Tris, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM mercaptoethanol, 0.05% NaN_3, pH 8.0) at 0°C. Thymosin β_4 was isolated from pig spleen as described (Hannappel et al., 1989). The purity of the preparation 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 hour) and pre-column derivatisation with o-phthaldialdehyde/3-mercaptopropionic acid (Hannappel et al., 1988).

Fluorescently labelled thymosin β_4 was prepared by incubation of 240 μg thymosin β_4 (200 μM) with 120 μg Oregon Green cadaverine (OGC) (1 mM) and 0.2 U guinea pig transglutaminase at room temperature in 240 μl buffer consisting of 10 mM Tris-HCl, pH 7.4, 15 mM $CaCl_2$, 3 mM DTT. After 1 and 2 hours, 5 μl of the reaction mixture were subjected to HPLC analysis. The reaction was stopped after 4 hours by addition of 5 μl trifluoroacetic acid (TFA). Then the reaction mixture was subjected to preparative HPLC. Separated peptides were concentrated in vacuo and then characterised by amino acid analysis and MALDI-TOF mass spectrometry.

Proteolytic fragments of OGC-labelled thymosin β_4 were prepared by the following procedure: 50 μg of peptide was incubated with 20 μU AsnC-endoproteinase in 100 μl reaction buffer (50 mM sodium acetate, pH 5.0, 0.2 mM DTT, 0.2 mM EDTA) for 16 hours at room temperature. Then the reaction was stopped by adding 5 μl 10% TFA and products were separated by preparative HPLC. Prior to analysis by MALDI-TOF-MS the samples were concentrated in vacuo.

In order to avoid intracellular dissociation of microinjected actin-thymosin β_4 complex and/or its polymerisation, we chemically crosslinked thymosin β_4 to ADP-ribosylated actin which is known to be polymerisation incompetent on its own (Schering et al., 1988). Therefore, fluorescently labelled thymosin β_4 was crosslinked to ADP-ribosylated actin using EDC as described previously (Ballweber et al., 2002). ADP-ribosylated actin was generated by treatment of rabbit skeletal muscle actin with iota toxin and nicotinamide-adenosine dinucleotide (NAD) as detailed (Ballweber et al., 2001).

HPLC

Chromatographic conditions were controlled by a Merck-Hitachi L-6200 system supplemented with a diode-array UV detector (L-7450A, Merck-Hitachi), a reaction pump for post-column derivatisation (655A-13, 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). The flow rate was 0.75 ml / minute in 0.1% TFA (trifluoroacetic acid) with a 0-40% acetonitrile gradient over 60 minutes in a Beckman ODS Ultrasphere (5 μm, 4.6×250 mm) column. UV detection was at 205 nm and fluorescence was detected after post-column derivatisation with fluorescamine (Huff et al., 1997).

Matrix-assisted laser desorption mass spectrometry

Mass determinations were performed with a Biflex™ III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The instrument is equipped with a nitrogen laser (λ=337 nm) and a reflectron. Laser-desorbed positive ions were analysed after being accelerated by 19 kV in the reflection mode. External calibration was performed by use of a standard peptide mixture. Thirty 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-hydroxy-cinnamic acid (Sigma, Germany) in 0.1% TFA, 33% acetonitrile and 1 μl of this mixture was spotted onto a stainless steel target.

Determination of dissociation constants

Actin:thymosin β_4 complex was generated by mixing equimolar concentrations of native or labelled thymosin β_4 with G-actin. These mixtures were used for a comparative determination of dissociation constants of G-actin in complex with either thymosin β_4 or its fluorescently labelled derivatives by equilibrium centrifugation as described (Huff et al., 1995).

Viscometry

Viscometric measurements were done with a falling-ball viscometer according to Cooper and Pollard (Cooper and Pollard, 1982) at an angle of 40° relative to the horizontal over a distance of 45 mm with a 0.794 mm diameter ball. Forty-eight microliters G-actin solution (0.18 mg/ml in G-buffer) was incubated with or without thymosin β_4 or Oregon Green cadaverine-labelled peptides for 15 minutes at room temperature and then 2 μl 50 mM $MgCl_2$ were added. The mixture was filled into a glass capillary (diameter 0.92 mm, 50 μl micro pipettes), sealed at one end and incubated for 4 hours before measuring.

Generation of polyclonal antibody

A synthetic decapeptide representing the nine C-terminal amino acids of thymosin β_4 with an additional cysteine residue at the N-terminus was conjugated to keyhole limpet hemocyanin (KLH, Sigma, Germany). New Zealand White rabbits were immunised with the KLH conjugate, with an amount corresponding to about 63 μg of the synthetic peptide emulsified with complete Freund's adjuvant (Sigma, Munich, Germany). Following a second immunisation, serum was collected and the anti-thymosin β_4 antibody was partially purified from the serum by precipitation with 50% ammonium sulfate. The precipitate was dissolved in 5 mM phosphate buffer, pH 6.5, dialyzed against PBS and adsorbed with a 1% suspension of acetone powder from bovine heart in order to remove antibodies reacting non-specifically with cytoskeletal components. The antibody was affinity purified by passing it over a column coupled with immunogenic decapeptide. The resulting antiserum showed no cross-reactivity with other β-thymosins, actin, or any other cellular proteins of molecular weights in the range from 10-50 kDa as judged by western blot analysis and ELISA.

Cell culture

Kidney cells from African green monkey (Vero cells) and the human mammary cancer cell line MCF-7 were maintained in Dulbecco's MEM/F12 (Gibco, UK) supplemented with 10% (v/v) foetal calf serum (Gibco). The rat fibroblastic NRK, the human cervical cancer HeLa and epidermoid cancer A431 cell lines were maintained in DMEM supplemented with 10% FCS-gold (Invitrogen, Karlsruhe, Germany).

Microinjection experiments

Microinjection was performed with an ECET cell injection system (Eppendorf, Hamburg, Germany) consisting of the micromanipulator 5170 and the microinjector 5242 adapted to an Axiovert 100 inverted microscope (Zeiss, Göttingen, Germany). Microinjections were visually controlled by a CCD camera on a TV monitor (SSM 121CE, Sony, Tokyo, Japan). Fluorescent thymosin β_4 and crosslinked ADP-ribosylated actin:thymosin β_4 complex were injected into the cytoplasm at a concentration of 32 μM and 8.27 μM respectively, in 135 mM KCl, 5 mM Na_2HPO_4, pH 7.2. The injection pressure was between 65 and 80 hPa (1 hPa=0.1 kPa) and the injection time between 0.5 and 0.7 seconds.

In vitro nuclear translocation experiments

HeLa cells were used for in vitro nuclear translocation experiments, as most previous nuclear translocation experiments have been performed with this cell line. HeLa cells were grown on coverslips until confluence was almost reached. Subsequently the cells were treated as described (Adam et al., 1990) with slight modifications (Jäkel and Görlich, 1998), i.e. they were washed three times with ice-cold PBS, placed on ice, and treated for 12 minutes with permeabilisation buffer (0.11 M potassium acetate, 5 mM magnesium acetate, 0.25 M sucrose, 0.5 mM EGTA and 20 mM HEPES, pH 7.5) supplemented with 40 μg/ml digitonin. Then the cells were washed three times with ice-cold permeabilisation buffer for 2, 5 and 10 minutes. In order to permeabilise the nuclear envelope, the digitonin extracted cells were incubated with 0.2% Triton X100 for 10 minutes and subsequently washed three times with permeabilisation buffer. Then the cells were incubated for 2 hours at room temperature with 3 μM fluorescent thymosin β_4 in import buffer (120 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM EGTA, 0.25 mM sucrose and 20 mM $KHPO_4$, pH 7.2).

Fluorescence microscopy

For immunofluorescence staining of MCF-7 cells with anti-thymosin β_4 antibody cells were fixed with 1.2% paraformaldehyde, permeabilised with 0.2% Triton X100, washed in PBS and stained using anti-thymosin β_4 antibody. Cell nuclei were counterstained by treating fixed cells with 1 μg/ml Hoechst 33258. Oregon Green labelled DNase I was employed to specifically stain monomeric actin. The microfilament system was visualised by staining paraformaldehyde fixed cells with TRITC-phalloidin as described (Paddenberg et al., 2001). Fluorescence microscopy and visualisation of microinjected fluorescent thymosin β_4 was achieved either using standard or confocal microscopy with a Zeiss Axiophot equipped with epifluorescence optics, or with a Zeiss LSM510 confocal microscope (Zeiss, Göttingen, Germany).

Results

Immunofluorescent staining for thymosin β_4 in MCF-7 cells reveals nuclear localisation

In the course of our studies on the possible role of thymosin β_4 in carcinogenesis we studied the intracellular localisation of this peptide in MCF-7 cells using an anti-thymosin β_4 antibody in indirect immunofluorescence. In addition to the expected cytoplasmic localisation we detected a distinct staining of a number of nuclei (Fig. 1A) as verified by their simultaneous staining with the chromatin specific dye Hoechst 33258 (Fig. 1C). As the nuclear staining with anti-thymosin β_4 varied, we also tested the possibility that there might have been an unequal distribution of monomeric actin in these cells. Therefore, we counterstained the cells with Oregon Green-labelled deoxyribonuclease I (DNase I), which is known to bind with high affinity and specificity to monomeric actin (Mannherz et al., 1980). As shown in Fig. 1B almost all nuclei were stained by the labelled DNase I indicating the presence of considerable amounts of G-actin in the nuclei of these cells. To further confirm the unexpected distribution of thymosin β_4 we analysed its distribution with an alternative but more direct method. Therefore, microinjection of fluorescently labelled thymosin β_4 was attempted.

Table 1. Characterisation of fluorescently labelled peptides and fragments thereof by MALDI-TOF mass spectrometry

Peptide	m/z (observed)	Composition	m/z (calculated)	Deviation (‰)
Thymosin β_4	4963.0	$[T\beta_4\text{-}H^+]$	4964.4	0.28
Labelled peptide 1	5445.4	$[T\beta_4(OGC)_1\text{-}H^+]$	5443.9	0.28
Labelled peptide 2	5924.4	$[T\beta_4(OGC)_2\text{-}H^+]$	5923.4	0.17
Fragment 1	2350.2	$[T\beta_4^{27\text{-}43}(OGC)_1\text{-}H^+]$	2350.4	0.09
Fragment 2	3591.4	$[T\beta_4^{1\text{-}26}(OGC)_1\text{-}H^+]$	3590.1	0.36

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

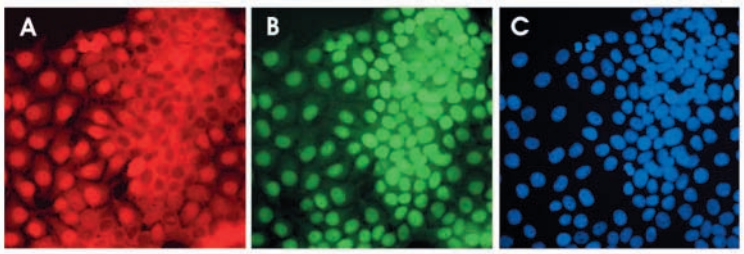

Fig. 1. Intracellular localisation of thymosin β_4 in MCF-7 cells detected by immunofluorescence. (A) MCF-7 cells grown to near confluence were stained with the affinity-purified anti-thymosin β_4 antibody. The cells were counterstained with Oregon Green DNase I (B) in order to label specifically monomeric actin and with the chromatin-specific dye Hoechst 33258 (C). Magnification, ×400.

Fluorescent labelling of thymosin β_4 does not influence its G-actin sequestering activity

Oregon Green cadaverine (OGC)-labelled thymosin β_4 was prepared as described in the experimental section. After preparative HPLC, we isolated the two main products showing Oregon Green fluorescence. Amino acid analysis of the isolated peptides showed identical amino acid compositions for both isolated products (data not shown). The peptides were then characterised by mass spectroscopy. Table 1 summarises the observed molecular masses, assigned molecular formula, calculated masses and its deviation. These data showed, that one of the labelled products was a mono-OGC $[T\beta_4(OGC)_1]$ and the other was a bis-OGC thymosin β_4 $[T\beta_4(OGC)_2]$ derivative. Further analysis using enzymatic cleavage with AsnC-endoproteinase or trypsin and subsequent mass spectroscopy revealed that in the mono-labelled derivative Gln-36 and in the bis-labelled derivative Gln-23 and Gln-36 had been derivatised (data not shown). For microinjection studies only the bis-labelled peptide was used.

To ensure that this labelling does not influence the G-actin sequestering activity the dissociation constant for the complex of bis-labelled thymosin β_4 and bovine cardiac G-actin as well as its capacity to inhibit the salt-induced actin polymerisation were determined. Using equilibrium centrifugation, we found that the dissociation constant for the complex of bis-labelled thymosin β_4 with G-actin (0.47±0.1 μM) did not differ significantly from that determined for unlabelled thymosin β_4 (0.59±0.08 μM). Inhibition of salt-induced actin polymerisation was tested using falling ball viscometry and showed inhibition of actin polymerisation by the labelled thymosin β_4 at equimolar concentrations (Table 2).

Microinjection of labelled thymosin β_4 into different cell lines reveals translocation into the nucleus

To assay the intracellular distribution, bis-labelled thymosin β_4 was microinjected into the cytoplasm of MCF-7 cells. As expected, directly after microinjection the labelled peptide was evenly distributed throughout the cytoplasm (data not shown). After incubation for 1 hour a pronounced staining of the cell nucleus was detected (Fig. 2A,B). To ensure that the nuclear localisation was not a cell-specific artefact of the MCF-7 cells, microinjection experiments were also performed with Vero cells, where a comparable pattern was observed (Fig. 2C,D).

Next we analysed the distribution of fluorescently labelled intact thymosin β_4 microinjected into additional cell lines. As in Vero and MCF-7 cells, thymosin β_4 was also found within the nuclei of fibroblastic NIH-3T3 and NRK as well as the human epidermoid A431 cells after microinjection. One hour after microinjection, confocal sections of NIH-3T3 cells revealed a clear accumulation within the cell nucleus (Fig. 3A,B). Fixation of the microinjected cells after 30 minutes demonstrated nuclear accumulation together with varying fluorescence intensity within the cytoplasm. Three hours after microinjection we still observed a remaining although weaker cytoplasmic staining (data not shown). Within the nuclei we observed a homogenous distribution of the microinjected thymosin β_4 with the exception that regions of presumed nucleoli were void of thymosin β_4 (inset in Fig. 3L). A similar staining pattern was obtained with Oregon Green-labelled DNase I specific for G-actin (see Fig. 1B).

In addition, we counterstained microinjected NIH-3T3 (Fig. 3C-E) or NRK cells (Fig. 3F-H) after paraformaldehyde

Table 2. Inhibition of salt-induced actin polymerisation by thymosin β_4 and its labelled derivatives assayed by falling ball viscometry

Sample	Time of fall (seconds)
Buffer	10±2 (n=5)
Actin	155±9 (n=5)
Actin + thymosin β_4	14±4 (n=5)
Actin + thymosin $\beta_4(OGC)_2$	12±3 (n=5)

G-actin (4 μM) was incubated with equimolar amounts of the investigated peptides for 15 minutes at room temperature. Polymerisation was started by adding $MgCl_2$ to a final concentration of 2 mM. Immediately after adding $MgCl_2$ the mixture was filled into a glass capillary. After incubation for 4 hours at room temperature, viscosity was determined by falling ball viscometry.

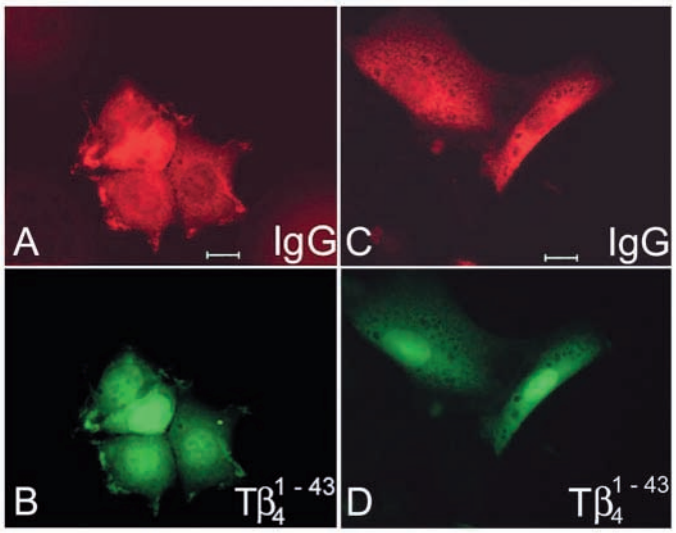

Fig. 2. Intracellular localisation of thymosin β_4 in MCF-7 and Vero cells. (A-D) Oregon Green cadaverine (OGC) labelled thymosin β_4 ($T\beta_4^{1-43}$) with rabbit IgG (controls) was microinjected into either MCF-7 (A,B) or Vero cells (C,D). Bars, 20 μm.

fixation at various time points with TRITC-phalloidin to visualise actin filaments and to analyse the distribution of thymosin β_4 in relation to the microfilament system. Besides its accumulation within the nucleus, the data obtained showed in many cases a faint punctuate staining along the cytoplasmic stress fibres of both fibroblastic NIH-3T3 and NRK cells together with a diffuse cytoplasmic localisation.

The immunohistochemical staining using the monospecific anti-thymosin β_4 antibody had indicated in a number of cases that the nuclear staining was dependent on cell density, being more intense in isolated cells than in cells within cell clusters (see also Fig. 1). Therefore, we microinjected contacting A431 cells in the middle and periphery of cell clusters. However, we did not find a dependence of the nuclear localisation of microinjected thymosin β_4 on the intensity of their cell-cell contacts (Fig. 3L-N).

The low molecular mass of thymosin β_4 (5 kDa) might suggest that it diffused through the nuclear pores by simple diffusion. To analyse its mode of nuclear translocation and accumulation, we incubated digitonin permeabilised and extracted HeLa cells with labelled thymosin β_4. Digitonin treatment of cultured HeLa cells has recently been shown to permeabilise the plasma membrane for macromolecules, but to leave the nuclear envelopes structurally intact and competent for active transport (Adam et al., 1990). Fluorescence microscopy of HeLa cells thus treated revealed a solely cytoplasmic distribution of the labelled peptide, i.e. no staining of the cell nuclei (Fig. 4A′). However, only after an additional treatment of the digitonin-extracted cells with 0.2% Triton X-100 for 10 minutes to also permeabilise the nuclear membranes was it possible to detect thymosin β_4 within the nuclei (Fig. 4B′). These data clearly indicate that the pore complexes of an intact nuclear envelope prevent the passage of thymosin β_4 through the nuclear pores in the absence of soluble cytoplasmic factors. An identical mode of thymosin β_4 exclusion was obtained with freshly isolated MCF-7 nuclei (data not shown).

The N-terminal portion of thymosin β_4 contains a sequence stretch enriched in lysine residues (^{14}KSKLKK^{19}) suggestive of a functional nuclear localisation signal. As an initial test as to whether translocation into the nucleus depends on this basic sequence, we digested bis-labelled thymosin β_4 using an AsnC-endoproteinase. Because thymosin β_4 possesses only one asparagine residue at position 26, this digestion produced two fragments: an N-terminal fragment (thymosin β_4^{1-26}) and a C-terminal fragment (thymosin β_4^{27-43}) each bearing one

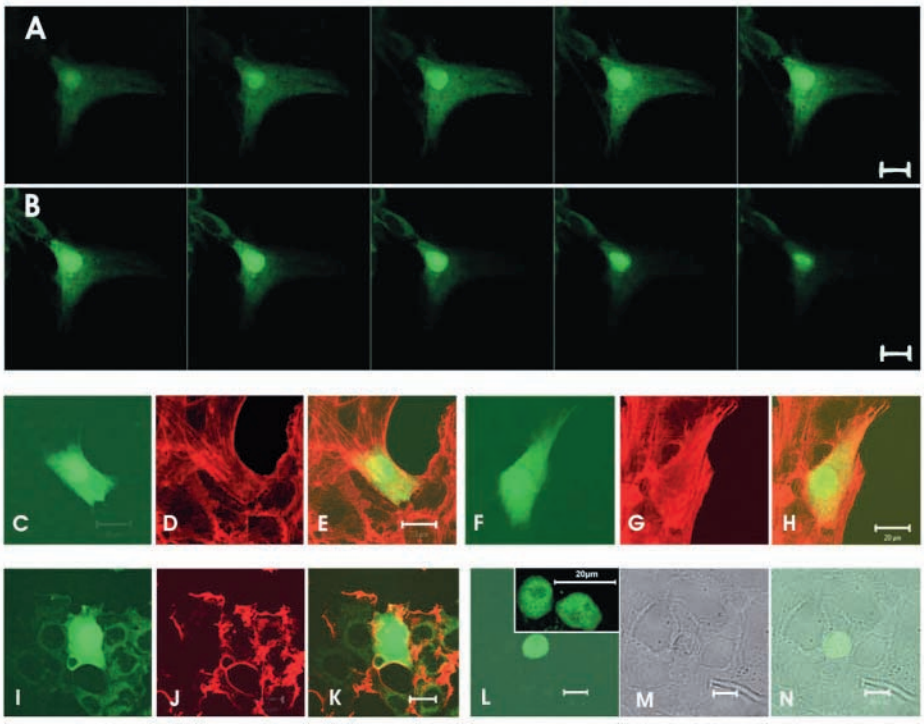

Fig. 3. Distribution of microinjected labelled thymosin β_4 in different cell lines. (A,B) Ten slices of 28 μm thickness of a continuous *Z*-scan one hour after microinjection of labelled thymosin β_4 into NIH 3T3 cells; note its presence in the cytoplasm and its accumulation within the nucleus. (C-E and F-H) NIH 3T3 cells fixed 30 minutes after microinjection with 4% paraformaldehyde (C,F) and counterstained with TRITC-phalloidin (D,G); note the high concentration of labelled thymosin β_4 within the cytoplasm and its colocalisation along the stress fibres (yellow in merged images E,H). (I-K) MCF-7 cell fixed 30 minutes after microinjection of labelled thymosin β_4 (I) and counterstained with TRITC-phalloidin (J), with the merged image (K); note the almost even distribution between cytoplasm and nucleus. (L-N) Microinjection of labelled thymosin β_4 into a single A431 cell grown within a cell cluster: preferential staining of the nucleus after two hours (L), phase-contrast image (M) and merged image (N). Insert in L shows nuclei of microinjected cells at higher magnification; note that the nucleoli appear almost unstained. Bars, 20 μm.

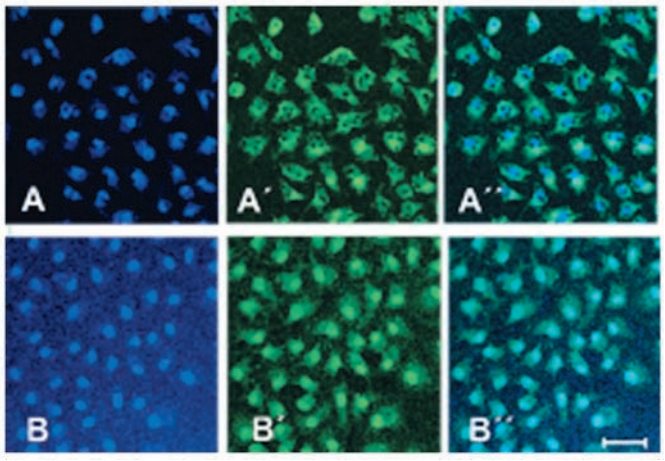

Fig. 4. Treatment of digitonin-permeabilised cells with fluorescently labelled thymosin β_4. (A-A″) HeLa cells were permeabilised with digitonin and thereafter incubated with fluorescently labelled thymosin β_4 (A′) and the chromatin specific dye chromomycin (A) with the merged image shown in A″. Note the pure cytoplasmic distribution of thymosin β_4. (B-B″) HeLa cells were permeabilised with digitonin and further treated using 0.2% Triton X-100. (B) chromomycin staining, (B′) labelled thymosin β_4, (B″) merged image. Bar, 50 μm.

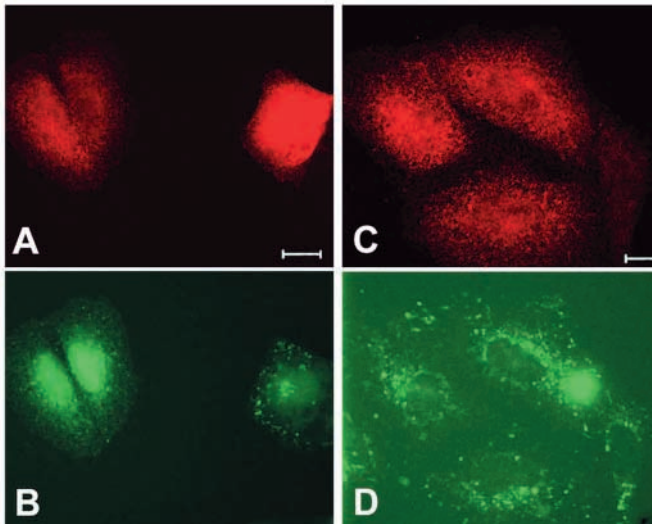

Fig. 5. Microinjection of labelled fragments of thymosin β_4 into Vero cells. (A-D) The labelled fragments thymosin $\beta_4^{1\text{-}26}$OGC and thymosin $\beta_4^{27\text{-}43}$OGC were generated by enzymatic cleavage using AsnC-proteinase. After separation and characterisation thymosin $\beta_4^{1\text{-}26}$OGC was microinjected alone (B) or together with rabbit IgG (A). Similarly thymosin $\beta_4^{27\text{-}43}$OGC was microinjected alone (C) or together with rabbit IgG (D) into Vero cells. Note the predominantly cytoplasmic staining with the thymosin $\beta_4^{27\text{-}43}$OGC fragment. Bars, 20 μm.

fluorescent label. This was confirmed by HPLC analysis with detection of Oregon Green fluorescence. After isolation and characterisation of the labelled fragments by amino acid analysis and mass spectrometry (Table 1), they were microinjected into Vero cells. The N-terminal fragment containing the ^{14}KSKLKK^{19} sequence exhibited a pronounced nuclear localisation (Fig. 5A,B), whereas the C-terminal fragment was restricted to the cytoplasm (Fig. 5C,D).

The aforementioned sequence motif ^{14}KSKLKK^{19} partially overlaps with the putative actin binding sequence of thymosin β_4 (^{17}LKKTET^{22}). To prove the assumption that the former motif is involved in nuclear translocation and to elucidate whether thymosin β_4 is translocated into the nucleus in complex with actin, fluorescent actin:thymosin β_4 complex was generated by chemical crosslinking with EDC. ADP-ribosylated rabbit skeletal muscle actin was used, as it has been previously shown to bind thymosin β_4 but not to polymerise, in order to secure its monomeric state after microinjection (Ballweber et al., 2001). Successful crosslinking was verified by UV examination of the treated material after SDS-PAGE (data not shown). Confocal microscopy of A431 cells after microinjection of the crosslinked complex showed that actin:thymosin β_4 remained confined to the cytoplasm even after 3 hours (Fig. 6).

Discussion

It is now well accepted that thymosin β_4 is the main G-actin sequestering peptide in the cytoplasm of mammalian cells. Together with other actin binding proteins that have F-actin severing and capping activities it is involved in the regulation of the ratio between monomeric (G-) and filamentous (F-) actin in the cytoplasm. Despite many publications dealing with the interaction of thymosin β_4 with G-actin, there are few reports focussing on its intracellular localisation. In an early study the intracellular localisation of thymosin β_4 was scrutinised by subcellular fractionation of rat spleen (Erickson-Viitanen et al., 1983), and described to be mainly cytosolic with negligible amounts in the nuclear, mitochondrial/lysosomal or microsomal fractions. Subsequently the intracellular localisation of thymosin β_4 and thymosin β_{10} in mouse peritoneal macrophages has been studied using specific antibodies raised against these two peptides (Yu et al., 1994). These authors reported that thymosin

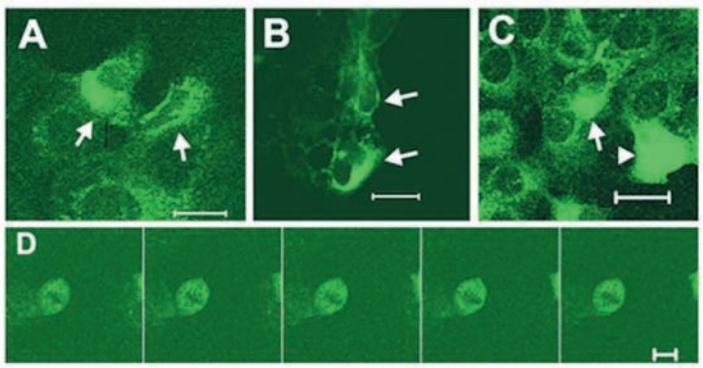

Fig. 6. Microinjection of EDC crosslinked thymosin β_4:actin complex into A431 cells. (A-D) A431 cells were microinjected with crosslinked complex of thymosin β_4 and ADP-ribosylated actin; note the cytoplasmic distribution of this complex at two hours (A,B) and three hours (C) after microinjection. Microinjected cells are marked with arrows. In C, a microinjected cell is shown whose nucleus is outside the confocal plane (arrow head). (D) A confocal *Z*-stack image series of a microinjected cell (plane distance 6.4 μm). Bars, 20 μm.

β_4 immunofluorescence was most intense in the centre of the cell and lower in the periphery and the filopodia, with no staining of the nucleus. Because of its known function as a G-actin sequestering peptide, this cytoplasmic localisation seemed to be reasonable. More recently, the influence of polyamine depletion onto the actin cytoskeleton of migrating IEC-6 cells has been studied (McCormack et al., 1999). These authors used a rabbit polyclonal antibody against thymosin β_4 and described a primarily cytoplasmic staining in control cells, punctuate in appearance and close to or on the nuclear membrane, but no staining of the nucleus itself. In contrast, they found a prominent staining of the nucleus in polyamine-deprived cells or two minutes after treatment of control cells with epidermal growth factor. They concluded that the nuclear appearance resulted from a treatment-induced translocation of thymosin β_4 into the nucleus. Our results on thymosin β_4 localisation in MCF-7 cells using indirect immunofluorescent labelling of an affinity-purified antibody indicate that cells with either cytoplasmic or nuclear accumulation can be found within a growing cell population. The differences between previous studies and our results showing variable degrees of cytoplasmic and nuclear localisation of thymosin β_4 by using immunolocalisation may be caused by variations in the preservation of the original localisation of this highly soluble and diffusible peptide. Another possible explanation may be that recognition of thymosin β_4 within the nucleus may depend on the epitope detected by the anti-peptide antibody. The polyclonal anti-peptide antibody used in this study recognises an epitope comprising of the four C-terminal amino acid residues of thymosin β_4.

Recently, the nuclear localisation of actin itself (Scheer et al., 1984; Gonsior et al., 1999), actin-related proteins (Arps) (Cairns et al., 1998; Harata et al., 1999; Harata et al., 2000) and a number of actin binding proteins (Nowak et al., 1997; Giesemann et al., 1999; Pestic-Dragovich et al., 2000; Skare et al., 2003) has been reported. Although the functions of nuclear actin are far from being fully understood, it has been proposed that it might be involved in chromatin remodelling (Zhao et al., 1998; Shen et al., 2003), mRNA processing and transport (Hofmann et al., 2001; Percipalle et al., 2001; Percipalle et al., 2002). It has been repeatedly reported that in contrast to the cytoplasm, the nuclei are devoid of phalloidin-stainable actin filaments (data also obtained during this study). Indeed, it has been shown that a monoclonal antibody that presumably recognises a particular G-actin conformation yielded a punctuate nuclear staining pattern (Gonsior et al., 1999). Thymosin β_4 was evenly distributed within the nucleus except for in nucleoli, which appeared to be free of this peptide. This distribution was observed after microinjection and immunostaining (see Fig. 1 and Fig. 3L). This staining pattern coincided with the Oregon Green-DNase I staining (Fig. 1B) and was also reported by Gonsior et al. (Gonsior et al., 1999) and is suggestive of an intranuclear co-distribution of both thymosin β_4 and G-actin. DNase I binds G-actin with high affinity and practically irreversibly (Mannherz et al., 1980). We are therefore confident that the nuclear staining and retention of Oregon Green-labelled DNase I was mainly due to actin binding, otherwise chromatin staining by Hoechst 33258 should have diminished or vanished owing to endonucleolytic activity of DNase I (see Fig. 1C).

The fact that nuclear actin seems to be maintained in its monomeric form will necessitate G-actin sequestering factors inside the nucleus. However, the lack of phalloidin staining does not exclude the presence of special forms of F-actin in the nucleus. Indeed, cellular stress frequently induces the formation of nuclear actin rods (Nishida et al., 1987) that are composed of ADF/cofilin decorated actin filaments, which were shown not to bind phalloidin (McGough et al., 1997). In addition, it has also been proposed that F-actin may be present in the nucleus in the form of very short and/or highly branched filaments (Pederson and Aebi, 2003).

Our data obtained by microinjection of fluorescent thymosin β_4 into several different cell lines show a pronounced nuclear localisation of the labelled thymosin β_4. After enzymatic digestion of bis-labelled thymosin β_4 into two labelled fragments, only the larger N-terminal fragment, thymosin $\beta_4^{1\text{-}26}$, was translocated to the nucleus, whereas the smaller C-terminal fragment thymosin $\beta_4^{27\text{-}43}$ remained in the cytoplasm. The amino acid sequence of thymosin β_4 does not contain a canonical nuclear localisation signal, but a cluster of positively charged amino acid residues (^{14}KSKLKK^{19}) suggestive of a functional nuclear localisation signal, which partially overlaps with the proposed actin binding site of thymosin β_4. Indeed analysis by SubLoc v1.0 (Hua and Sun, 2001) predicted a nuclear localisation of intact thymosin β_4 (accuracy 74%) as well as of its N-terminal fragment thymosin $\beta_4^{1\text{-}26}$ (accuracy 94%), whereas removal of just the above mentioned cluster from the amino acid sequence changes the prediction to cytoplasmic localisation (accuracy 95%). This assumption was further confirmed by the fact that chemically crosslinked actin:thymosin β_4 complex, in which the ^{14}KSKLKK^{19} motif might be sterically blocked by actin binding, is not translocated into the nucleus. Moreover, this result argues against a possible transport of thymosin β_4 to the nucleus in complex with actin. Our data suggest that this cluster of charged amino acid residues (^{14}KSKLKK^{19}) may be involved in the translocation of entire thymosin β_4, as well as its N-terminal fragment into the nucleus.

As treatment of digitonin-permeabilised HeLa cells with labelled peptide resulted in a solely cytoplasmic localisation of the peptide, the translocation of thymosin β_4 to the nucleus cannot be explained by a simple diffusion mechanism of the 5 kDa peptide, nor can it be caused by the fluorescent labelling. Therefore, we propose that thymosin β_4 is translocated into the nucleus by an active transport mechanism, requiring an as yet unknown soluble cytoplasmic factor. It appears surprising that a peptide of only 5 kDa molecular mass does not freely diffuse through nuclear pores. However, structural studies have implicated that thymosin β_4 is an elongated molecule (Zarbock et al., 1990; Czisch et al., 1993; Ballweber et al., 2002) and data from gel filtration experiments have shown that it migrates like a protein of considerably higher molecular mass (Haritos et al., 1989). Future experiments will aim to identify the cytoplasmic factors involved in its nuclear translocation and binding partners.

Our data clearly indicate that distinct amounts of the G-actin sequestering peptide thymosin β_4 are translocated into the nucleus of cells by an active transport mechanism. When considered with the recent detection of G-actin and other actin binding proteins in the cell nucleus, our results suggest that thymosin β_4 is not only the main G-actin sequestering peptide

in the cytoplasm of mammalian cells, but may also account for the G-actin sequestering activity within the nucleus. As well as this presumed nuclear activity of thymosin β_4 it is tempting to speculate that it may have additional functions in the nucleus as also proposed for actin itself and other actin binding proteins. Further studies into these nuclear functions may give additional insight into the proposed role of thymosin β_4 in events like carcinogenesis or apoptosis.

Alternatively, a specific function for nuclear actin has been questioned as it was shown that actin itself contains two nuclear export sequence stretches (Wada et al., 1998). Indeed, it has also been shown that nuclear accumulation of the actin binding protein profilin only serves to facilitate its nuclear export (Stüven et al., 2003). In view of these data, it might also be possible that the nuclear accumulation of thymosin β_4 stabilises actin in its monomeric state by forming a G-actin:thymosin β_4 complex that is subsequently transformed into a G-actin:profilin complex (as during actin cycling in the cytoplasm) ready for nuclear export. Thus the supposed sequestering activity of thymosin β_4 within the nucleus might also support the process of nuclear export of actin, which probably occurs after mixing cytoplasmic and nuclear contents during the open mitosis of mammalian cells.

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