J Cancer Res Clin Oncol (2002) 128: 247–256
DOI 10.1007/s00432-002-0332-7

ORIGINAL PAPER

A.M. Otto · C.S.G. Müller · T. Huff · E. Hannappel

Chemotherapeutic drugs change actin skeleton organization and the expression of β-thymosins in human breast cancer cells

Received: 2 October 2001 / Accepted: 22 January 2002 / Published online: 19 March 2002

Abstract *Purpose*: Elevated expression of the β-thymosin isotypes $T\beta_4$, $T\beta_{10}$, and T_{15} appears to be involved in the manifestation of a malignant phenotype of human tumor cells, including those of mammary carcinomas. This has evoked an interest in these peptides as diagnostic/prognostic tumor markers. If increased levels of β-thymosins correspond to tumor malignancy, the question arises whether tumor growth inhibition induced by chemotherapeutic drugs would reduce their expression. *Methods*: Two human breast cancer cell lines, the estrogen receptor(ER)-positive MCF-7 and the ER-negative MDA-MB231, were thus analyzed for the amount of β-thymosin mRNAs by RNase protection assay and for the respective peptide levels by HPLC following different hormonal and drug treatments. *Results*: Both cell lines, growing in medium with 10% FCS, contain $T\beta_4$ (400–500 fg/cell) and $T\beta_{10}$ (about 100 fg/cell), but no $T\beta_{15}$. Incubating MCF-7 cells with tamoxifen (1 μM) for 5 days resulted in about 80% growth inhibition and in reduction of intracellular $T\beta_4$ and $T\beta_{10}$ concentrations by about 40%. Levels of $T\beta_4$ and $T\beta_{10}$-mRNA were reduced by about 60%. In contrast, cisplatin (2 μM) changed neither the peptide concentrations nor the mRNA levels of β-thymosins, in spite of marked growth inhibition. In addition, no changes in β-thymosin expression were observed in MDA-MB231 cells treated with either drug. MCF-7 cells maintained in estrogen-poor medium (10% horse serum) or stimulated to grow with estradiol (1 nM) had $T\beta_4$ and $T\beta_{10}$ concentrations reduced by about 30%, but changes in $T\beta_4$- and $T\beta_{10}$-mRNA levels did not correspond to those of the peptide. *Conclusion*: Expression of $T\beta_4$ and $T\beta_{10}$ mRNAs and their peptides is differentially regulated and does not correlate with growth. Instead, reduced β-thymosin expression may be linked to more intensive TRITC-phalloidin staining of F-actin lining the membrane at sites of intimate cell-cell contacts, while increased β-thymosin levels appear in cells with more extensive substrate adhesion. This suggests that β-thymosins play a role in cell surface dynamics.

Keywords Actin skeleton · Breast cancer cells · Cisplatin · Tamoxifen · β-thymosins

Abbreviations *FCS* fetal calf serum · *HS* adult horse serum · *$T\beta_4$* thymosin β_4 · *$T\beta_{10}$* thymosin β_{10}

Introduction

Cancer cells possess a cytoskeletal organization different to that of normal cells from which they are derived (Pollack et al. 1975; Gabbiani 1979). Cell transformation, reflecting a step in tumorigenesis, is accompanied by a loss of growth regulation as well as by changes in cell morphology, reduced cell-cell contact and substrate adhesion, and, in particular, by a loss of actin filaments (Decloitre et al. 1991). Even though the precise mechanisms of how actin is involved in tumorigenic transformation are not yet completely delineated, it is becoming evident that a myriad of actin-binding proteins governing the organization of the actin structures is involved (Pollard 1986; Kreis and Vale 1993). While the interaction of such proteins with different structural states of actin has in some cases been quite well studied, their relationships to signal transduction and subsequent cellular functions has more recently become a major subject of intensive investigations (Ayscough 1998).

This work was funded by a grant from the Mildred Scheel-Stiftung für Krebsforschung H10-1262-HA-1

A.M. Otto (✉) · C.S.G. Müller · T. Huff · E. Hannappel
Institute of Biochemistry,
University of Nuremberg-Erlangen,
Fahrstr.17, 91054 Erlangen, Germany
E-mail: angela.otto@ei.tum.de
Tel.: +89-289-22913
Fax: +89-289-22950

Present address: A.M. Otto
Heinz-Nixdorf-Lehrstuhl für
Medizinische Elektronik,
Technische Universität München,
Arcisstr. 21, 80333 Munich, Germany

One group of actin-binding proteins comprises the β-thymosins, a class of peptides with a molecular weight of about 5 kD (Nachmias 1993; Huff et al. 2001). They are ubiquitous molecules in the mammalian organism, being present in all cell types except erythrocytes, and thus are probably endowed with some common functions. β-Thymosins exist in numerous highly homologous isoforms, of which two to three types can be detected in human tissues and cells: thymosin β_4, β_{10} and, more recently, β_{15} ($T\beta_4$, $T\beta_{10}$, $T\beta_{15}$, respectively) (Hannappel and Leibold 1985; Erickson et al. 1983; Hall 1990; Hall 1991; Bao et al. 1996; Bao et al. 1998). The intracellular amounts of β-thymosins calculated in several mammalian tissues and cells range from 5–30 fg $T\beta_4$ per platelet (Hannappel and van Kampen 1987) up to 1 ng in an EBV-transfected B-cell (Hannappel and Leibold 1985). In most cells the intracellular concentration of β-thymosins appears to be comparable to or even greater than that of actin (Hannappel and van Kampen 1987; Yu et al. 1994). With a dissociation constant in the range of 1 μM, $T\beta_4$ and $T\beta_{10}$ together could bind about 30–50% of monomeric actin in the cell (Sanger et al. 1995). A major function of β-thymosins is thus thought to be the sequestering of actin monomers (G-actin) (Yu et al. 1993; Cassimeris 1992), which is held responsible for maintaining a pool of G-actin under cellular conditions favoring actin polymerization.

The actin skeleton is a sensitive structure involved in many dynamic processes regulating growth and motility, including tumorigenesis and metastasis. There is evidence for β-thymosins being involved in regulating these processes. Overexpression of $T\beta_4$ in fibroblasts in the short term (hours) can lead to a loss of actin stress fibers and a decrease in focal adhesions (Sanders et al. 1992; Yu et al. 1994). In the long-term (days), however, overexpressed $T\beta_4$ results in increased levels of both G- and F-actin as well as of other cytoskeletal proteins (Golla et al. 1997). The overexpression of $T\beta_{10}$, on the contrary, caused an increase in actin bundles. Such cells were more motile, spread more rapidly on the substratum than their normal counterparts (Sun et al. 1996), and thus showed properties also characteristic of transformed and cancer cells.

Increased expression of $T\beta_4$ and $T\beta_{10}$, detected either as mRNA or as immunologically recognizable peptides, has indeed been found in various tumors, e.g. of the breast, ovary, uterus, colon, and thyroid (Yamamoto et al. 1993; Verghese Nikolakaki et al. 1996; Santelli et al. 1999). The mRNA of $T\beta_{15}$ was recently identified in a metastatic rat prostate carcinoma cell line (Bao et al. 1996). $T\beta_{15}$ was also found expressed in human tumor tissue and cell lines of the breast, and immunohistochemical analysis localized the peptide preferentially in malignant tumors (Bao et al. 1998; Gold et al.1997). The levels of these β-thymosins in tumor tissues have thus been proposed to constitute a potential diagnostic marker for the state of a tumor.

If the level of β-thymosins corresponds to the malignancy of a tumor, then in turn these peptides may also constitute a marker for tumor regression following a chemotherapeutic treatment. To study this aspect, two human breast cancer cell lines, the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB231 cells, differing in their morphology, their estrogen and growth factor receptor as well as oncogene expression, and metastasizing potential (Gierthy et al. 1991; Mohamood et al. 1997), constitute a useful model system. These cell lines have been previously used to study the effect of estrogens and chemotherapeutic drugs on growth and cell death in culture (Otto et al. 1991; Otto et al. 1996). Using these cells, we now investigated whether estrogenic growth stimulation or inhibition evoked by the antiestrogen tamoxifen and the DNA-damaging drug cisplatin changed the expression of the β-thymosins both at the level of the mRNA and the peptides. $T\beta_4$ and $T\beta_{10}$ were quantified by HPLC of acid-soluble cell extracts, while mRNAs were detected with the respective human cDNA using RNase protection assay. Cells were also stained for F-actin with TRITC-phalloidin to detect changes in actin organization. With these experimental procedures we provide evidence for differential expression of $T\beta_4$ and $T\beta_{10}$ in MCF-7 cells, which is not related to growth, but appears linked to changes in cell morphology, including cell-cell contacts.

Material and methods

Cell culture

The human mammary cancer cell lines MCF-7 and MDA-MB231 were maintained in Dulbecco's MEM/F12 supplemented with 10% fetal calf serum (FCS), without phenol red and antibiotics. Cells were plated at a titer of 2×10^4/ml in medium with 10% FCS in 10-cm or 15-cm dishes. Tamoxifen was dissolved at a concentration of 1 mM in 70% ethanol and diluted to 0.1 mM in 0.9% saline. Cisplatin was prepared as a stock solution of 1 mM in 0.9% saline and dissolved upon dilution to 0.1 mM. The drugs were added to the cultures without medium change 1 day after plating. Cells were incubated for 5 days after addition of the drugs.

For growth under estrogen-poor conditions MCF-7 cells were plated in medium containing 10% adult horse serum (HS) instead of FCS. Cell growth was stimulated by adding estradiol at a final concentration of 1 nM 1 day after plating and incubating for 5 days.

Preparation of cells for HPLC analysis

Cell extracts were prepared either from lysates or from cells directly scraped from the dish, with similar results. For lysis, cells were allowed to swell in a hypotonic buffer (20 mM HEPES, pH7.4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$) on ice for 15 min and scraped off the dish. From the resulting lysate, aliquots were taken each for determination of cell number and protein content.

Determination of the cell number

A nuclei suspension was counted, which was prepared by adding 0.5% benzalkonium chloride in 10% acetic acid to an aliquot of the cell lysate, as described before (Otto et al. 1991).

Determination of protein content

In an aliquot of the cell lysate, proteins were solubilized by adding an equal volume of 2.5 N NaOH. Protein content was determined with a Coomassie solution (Biorad).

HPLC analysis

The procedure was basically as described (Hannappel 1986) with some modifications. Briefly, the cell lysates were precipitated in 0.4 M perchloric acid on ice and pelleted by centrifugation. The supernatant was adjusted to a pH of 4–6 using 10 M KOH, and immediately thereafter 1/10 volume of a formate buffer (1 M formic acid, 0.2 M pyridine) was added. The dipeptide Phe_2 was added as an internal marker. Following centrifugation to remove precipitated perchlorate, the supernatant was analyzed by HPLC.

The chromatographic setup and detection were as described before (Huff et al. 1997; Huff et al. 1999). Briefly, samples were injected on a Beckman ODS Ultrasphere column (5 µM, 4.6×250 mm), and β-thymosins were separated by eluting with 0.1% trifluoroacetic acid using a linear gradient of 0–40% acetonitril over 60 min at a flow rate of 0.75 ml/min. Detection was at 205 nm or by fluorescence following a post-column derivatization of eluted peptides with fluorescamine. The retention times of Tβ_4 and Tβ_{10} were at 47 min and 49 min, respectively. The peak areas of defined amounts of Phe_2 and of Tβ_4 and Tβ_{10} isolated from bovine and rabbit spleen, respectively, (Hannappel et al. 1989) were used for quantitation of the experimental samples.

RNA Isolation

MCF-7 cells were washed once with PBS and scraped from the dish using a rubber policeman. The collected cells were carefully suspended to be able to take aliquots for determining the cell number, protein content, and β-thymosin contents, as described above. RNA from 0.5–7×10^6 cells was isolated using the RNeasy Mini Kit (Qiagen) according to the protocol for animal cells grown in a monolayer. The basic procedure included cell lysis with guanidine isothiocyanate, addition of ethanol and binding of the RNA to a silica-based membrane, from which RNA was eluted with water.

Preparation of the plasmids

Clones of E. coli containing plasmids with the human cDNA sequences of the proteins thymosin β_4 (IMAGp998P211900; designated as pAT4), thymosin β_{10} (IMAGp998B192260; pAT10), β-actin (IMAGp998L082496; pAB), and GAPDH (IMAGp998I21212; pAG) were obtained from the Resource Center of the German Human Genome Project at the Max Planck Institute for Molecular Genetics (Berlin, Germany). These plasmids were modified as follows: the size of the pAT4 was reduced by digestion with XbaI, then purified (Qiaquick Nucleotide Removal Kit, Qiagen) and religated. The specific regions of the other three plasmids were amplified by PCR using oligonucleotide primers which include either a BamHI or EcoRI restriction site (MWG, Ebersberg, Germany). The PCR products were purified, digested with BamHI or EcoRI restriction enzymes to generate cloning sites and then cloned into BamHI and EcoRI sites of the pAMP10 plasmid (Life Technologies). These new plasmids were transfected into competent DH5α-E. coli cells. Positive transformants were grown in LB broth containing ampicillin (0.1 g/l), and recombinant plasmids were prepared with the Nucleobond Kit (Machery-Nagel, Düren, Germany). The identity of each cDNA was confirmed by sequencing.

Preparation of radioactively labeled riboprobes

The plasmids pAT10, pAB, and pAG were linearized by *BamHI* digestion, purified with the RNeasy Mini Kit (Qiagen) using the cleanup protocol, and were transcribed using SP6 RNA polymerase. The pAT4 plasmid was linearized by *EcoRI* digestion and transcribed using T7 RNA polymerase. The transcription reactions were performed with [α-^{32}P]CTP (initial specific activity: 10 mCi/ml, 800 Ci/mmol) and the reagents supplied with the transcription kit Riboprobe Combination System SP6/T7 (Promega, Madison, USA). Transcription was initiated by the addition of RNA polymerase [20 U/µl] and was allowed to proceed for 2 h at 37 °C. To digest the DNA, RNase-free DNase [1 U/ml] was added, and the tubes were incubated for a further 15 min at 37 °C. An aliquot of the reaction mix was counted to determine the total radioactivity. After the probe was purified with the RNeasy Mini Kit, an aliquot of the eluted RNA solution was used to determine the fraction of radioactivity incorporated and to estimate the specific activity of the probe. The reaction conditions described for the standard assay produced probes with high specific activity from each plasmid (≥10^8 cpm/µg RNA). The integrity of the RNA was checked by denaturing PAGE gel electrophoresis (5% polyacrylamide, 8 M urea, in 60 mM Tris, 60 mM boric acid, 1 mM EDTA, pH 8.3).

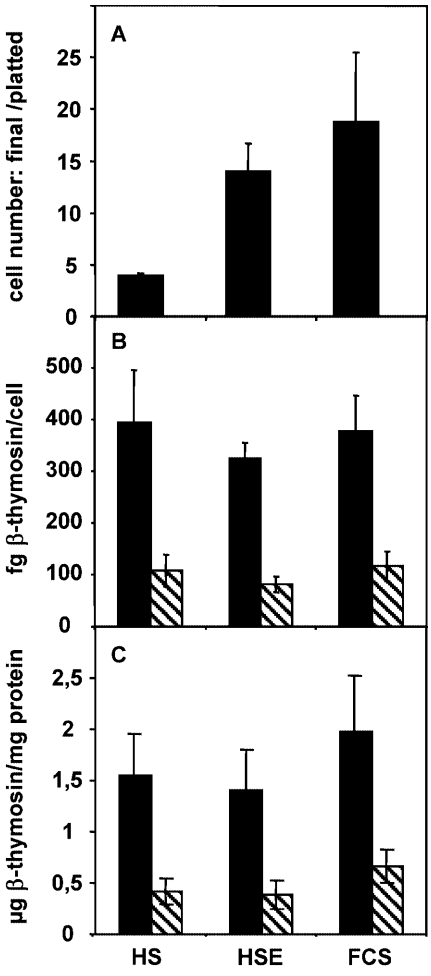


Fig. 1a–c. Effect of estrogens on β-thymosin content in MCF-7 cells. Cells were plated in medium containing either 10% FCS or 10% estrogen-poor HS and supplemented with 1 nM E_2 for 5 days. **A** Increase in cell numbers over the initial number of cells plated; **B** Amount of β-thymosins calculated per cell number; **C** Amount of β-thymosins calculated per mg protein (*filled bars*: Tβ_4, *hatched bars* Tβ_{10}). Results are averages and ranges of four independent experiments, all showing similar changes

RNase protection assay

Hybridization with cellular RNA was performed using the protocol and reagents supplied in the RNase Protection Assay (RPA) II Kit (Ambion) as described for the standard procedure. The RNA samples (10 µg) were combined with 4×10^5 cpm of each probe and coprecipitated. As a negative control, RNA from yeast supplied by the RPA II kit was used. The pellets were dissolved in 20 µl of the hybridization buffer (provided in the kit) and incubated for 4 min

Table 1. Protein- and RNA- content of MCF-7 cells under various culture conditions. Cells were grown for 5 days in media containing: 10% horse serum alone or with 1 nM E_2; 10% fetal calf serum alone or with either 1 μM tamoxifen or 2 μM cisplatin. Averages and standard deviations are values from 3–6 experiments

	Protein (pg/cell)	RNA (pg/cell)
HS	309 + 25	13.31 + 0.86
HS + E_2	263 ± 59	16.39 ± 1.94
FCS	220 ± 54	18.05 ± 3.31
Tamoxifen	269 ± 62	11.07 ± 1.42
Cisplatin	431 ± 91	22.73 ± 3.58

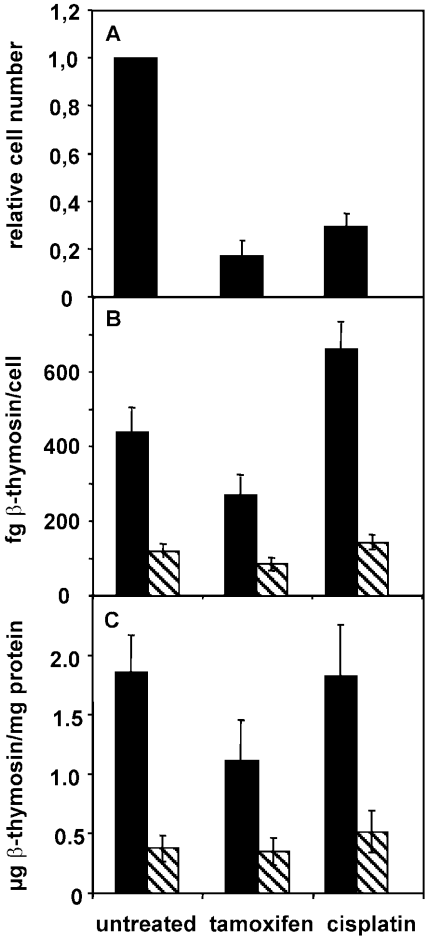


Fig. 2a–c. Effect of chemotherapeutic agents on β-thymosin content in MCF-7 cells. Cultures were incubated for 5 days in medium containing 10% FCS with 1 μM tamoxifen or 2 μM cisplatin. **A** Relative cell number compared to untreated cells (= 1.0); **B** Amount of β-thymosins calculated per cell number; **C** Amount of β-thymosins calculated per mg protein (*filled bars* $T\beta_4$, *hatched bars* $T\beta_{10}$). Results are averages and ranges of six independent experiments, all showing similar changes

at 95 °C to denature and solubilize the RNA. For hybridization the sample was incubated in a water bath at 42 °C overnight. Remaining single-stranded RNA was digested by adding an RNase cocktail (0.1 U RNase A, 4 U RNase T1) in 200 μl RNase digestion buffer (Ambion) and incubating for 30 min at 37 °C. Two hybridization samples with yeast RNA were also incubated without the RNase cocktail as controls. The protected hybridization

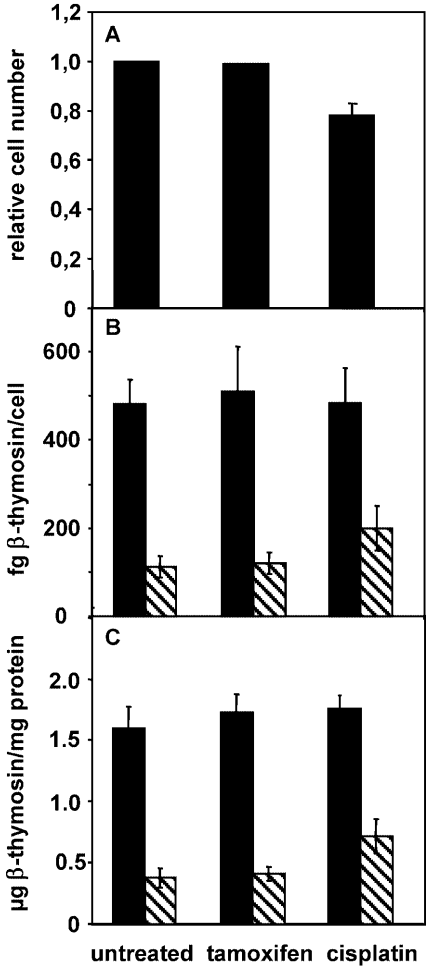


Fig. 3a–c. Effect of chemotherapeutic drugs on β-thymosin content in MDA-MB231 cells. Cultures were incubated for 5 days in medium containing 10% FCS with 1 μM tamoxifen or 2 μM cisplatin. **A** Relative cell number compared to untreated cells (= 1.0); **B** Amount of β-thymosins calculated per cell number; **C** Amount of β-thymosins calculated per mg protein (*filled bars* $T\beta_4$, *hatched bars* $T\beta_{10}$). Results are averages and ranges of three independent experiments, all showing similar changes

products were precipitated in the RPA II inactivation/precipitation mixture at –20 °C for 1 h and centrifuged. The pellets were resuspended in gel-loading buffer and separated by denaturing PAGE-electrophoresis as above. Radioactivity was detected by exposing the wet gel overnight to an intensifying screen (Molecular Dynamics, Sunnyvale, USA). The relative intensity of the radioactivity was quantified by Imagequant Software (Molecular Dynamics).

Histochemistry

Cells were plated on glass cover slips in the same culture conditions described above, fixed with 1% formaldehyde for 20 min and permeabilized with 0.2% Triton X100 in PBS for 2 min. Cells were incubated with TRITC-labeled phalloidin (1.5 μM; 45 min) for staining of actin filaments and with Hoechst 33258 (1 μg/ml) for DNA-staining, washed with PBS, and finally embedded in Mowiol. Fluorescent images were visualized using a fluorescence microscope (Axioskop, Zeiss) equipped with AMCA and Cy2 filters (Analysentechnik), and documented using a digital camera (Hamamatsu) and QED-Imaging software.

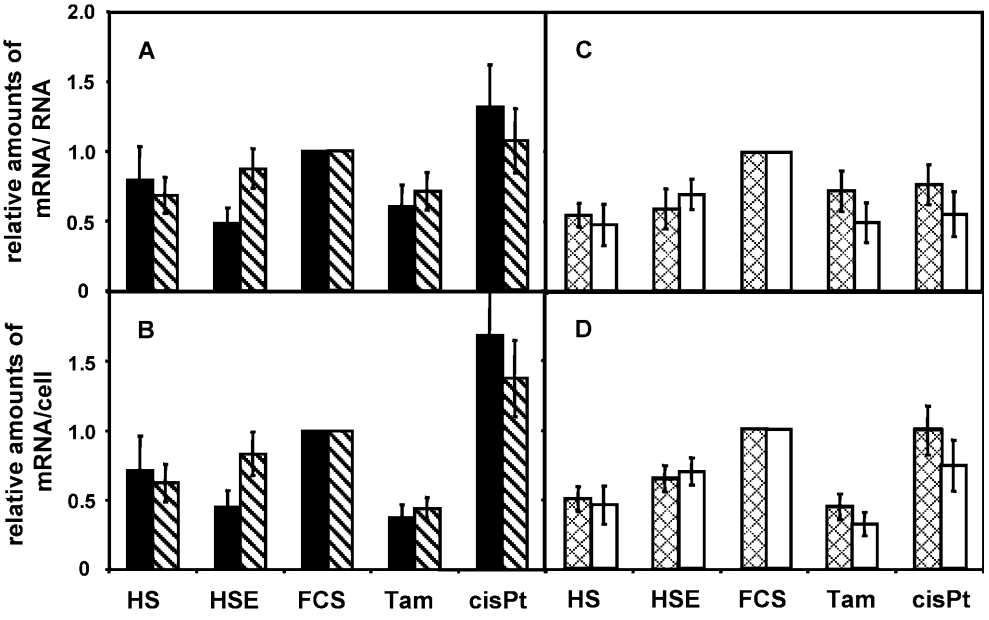


Fig. 4a–d. Analysis of mRNA expression for Tβ_4, Tβ_{10}, β-actin, and GAPDH in MCF-7 cells by RNase protection assay. Aliquots containing 10 μg of the total cellular RNA hybridized with the four radioactively labeled riboprobes were separated and quantified as described in Material and Methods. Values are the averages and the range from three experiments. **A**, **C** The relative amounts of mRNA (FCS = 1) based on equal amounts RNA; **B**, **D** The relative amounts of mRNA per cell, taking into account the different amounts of total RNA per cell (*filled bars* Tβ_4, *hatched bars* Tβ_{10}, *crossed bars* actin, *open bars* GAPDH)

Results

Identification and quantification of β-thymosins under various growth conditions

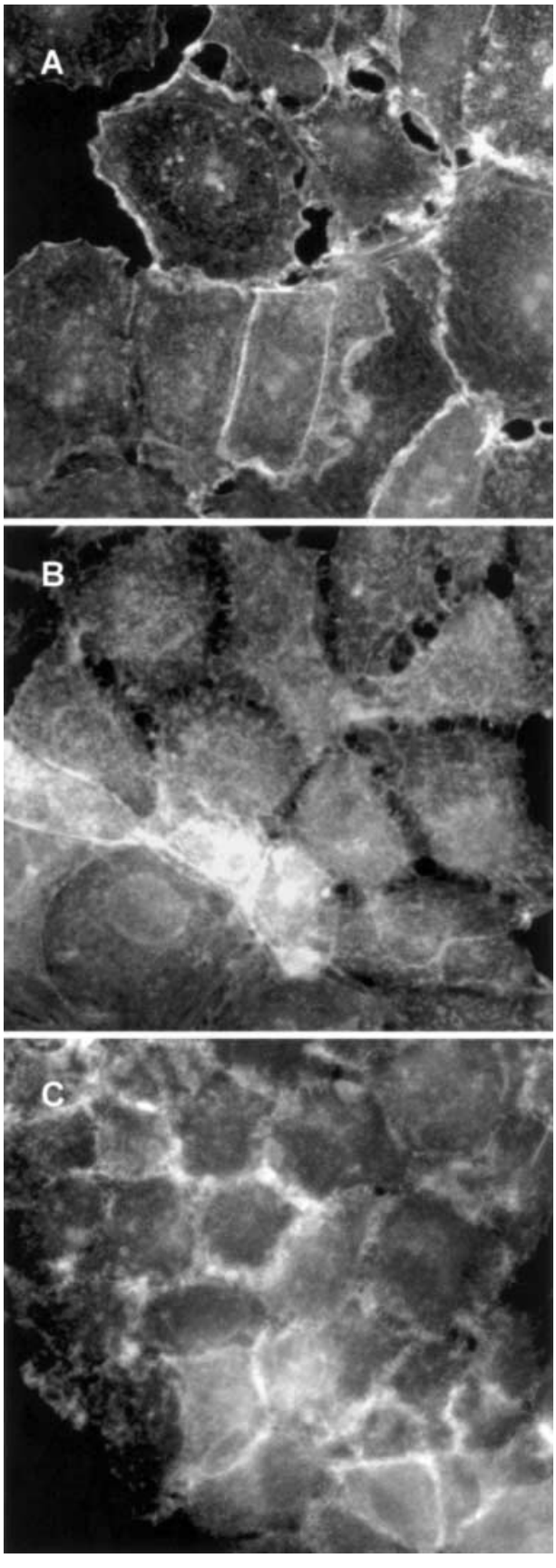
In MCF-7 cells both Tβ_4 and Tβ_{10} can be detected as peptides in acid-soluble extracts (Fig. 1). No Tβ_{15} was detectable in MCF-7 extracts even when using mass spectrometry [Bonk, Humeny, and Otto, unpublished data from MALDI-TOF (as described in Huff et al. 1999)]. The average amount of Tβ_4 was about 400 fg/cell and the amount of Tβ_{10} was about 100 fg/cell. With an average cellular volume of about 2 pl these amounts correspond to an intracellular concentration in the range of 40 μM and 10 μM, respectively. When β-thymosin levels were calculated with reference to the cellular protein content (Table 1), MCF–7 cells contain about 1.5 μg Tβ_4 and 0.4 μg Tβ_{10} per mg protein. This means that Tβ_4 and Tβ_{10} together constitute about 0.20% of the total cellular protein.

MCF-7 cells are usually grown in medium containing 10% FCS, which naturally contains estrogens at concentrations sufficient to exert a growth stimulatory effect. Under this condition the doubling time is about 32 h, resulting in an increase in cell number between 12- and 24-fold after a 5-day incubation (Fig. 1A). To investigate the effect of hormonal growth stimulation on the cellular content of Tβ_4 and Tβ_{10}, the growth of MCF-7 cells was retarded by growing them under estrogen-poor conditions which allowed the cell numbers to increase only about fourfold (Fig. 1A). In spite of this low proliferation rate, the levels of both Tβ_4 and Tβ_{10} per cell were comparable to those in cells growing in FCS (Fig. 1B). Addition of 1 nM estradiol (E_2) to cells growing in 10% HS increased the number of cells in the monolayer about 15-fold (Fig. 1A). Here, the amounts of β-thymosin per cell tended to be reduced by about 20%. β-Thymosin concentrations calculated per mg protein were generally about 30% lower in cells growing with 10% HS, regardless of estradiol content, compared to cells grown in FCS-containing medium (Fig. 1B, C). This is explained by the differences in cell size, i.e., in protein content, under the different conditions of growth (Table 1); MCF-7 cells growing in FCS are generally smaller and grow in more compact cell aggregates than those in HS. In conclusion, the results show that there is no correlation between the rate of cell proliferation, and the levels of the β-thymosins.

The growth of MDA-MB231 cells is estrogen-independent, and in medium with 10% FCS the growth rate of these cells is similar to that of MCF-7 cells. The levels of β-thymosins was about 500 fg Tβ_4 and 110 fg Tβ_{10} per cell, and 1.5 μg Tβ_4 and 0.4 μg Tβ_{10} per mg protein. These values are very similar to those obtained with MCF-7 cells.

Changes in β-thymosin levels upon drug-treatment

It was now of interest to investigate the effect of chemotherapeutic drugs on the β-thymosin levels in tumor cells. In MCF-7 cells the antiestrogen tamoxifen inhibits growth by reducing the proliferation rate, eventually leading to an accumulation of the cells in the G_1-phase of the cell cycle; there is little increase in cell death (Sutherland et al. 1984; Otto et al. 1996). After a 5-day incubation, the number of cells in the dish was about 20% compared to that of untreated cells, and the levels of both Tβ_4 and Tβ_{10} were reduced by about 40% (Fig. 2). Tamoxifen had no effect on the growth of the estrogen-insensitive MDA-MB231 cells, and no changes in the β-thymosin levels were observed (Fig. 3). The reduction in the β-thymosin levels can therefore be ascribed to the antiestrogenic effect of tamoxifen.



◀

Fig. 5a–c. TRITC-phalloidin staining of F-actin in MCF-7 cells grown for 5 days under various hormonal conditions as in Fig. 1. **A** 10% HS; **B** 10% HS + 1 nM E_2; **C** 10% FCS. Microscope magnification: 400×

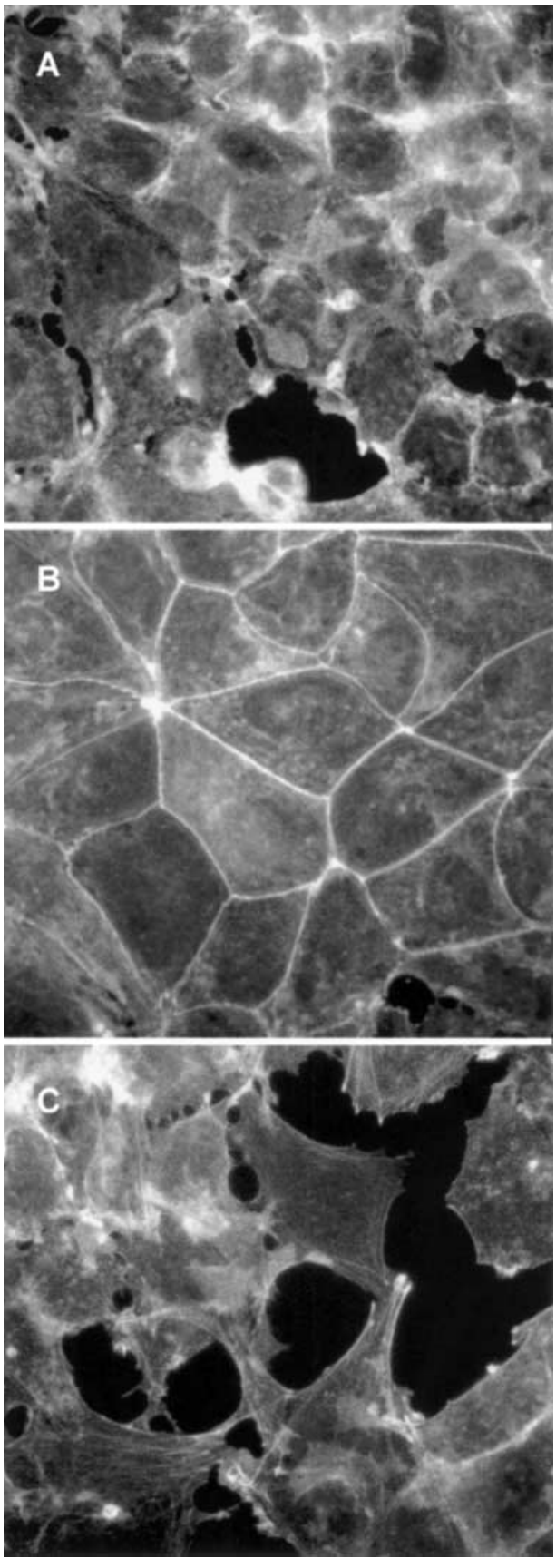
Cisplatin inhibits growth of MCF-7 cells not by inducing cell cycle arrest, but by increasing the rate of cell death (Otto et al. 1996). Cisplatin also inhibits the growth of MDA-MB231 cells, but to a lesser extent. Following cisplatin treatment of MCF-7 cells, the amounts of both Tβ_4 and Tβ_{10} per cell always increased 20–50% (Fig. 2B). This increase depended on the concentration of cisplatin (0.1–4 μM) and was coherent with a reduction in the fraction of surviving cells (Otto et al. 1997). When taking into account that cisplatin increased the total protein content of the surviving cells (Table 1), the cellular *concentration* of the β-thymosins actually remained constant (Fig. 2C). This means that the increase in β-thymosins parallels the increase in total cell protein.

In MDA-MB231 cells, cisplatin had only a weak effect on cell growth (Fig. 3A), and no increase in Tβ_4 level per cell was observed (Fig. 3B, C). However, the level of Tβ_{10} was increased by 65–80%, so that the ratio of Tβ_4/Tβ_{10} changed from 4,4 to 2,6. This could be related to the more malignant property of MDA-MB231 cells.

Expression of β-thymosin mRNA in MCF-7 cells

An important question is whether changes in β-thymosin levels reflected those of their respective mRNAs. Equal amounts of RNA extracted from MCF-7 cells following the various treatments were, therefore, analyzed by RNAse protection assay. The relative steady-state levels of Tβ_4- and Tβ_{10}-mRNA in tamoxifen-treated cells were reduced by 30–40% compared to untreated cells (Fig. 4A). In contrast, in cisplatin-treated cells there was no reduction in either Tβ_4- or Tβ_{10}-mRNA expression, even though growth was inhibited to the same extent as with tamoxifen (Fig. 2A). Cells growing in 10% HS also showed little or no decrease in the levels of Tβ_4 and Tβ_{10}-mRNAs, while cells stimulated to grow in 10% HS with estradiol showed an unexpected selective decrease in Tβ_4-mRNA of over 60%.

Taking into account that the RNA-content per cell varies depending on the treatment (Table 1), the relative levels of mRNAs were recalculated. The RNA-level is markedly reduced in cells growing in estrogen-poor conditions and in tamoxifen-treated cells; thus, the amount of Tβ_4- and Tβ_{10}-mRNA per cell is lower than observed when comparing equal amounts of RNA (Fig. 4B). On the other hand, cisplatin-treated cells have the highest RNA-content, which results in an even higher cellular level of Tβ_4- and Tβ_{10}-mRNA compared to untreated cells. With this evaluation the changes in the relative mRNA levels are greater than the changes in



◀

Fig. 6a–c. TRITC-phalloidin staining of F-actin in MCF-7 cells grown for 5 days with tamoxifen and cisplatin as in Fig. 2. **A** 10% FCS; **B** 1 μM tamoxifen; **C** 2 μM cisplatin. Microscope magnification: 400×

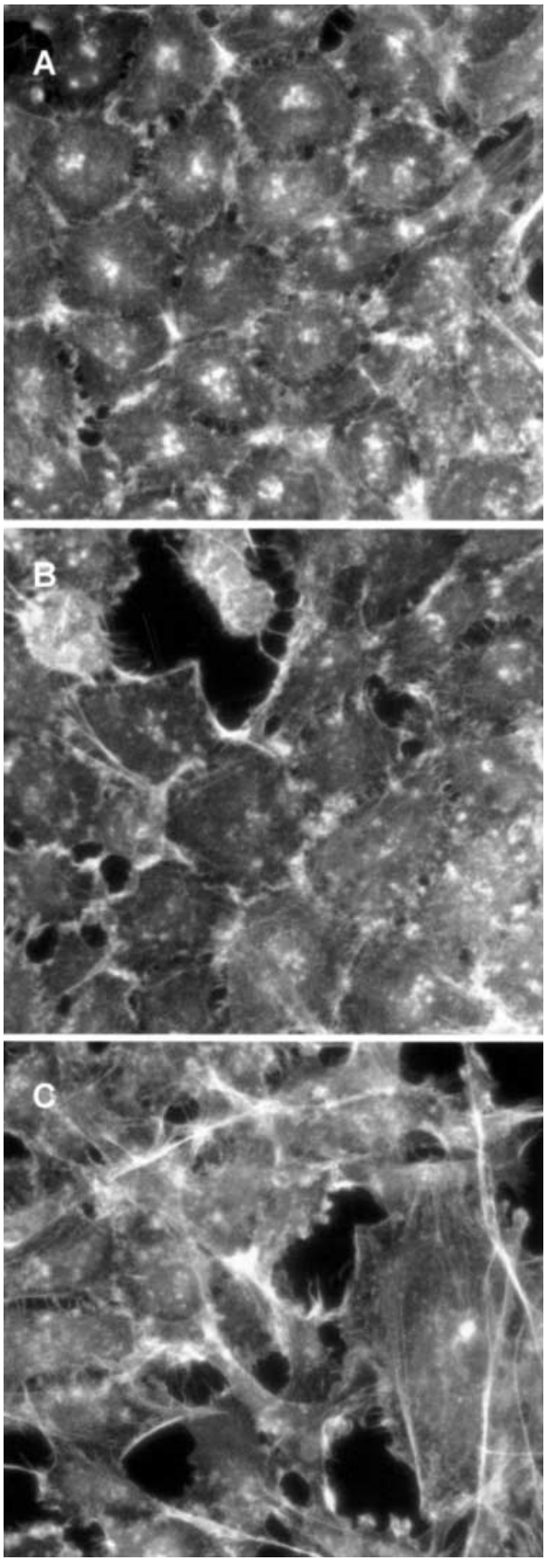
the cellular β-thymosin content. This is most pronounced in tamoxifen-treated cells. Taken together, these results suggest differences in the expression between $T\beta_4$ and $T\beta_{10}$ as well as differences between the transcriptional and translational regulation of their genes.

The mRNAs of two genes, β-actin and the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were analyzed for comparison, since their expressions are often used as independent references. The results (Fig. 4C, D) show, however, that the relative amounts of mRNA of both genes also vary depending on the growth conditions: the cellular amount of GAPDH-mRNA was reduced most prominently in estrogen-deficient and in drug-treated cells, reflecting a lower rate of glucose metabolism in growth-inhibited cells. β-Actin-mRNA was similarly reduced, but its cellular level was not affected by cisplatin. Since changes in the mRNA-levels of the β-thymosins do not follow the same pattern as those of the "housekeeping" genes, it is unlikely that β-thymosin expression is regulated by the metabolic state of the cell.

Cytochemical analysis of the F-actin distribution in MCF-7 and MDA-MB231 cells

Since β-thymosins are postulated to participate in the organization of the actin skeleton, cells were stained with TRITC-phalloidin to visualize actin filaments and thus detect possible differences which may correlate with the observed changes in β-thymosin levels. When MCF-7 cells grow in medium with 10% FCS, they show an epithelial, cobblestone-like morphology of heterogeneous density. Staining cells with TRITC-phalloidin reveals virtually no actin bundles but a fuzzy network of apparently very short filaments in the cytoplasm (Fig. 5C, and Fig. 6A). At the sites of cell-cell contacts, staining appears more intense, but is very irregular. Cells grown in 10% HS are larger, spread out more on the substratum, and have discernible filaments in the cytoplasm. Actin-staining at the sites of cell-cell contacts may appear as a fine border line (Fig. 5A). Estradiol renders cells more compact but with numerous protrusions between cells and a morphology similar to cells grown in FCS (Fig. 5B). Distinct actin fibers can be detected in only a few cells.

Following a 5-day treatment with tamoxifen, MCF-7 cells appeared smaller and more tightly associated than control cells (Fig. 6A, B). TRITC-phalloidin staining showed a mainly diffuse cytoplasmic staining, but with a distinct line of intense staining where cells are in intimate contact with each other. In contrast, cells which had



◀

Fig. 7. TRITC-phalloidin staining of F-actin in MDA-MB231 cells grown for 5 days with drugs as in Fig. 3. **A** 10% FCS; **B** 1 μM tamoxifen; **C** 2 μM cisplatin. Microscope magnification: 400×

been treated with cisplatin were increased in cell size, appeared more spread out on the substratum, and sometimes contained more than one nucleus (DNA-staining, not shown). In addition, their form was less epithelial-like and there were fewer sites of cell-cell contacts. Little TRITC-phalloidin staining was detectable in regions of cell contacts, but long actin filaments could be found arranged in loose bundles in the flat, peripheral regions of the cytoplasm (Fig. 6C). The sites of substrate adhesion and the sub-membrane region were also found stained. Thus, the actin skeleton in cisplatin-treated cells appeared to be less organized at sites of cell-cell contacts and was instead more pronounced in areas of substrate adhesion.

The morphology of MDA-MB231 cells is characterized by a fibroblastoid cell form devoid of intimate cell-cell contacts. The cells show actin filaments crossing the cytoplasm particularly in the cell periphery (Fig. 7A). Intense staining of an amorphous, granular structure is located adjacent to the nucleus (DNA-staining, not shown). Following incubations with tamoxifen, MDA-MB231 cells showed no discernible changes in the actin skeleton (Fig. 7B), while cisplatin-treated cells were increased in cell size and showed long actin bundles (Fig. 7C).

Discussion

A role for β-thymosins in the manifestation of the oncogenic phenotype has been proposed for various tumors. While Tβ_{10} expression appears to be generally increased in malignant cells (Verghese Nikolakai et al. 1996; Santelli et al. 1999), the observations with Tβ_4 are less coherent (Yamamoto et al. 1993). However, few studies have addressed the absolute amounts of β-thymosins and the relationship between these two peptides in tumor cells, which is a prerequisite for their use as diagnostic marker. In this paper we, therefore, compared the expression of Tβ_4 and Tβ_{10} at both the mRNA levels and the peptide in two breast cancer cell lines under various treatments.

It is noteworthy that the levels of both β-thymosins in MCF-7 and MDA-MB231 cells are basically similar even though these two cell lines differ in several cytological and biochemical parameters: while the MCF-7 cells possess a functional estrogen receptor, have epithelial-like morphology, and grow in cobblestone-like aggregates, the MDA-MB231 cells lack functional estrogen receptors, appear more mesenchymal, and grow as a homogenous monolayer. These two cell lines also differ in their metastasizing potential, with metastasis of MCF-7 cells being stimulated by estradiol, while MDA-MB231 cells metastasize without further stimulus (Bao

et al. 1998; Couissi et al. 1997). Thus, the absolute levels of Tβ_4 and Tβ_{10} do not correlate with the presence of the estrogen receptor, actin organization or metastasizing behavior. However, it must be borne in mind that both cell lines were cultivated under the same (artificial) growth conditions.

Since tamoxifen and cisplatin both elicited growth inhibition in MCF-7 cells to a similar extent (Fig. 2), it was an unexpected result that only tamoxifen reduced the expression of both β-thymosins upon growth inhibition of MCF-7 cells. In the non-responsive MDA-MB231 cells the β-thymosin levels were unaffected by tamoxifen (Fig. 3). At first site, a likely explanation is that estrogen receptor-mediated processes downstream of estrogen receptor-binding are involved. In line with this interpretation is the result that MCF-7 cells grown with estrogen-poor serum also had a lower cellular concentration of both Tβ_4 and Tβ_{10} than cells grown with FCS. However, the lack of available estrogens alone cannot explain this reduction, since cells stimulated to grow with estradiol had similarly reduced peptide concentrations.

Changes in morphology induced by antiestrogens may, nevertheless, be associated with the expression of Tβ_4 and Tβ_{10}. In MCF-7 cells estradiol supports the formation of lamellipodial structures, which is suppressed by the antiestrogen ICI 182,780 (DePasquale 1998). Estradiol also reduces both apical cell-cell contacts and basolateral cell-substrate adhesion, which is accompanied by reduced vinculin and talin-containing plaques as well as by a rearrangement of F-actin and plakoglobin (DePasquale et al. 1994). In line with these reports is our observation that in tamoxifen-treated MCF-7 cells F-actin staining changed from a fuzzy morphology to a quite well-defined lining at the sites of cell-cell contact, suggesting a restriction in cell surface dynamics. In view of these morphological observations, the growth inhibitory effect of tamoxifen is probably also a consequence of altered estrogen-mediated cell-cell-communication. Thus, enhanced cell-cell contacts appear to correlate with reduced levels of both Tβ_4 and Tβ_{10}.

Cisplatin inhibits growth of MCF-7 cells, albeit by different mechanisms than tamoxifen (Otto et al. 1996). Also contrary to tamoxifen, cisplatin increased the cellular amount of both β-thymosins in MCF-7 and in MDA-MB231 cells, while the intracellular concentrations remained unaltered. Since cisplatin leads to an increase in cell size, it suggests that β-thymosins are upregulated to accommodate for an enlarged actin network. More actin bundles crossing the cytoplasm are observed in cisplatin-treated MCF-7 cells, but this is particularly observed in MDA-MB231 cells, where the Tβ_{10} level is selectively increased. This histological observation combined with enhanced β-thymosin expression is compatible with reports on stable overexpression of Tβ_4 and Tβ_{10} in fibroblastic cells, which show stronger substrate adhesion.

One aspect of this investigation was the comparison of the β-thymosin peptide levels with the relative levels of mRNA under various treatments, and indeed, changes in peptide levels did not always correspond with those of the corresponding mRNA. This discrepancy was most pronounced in cells grown in HS with estradiol, which had a greater reduction in the level of Tβ_4-mRNA than in the level of the peptide, when compared to cells grown in FCS. This suggests that in MCF-7 cells Tβ_4 and Tβ_{10} may be differentially regulated with estrogenic treatment. Furthermore, our data on the divergence of mRNA and peptide expression of Tβ_4 support previous studies with stimulated thymocytes (Schöbitz et al. 1991), which show that there are different controls at the transcriptional and translational levels regulating Tβ_4 expression.

Histochemical studies on tissue samples from breast cancer patients using polyclonal antibodies against Tβ_{10} have suggested a correlation between malignancy and an increased number of cells expressing Tβ_{10} epitopes (Verghese Nikolakaki et al. 1996). Comparison of normal and tumor kidney tissues by genomic and HPLC analysis showed that malignant tumor tissue had increased levels of both Tβ_4 and Tβ_{10} (Hall 1991). In a preliminary HPLC analysis of extracts from various urogenital tumors, we found almost tenfold increases in the amount of Tβ_{10} and also increases in the level of the more abundant Tβ_4 over levels from non-cancerous tissue (Otto et al. 1999). Differences in the expression of Tβ_4 and Tβ_{10} have also been observed in other cellular systems, particularly during embryonic brain development (Lugo et al. 1991) and during induction of cell death in prostate carcinoma cells (Iguchi et al. 1998).

The results presented here show that the expression of Tβ_4 and Tβ_{10}-thymosins decreased in concert upon growth inhibition by tamoxifen, while no such changes were observed with cisplatin-treated cells. This indicates that changes of cell-cell contacts and/or substrate adhesion – which are important features of metastasis – are more plausible correlates with β-thymosin expression than growth inhibition per se. Their potential diagnostic value in malignant tumors, however, will require a more profound understanding of their cellular functions.

Acknowledgments We thank Doris Jaegers for her technical assistance and Dr. H.G. Mannherz, Ruhr University of Bochum, for critical reading of the manuscript.

References

Ayscough KR (1998) In vivo functions of actin-binding proteins. Curr Opin Cell Biol 10: 102–111

Bao L, Loda M, Janmey PA, Stewart R, Anand Apte B, Zetter BR (1996) Thymosin beta 15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. Nat Medicine 2: 1322–1328

Bao L, Loda M, Zetter BR (1998) Thymosin beta15 expression in tumor cell lines with varying metastatic potential. Clin Exp Metastasis 16: 227–233

Cassimeris L, Safer D, Nachmias VT, Zigmond SH (1992) Thymosin beta 4 sequesters the majority of G-actin in resting human polymorphonuclear leukocytes. J Cell Biol 119: 1261–1270

Couissi D, Dubois V, Remacle C, Schonne E, Trouet A (1997) Western immunoblotting and enzymatic activity analysis of cathepsin D in human breast cancer cell lines of different invasive potential. Regulation by 17beta-estradiol, tamoxifen and ICI 182,780. Clin Exp Metastasis 15: 349–360

Decloitre F, Cassingena R, Estrade S, Martin M (1991) Concomitant alterations of microfilaments and microtubules in human epithelial cells (HBL-100) in relation to their malignant conversion. Tumour Biol 12: 111–119

DePasquale JA (1998) Cell matrix adhesions and localization of the vitronectin receptor in MCF-7 human mammary carcinoma cells. Histochem Cell Biol 110: 485–494

DePasquale JA, Samsonoff WA, Gierthy JF (1994) 17-beta-Estradiol induced alterations of cell-matrix and intercellular adhesions in a human mammary carcinoma cell line. J Cell Sci 107: 1241–1254

Erickson Viitanen S, Ruggieri S, Natalini P, Horecker BL (1983) Thymosin beta 10, a new analog of thymosin beta 4 in mammalian tissues. Arch Biochem Biophys 225: 407–413

Gabbiani G (1979) The cytoskeleton in cancer cells in animals and humans. Methods Achiev Exp Pathol 9: 231–243

Gierthy JF, Lincoln DW, Roth KE, Bowser SS, Bennett JA, Bradley L, Dickerman HW (1991) Estrogen-stimulation of postconfluent cell accumulation and foci formation of human MCF-7 breast cancer cells. J Cell Biochem 45: 177–187

Gold JS, Bao L, Ghoussoub RA, Zetter BR, Rimm DL (1997) Localization and quantitation of expression of the cell motility-related protein thymosin beta15 in human breast tissue. Mod Pathol 10: 1106–1112

Golla R, Philp N, Safer D, Chintapalli J, Hoffman R, Collins L, Nachmias VT (1997) Co-ordinate regulation of the cytoskeleton in 3T3 cells overexpressing thymosin-beta4. Cell Motil Cytoskeleton 38: 187–200

Hall AK (1991) Differential expression of thymosin genes in human tumors and in the developing human kidney. Int J Cancer 48: 672–677

Hall AK, Hempstead J, Morgan JI (1990) Thymosin beta 10 levels in developing human brain and its regulation by retinoic acid in the HTB-10 neuroblastoma. Brain Res Mol Brain Res 8: 129–135

Hannappel E (1986) One-step procedure for the determination of thymosin beta 4 in small tissue samples and its separation from other thymosin beta 4-like peptides by high-pressure liquid chromatography. Anal Biochem 156: 390–396

Hannappel E, Leibold W (1985) Biosynthesis rates and content of thymosin beta 4 in cell lines. Arch Biochem Biophys 240: 236–241

Hannappel E, van Kampen M (1987) Determination of thymosin beta 4 in human blood cells and serum. J Chromatogr 397: 279–285

Hannappel E, Wartenberg F, Bustelo XR (1989) Isolation and characterization of thymosin beta 9 Met from pork spleen. Arch Biochem Biophys 273: 396–402

Huff T, Müller C, Hannappel E (1997) HPLC and post-column derivatization with fluorescamine isolation of actin-sequestering β-thymosins by reversed-phase HPLC. Analytica Chimica Acta 352: 239–248

Huff T, Ballweber E, Humeny A, Bonk T, Becker C, Muller CS, Mannherz HG, Hannappel E (1999) Thymosin beta(4) serves as a glutaminyl substrate of transglutaminase. Labeling with fluorescent dansylcadaverine does not abolish interaction with G-actin. FEBS Lett 464: 14–20

Huff T, Müller CS, Otto AM, Netzker R, Hannappel E (2001) beta-Thymosins, small acidic peptides with multiple functions. Int J Biochem Cell Biol 33: 205–220

Iguchi K, Hamatake M, Ishida R, Usami Y, Adachi T, Yamamoto H, Koshida K, Uchibayashi T, Hirano K (1998) Induction of necrosis by zinc in prostate carcinoma cells and identification of proteins increased in association with this induction. Eur J Biochem 253: 766–770

Kreis T, Vale R (eds) (1993) Guidebook to the cytoskeletal and motor proteins. Oxford University, Oxford

Lugo DI, Chen SC, Hall AK, Ziai R, Hempstead JL, Morgan JI (1991) Developmental regulation of beta-thymosins in the rat central nervous system. J Neurochem 56: 457–461

Mohamood AS, Gyles P, Balan KV, Hollis VW, Eckberg WR, Asseffa A, Han Z, Wyche JH, Anderson WA (1997) Estrogen receptor, growth factor receptor and protooncogene protein activities and possible signal transduction crosstalk in estrogen dependent and independent breast cancer cell lines. J Submicrosc Cytol Pathol 29: 1–17

Nachmias VT(1993) Small actin-binding proteins: the beta-thymosin family. Curr Opin Cell Biol 5: 56–62

Otto AM, Faderl M, Schönenberger H (1991) Dissociation of estrogenic and cytotoxic properties of an estrogen receptor-binding platinum complex in human breast cancer cell lines. Cancer Res 51: 3217–3223

Otto AM, Paddenberg R, Schubert S, Mannherz HG (1996) Cell-cycle arrest, micronucleus formation, and cell death in growth inhibition of MCF-7 breast cancer cells by tamoxifen and cisplatin. J Cancer Res Clin Oncol 122: 603–612

Otto AM, Huff T, Hannappel E (1997) Changes in the levels of β-thymosins in a human breast cancer cell line under various conditions of growth. Eur J Cell Biol 72[suppl 43]: A195

Otto AM, Huff T, Hannappel E (1999) Changes in the level of β-thymosins, small actin-binding proteisn, in tumor cells: a possible role in growth and malignancy. J Cancer Res Clin Oncol 125: S91

Pollack R, Osborn M, Weber K (1975) Patterns of organization of actin and myosin in normal and transformed cultured cells. Proc Natl Acad Sci 72: 994–998

Pollard TD (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. Ann Rev Biochem 55: 987–1035

Sanders MC, Goldstein AL, Wang Y-L (1992) Thymosin β4 (Fx peptide) is a potent regulator of actin polymerization in living cells. Proc Natl Acad Sci USA 89: 4678–4682

Sanger JM, Golla R, Safer D, Choi JK, Yu KR, Sanger JW, Nachmias VT (1995) Increasing intracellular concentrations of thymosin beta 4 in PtK2 cells: effects on stress fibers, cytokinesis, and cell spreading. Cell Motil Cytoskeleton 31: 307–322

Santelli G, Califano D, Chiappetta G, Vento MT, Bartoli PC, Zullo F, Trapasso F, Viglietto G, Fusco A (1999) Thymosin beta-10 gene overexpression is a general event in human carcinogenesis. Am J Pathol 155: 799–804

Schobitz B, Netzker R, Hannappel E, Brand K (1991) Cell-cycle-regulated expression of thymosin beta 4 in thymocytes. Eur J Biochem 199: 257–262

Sun H-Q, Kwiatkowska K, Yin HL (1996) β-Thymosins are not simple actin monomer buffering proteins. Insight from overexpression studies. J Biol Chem 271: 9223–9230

Sutherland RL, Green MD, Hall RE, Reddel RR, Taylor IW (1984) Tamoxifen induces accumulation of MCF-7 human mammary carcinoma cells in the G0/G1 phase of the cell cycle. Eur J Cancer Clin Oncol 19: 615–621

Verghese Nikolakaki S, Apostolikas N, Livaniou E, Ithakissios DS, Evangelatos GP (1996) Preliminary findings on the expression of thymosin beta-10 in human breast cancer. Br J Cancer 74: 1441–1444

Yamamoto T, Gotoh M, Kitajima M, Hirohashi S (1993) Thymosin beta-4 expression is correlated with metastatic capacity of colorectal carcinomas. Biochem Biophys Res Commun 193: 706–710

Yu FX, Lin SC, Morrison Bogorad M, Atkinson MA, Yin HL (1993) Thymosin beta 10 and thymosin beta 4 are both actin monomer sequestering proteins. J Biol Chem 268: 502–509

Yu FX, Lin SC, Morrison Bogorad M, Yin HL (1994) Effects of thymosin beta 4 and thymosin beta 10 on actin structures in living cells. Cell Motil Cytoskeleton 27: 13–25