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## The antiproliferative activity of the tetrapeptide Acetyl-N-SerAspLysPro, an inhibitor of haematopoietic stem cell proliferation, is not mediated by a thymosin $\beta$ 4-like effect on actin assembly

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**Abstract.** Acetyl-N-SerAspLysPro (AcSDKP), known as a negative regulator of haematopoiesis, has been principally reported as an inhibitor of haematopoietic pluripotent stem cell proliferation. The tetrapeptide sequence is identical to the N-terminus of thymosin  $\beta$ 4 (T $\beta$ 4), from which it has been suggested that it may be derived. Recently, evidence was shown that T $\beta$ 4 plays a role as a negative regulator of actin polymerization leading to the sequestration of its monomeric form. The structural similarity between the N-terminus of T $\beta$ 4 and AcSDKP has raised the possibility that AcSDKP may also participate in intracellular events leading to actin sequestration.

The effect of T $\beta$ 4 on the proliferation of haematopoietic cells was compared to that of AcSDKP. The results revealed that T $\beta$ 4, like AcSDKP, exerts an inhibitory effect on the entry of murine primitive bone marrow cells into cell cycle *in vitro*. Qualitative electrophoretic analysis and quantitative polymerization assays were used to investigate the role of AcSDKP in actin polymerization. AcSDKP does not affect actin assembly at concentrations up to 50  $\mu$ M, and does not compete with T $\beta$ 4 for binding to G-actin. These results suggest that AcSDKP is not involved in cell cycle regulation via an effect on the process of actin polymerization.

Haematopoiesis is regulated by a set of factors that affect cell proliferation and differentiation. Among these mainly humoral factors, the tetrapeptide Acetyl-N-SerAspLysPro (AcSDKP) remains a molecule of fundamental importance and clinical interest.

AcSDKP, a tetrapeptide purified from fetal calf bone marrow, has been reported to inhibit the cycling of the murine haematopoietic pluripotent stem cell *in vivo* following cytotoxic drug administration (Lenfant *et al.* 1989, Bogden *et al.* 1991) and, as a consequence, to increase the survival of mice treated with lethal doses of chemotherapeutic agents (Bogden *et al.* 1991). AcSDKP has also been shown to block the entry of rat hepatocytes into S phase *in vivo* (Lombard *et al.* 1990) and to inhibit the entry of normal murine and human haematopoietic progenitors (Monpezat & Frindel 1989, Guigon *et al.* 1990, Cashman *et al.* 1994) and human and chicken lymphocytes (Volkov *et al.* 1996) into S phase *in vitro*. *In vivo* studies in

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mice (Monpezat *et al.* 1989) demonstrated that AcSDKP acts on haematopoietic stem cells at the transition between G<sub>0</sub> and G<sub>1</sub> or in early G<sub>1</sub> phase of cell cycle. The ability of AcSDKP to maintain primitive stem cells in a quiescent state has led to the investigation of the role of this molecule as a potential haematoprotective agent during anticancer chemotherapy. Results obtained during phase I–II clinical trials have demonstrated the haematoprotective potential of AcSDKP during chemotherapeutic regimen (Carde *et al.* 1992).

AcSDKP is constitutively produced in mice and synthesized by bone marrow cells in long-term culture (Wdzieczak-Bakala *et al.* 1990). It has been reported that the tetrapeptide may be derived from thymosin  $\beta$ 4 (T $\beta$ 4) which contains the complete N-terminal AcSDKP amino acid sequence. Incubation of [<sup>3</sup>H]T $\beta$ 4 with either intact or lysed bone marrow cells lead to the formation of [<sup>3</sup>H]AcSDKP (Grillon *et al.* 1990). Labelled tetrapeptide was not degraded under these conditions (Grillon *et al.* 1993). However, T $\beta$ 4 may not be the only potential precursor of AcSDKP. AcSDKP has been detected in the rabbit (Pradelles *et al.* 1991) where T $\beta$ 4 is present only in a T $\beta$ 4<sup>Ala</sup> form with an N-terminal AcADKP sequence.

T $\beta$ 4, a 5-kDa polypeptide, was first isolated from an extract of calf thymus and identified as an immunomodulatory factor (Low *et al.* 1981). T $\beta$ 4 appears to be present in large amounts (0.1–1% of total protein) in most tissues (Hannapel *et al.* 1982, Horecker & Morgan 1984), and although it is synthesized at a high rate (Hannapel & Leibold 1985) it is secreted at relatively low levels (Schöbitz *et al.* 1991). The presence of T $\beta$ 4 at micromolar concentrations in bone marrow, which is considered as a target tissue for AcSDKP, has been demonstrated (Grillon *et al.* 1990, Pradelles *et al.* 1991). Recently, T $\beta$ 4 has been identified as a major sequestering molecule of globular actin (G-actin) in motile and proliferating cells. It forms a 1:1 complex with actin monomers and inhibits polymerization of G-actin (Safer *et al.* 1991, Cassimeris *et al.* 1992) depending on the ATP/ADP ratio (Carrier *et al.* 1993). Since the polymerization and depolymerization of actin plays a fundamental role in cell locomotion, cell attachment and remodelling of the cytoskeleton during cell division, T $\beta$ 4 appears to be a key protein in the maintenance of cellular growth and differentiation. It has been reported that T $\beta$ 4, similarly to AcSDKP, is able to protect mice from LD<sub>50</sub> doses of cytosine arabinoside (Moscinski *et al.* 1993), possibly by inhibiting stem cell proliferation as has previously been demonstrated for AcSDKP (Bogden *et al.* 1991).

Observations which suggest that T $\beta$ 4 could inhibit stem cell proliferation, in a manner similar to AcSDKP, and recent evidence that this polypeptide acts as an intracellular inhibitor of actin polymerization has allowed us to speculate about the possible relationship between the inhibition of normal mitotic events by AcSDKP and actin polymerization. Firstly, the ability of both T $\beta$ 4 and AcSDKP to inhibit the progression of primitive murine haematopoietic cells with a high proliferative potential (HPP-CFC) into cell cycle was demonstrated. Secondly, the direct effect of AcSDKP on actin polymerization and the inhibition of T $\beta$ 4 binding to G-actin were evaluated. Under the experimental conditions used, AcSDKP was shown to exert no effect either on actin assembly or on T $\beta$ 4 binding to actin monomer. This suggests that the ability of AcSDKP to modulate proliferation of haematopoietic stem cells is not mediated by a T $\beta$ 4-like effect on actin assembly.

## MATERIALS AND METHODS

### Materials

The synthetic AcSDKP was kindly provided by IPSEN-Biotech (Paris, France). Muscle actin was purified as described by Spudich & Watt (1971) and isolated as CaATP-G-actin by gel filtration chromatography on Sephadex G-200 in buffer G (5 mM Tris HCl/0.1 mM CaCl<sub>2</sub>/

0.2 mM ATP/0.01% NaN<sub>3</sub>, pH 7.6) (Carlier *et al.* 1985) and next labelled at 80–92% with N-(1-pyrenyl)iodoacetamide as a fluorophor (Kouyama & Mihashi 1981). CaATP-G-actin was converted into MgATP-G-actin by addition of 0.2 mM EGTA and MgCl<sub>2</sub> at a concentration of one molar equivalent to G-actin and 10  $\mu$ M excess (Carlier *et al.* 1985). Thymosin  $\beta$ 4 was purified from sheep spleen according to the procedure described by Hannappel (1986) and adapted in our laboratory (Carlier *et al.* 1993). The final solutions of AcSDKP and T $\beta$ 4 were prepared in saline. G-actin used in all experimental procedures was diluted in buffer G.

#### HPP-CFC proliferation assay

The assay was carried out as described previously (McNiece *et al.* 1987, Robinson *et al.* 1992). Bone marrow cells from CBA/J mice were incubated in Dulbecco's medium ( $5 \times 10^6$  cells/ml) for 2 h at 37°C either with culture medium as control or with 3-h-conditioned medium of bone marrow cells from mice sublethally irradiated 7 days before as a crude source of stimulator. T $\beta$ 4, AcSDKP ( $2 \times 10^{-9}$  M final concentration) or medium as control were added at the beginning of the incubation. Cytosine arabinoside (AraC, 25  $\mu$ g/ml final concentration) or culture medium were then added for a 1-h incubation. Cells were washed twice prior to HPP-CFC assay. HPP-CFC were cultured using a bilayer semi-solid agar assay. The underlayer was aliquoted into 55-mm diameter petri dishes and contained complete medium (Dulbecco's medium with 20% horse serum, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin) supplemented with 0.5% melted agar, 10% conditioned medium from the WEHI 3B myelomonocytic leukaemic cell line and 10% conditioned medium from L929 fibroblast cell line. The upper layer contained  $4 \times 10^4$  bone marrow cells in 2 ml of complete medium. Quadruplicate cultures were incubated for 14 days at 37°C in a fully humidified atmosphere with 5% CO<sub>2</sub>. Twelve hours before the end of the culture, 1 ml of 0.1% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution in saline was added. Viable cells metabolize the colourless INT into an insoluble red salt which precipitates inside the cells and allows macroscopic assay of colony numbers. HPP-CFC derived macroscopic colonies (>1.5 mm) were scored. The proportion of cells in S phase (mean  $\pm$  SE) was estimated from quadruplicate cultures with addition of AraC or culture medium, in each condition (control, stimulated cells, stimulated cells in the presence of T $\beta$ 4 or AcSDKP) during the pre-incubation prior to plating. The whole experiment was repeated four times.

The effect of stimulating medium *v.* control and of the addition of T $\beta$ 4 or AcSDKP *v.* stimulating medium alone was evaluated through an analysis of variance performed on the proportion of cells in S phase, taking into account the matching of data arising from the same experiment, the intra- and inter-experiment variations of the data and the number of comparisons performed through Dunnett's *t*-test.

#### Actin polymerization measurements

Spontaneous polymerization of actin was monitored by the increase in pyrenyl actin fluorescence ( $\lambda_{exc}$ : 336 nm;  $\lambda_{em}$ : 387 nm) using a Spex Fluorolog 2 spectrofluorimeter, at a temperature of 20°C. To evaluate the direct effect of AcSDKP on actin polymerization, 10  $\mu$ M MgATP-G-actin was briefly incubated in the absence (control) or presence of either AcSDKP (10  $\mu$ M or 50  $\mu$ M) or T $\beta$ 4 (10  $\mu$ M). Polymerization measurements were carried out as described by Carlier *et al.* (1985).

To examine whether AcSDKP interfered with the sequestering effect of T $\beta$ 4 on G-actin, the polymerization assays were carried out with 10  $\mu$ M MgATP-G-actin in the presence of

two different concentrations of T $\beta$ 4 (5 and 10  $\mu$ M). The effect of AcSDKP addition to the final concentration of 10 and 100  $\mu$ M into the preparations of actin/T $\beta$ 4 mixtures was evaluated.

#### Non-denaturing PAGE

Electrophoretic analysis of the formation of the complex actin-T $\beta$ 4 in the absence or presence of AcSDKP was carried out in order to determine whether this tetrapeptide inhibits the interaction of T $\beta$ 4 with G-actin. Variable amounts of AcSDKP (0–40  $\mu$ M) were added to the mixture of 4  $\mu$ M actin/8  $\mu$ M T $\beta$ 4. Control samples contained 4  $\mu$ M actin with or without 8  $\mu$ M T $\beta$ 4. Non-denaturing polyacrylamide gels were prepared and run as described by Safer *et al.* (1990). Briefly, samples prepared as described above were supplemented with 2 M sucrose and 0.01% bromophenol blue (vol/vol) and loaded onto 7.5% gel. Electrophoresis was performed at 4°C in Tris/glycine buffer pH 8.3 containing 0.2 mM ATP at 150 V for 4 h, after a pre-run of 1 h. Proteins were visualized by staining the gels for 1 h with 0.2% Coomassie blue.

## RESULTS

#### *In vitro* effect of T $\beta$ 4 on the recruitment of HPP-CFC into S phase

An *in vitro* assay of a primitive haematopoietic precursor, characterized by its high proliferative potential, was used to evaluate the ability of T $\beta$ 4 to block the action of a stem cell proliferation stimulator present in regenerating haematopoietic tissue. As shown in Figure 1, the percentage of HPP-CFC population in S phase in normal murine marrow was significantly increased from 9% to 23% ( $P < 0.05$ ) in the presence of stimulating medium. The simultaneous presence of stimulator and  $2 \times 10^{-9}$  M T $\beta$ 4 led to a significant decrease of HPP-CFC entry into S phase (20%,  $P < 0.05$ ). Comparatively, in the presence of stimulating medium and  $2 \times 10^{-9}$  M AcSDKP, a significant decrease of HPP-CFC percentage in S phase (9%,  $P < 0.01$ ) was observed. In these experiments, the absolute number of HPP-CFC was constant in AraC non-treated cells and corresponded to  $32.1 \pm 1.4$  (mean  $\pm$  SE) for  $4 \times 10^4$  plated murine bone marrow cells.

#### Polymerization assay

Polymerization kinetics of 10  $\mu$ M MgATP-G-actin in the presence of 10 or 50  $\mu$ M AcSDKP or 10  $\mu$ M T $\beta$ 4 are presented in Figure 2a. It was shown that both a molar equivalent as well as a fivefold molar excess of AcSDKP over actin (curves 2 and 3) does not cause any change in actin polymer formation as compared with control (curve 1) while one molar equivalent of T $\beta$ 4 (curve 4) leads to a significant inhibition of actin polymerization.

In a second set of experiments the possibility that AcSDKP inhibits binding of T $\beta$ 4 to MgATP-G-actin was studied. As shown in Figure 2b, a significant inhibition of actin polymerization was noted when a molar equivalent of T $\beta$ 4 (10  $\mu$ M) was added (curve 3). A lower amount of this polypeptide (5  $\mu$ M, half molar equivalent) led only to a partial inhibition (curve 2). The presence of 10  $\mu$ M or 100  $\mu$ M AcSDKP (curves 4 and 5, respectively) in the samples of actin/T $\beta$ 4 does not change the T $\beta$ 4-induced inhibition of polymerization kinetics, indicating clearly that AcSDKP does not interfere with T $\beta$ 4 binding to G-actin.

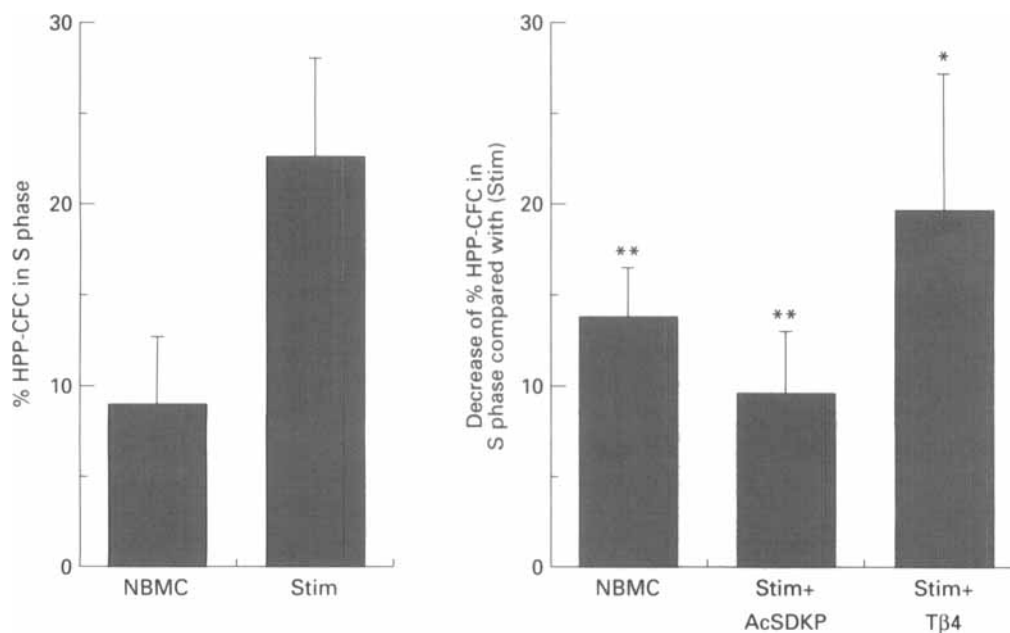
#### Electrophoretic analysis

Binding of T $\beta$ 4 to G-actin alters the electrophoretic migration of G-actin and thus can be visualized by electrophoretic analysis. Therefore, the non-denaturing gel system has been

used to evaluate the putative AcSDKP effect on T $\beta$ 4 binding to G-actin. As shown in Figure 3, the actin/T $\beta$ 4 complex (lanes 2, 5 and 8) migrates at a faster rate than actin alone (lanes 1 and 10). No alteration of actin/T $\beta$ 4 complex electrophoretic mobility was observed in the presence of AcSDKP at concentrations up to 40  $\mu$ M. The bands corresponding to the actin/T $\beta$ 4 complex (lanes 2, 5 and 8) run at a rate similar to that observed for samples composed of actin, T $\beta$ 4 and AcSDKP at 0.5  $\mu$ M (lane 3), 1  $\mu$ M (lane 4), 4  $\mu$ M (lane 6), 8  $\mu$ M (lane 7) and 40  $\mu$ M (lane 9). AcSDKP (200  $\mu$ M) alone had no effect on the electrophoretic migration of actin (data not shown).

## DISCUSSION

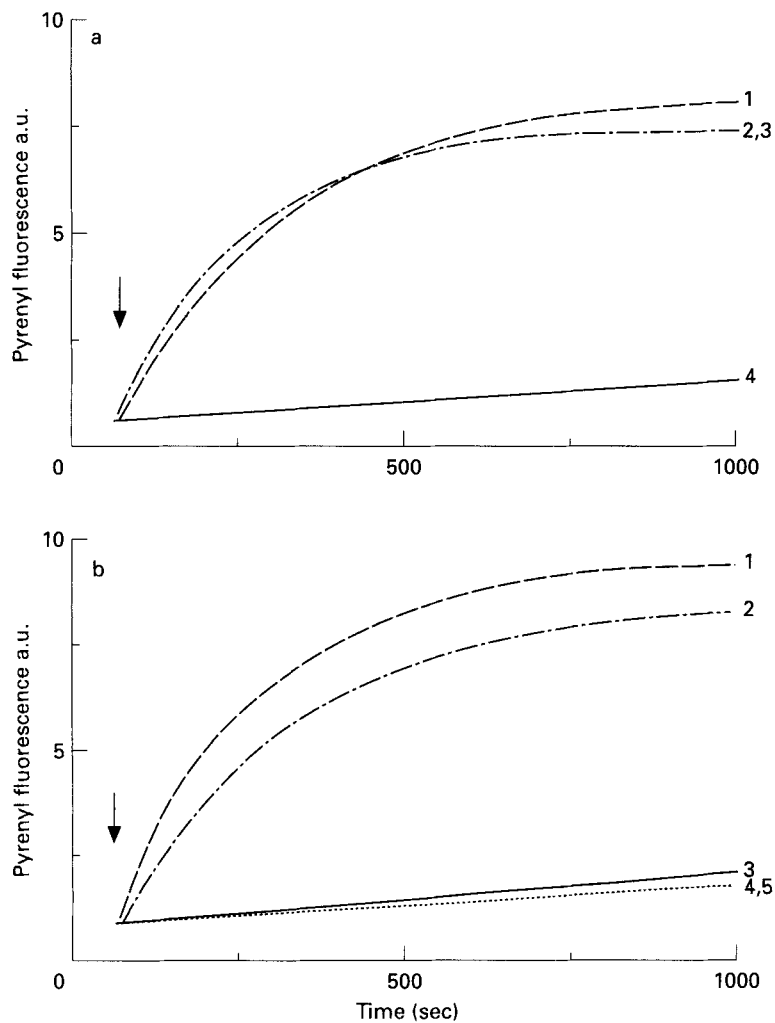
Haematopoiesis is under the control of a large number of positive and negative regulatory molecules. The tetrapeptide AcSDKP, an inhibitor of haematopoietic pluripotent stem cell proliferation, blocks the progression of stem cells into S phase *in vivo* (Lenfant *et al.* 1989, Frindel *et al.* 1992) and *in vitro* (Bonnet *et al.* 1993). This inhibitory effect is dose dependent, and is maximal at  $10^{-9}$  M. At less than  $10^{-12}$  M and in excess of  $10^{-7}$  M (Bonnet *et al.* 1995) no effects are observed. The tetrapeptide has been demonstrated to spare the stem cells from chemotherapy-induced depletion. Recent observations have also shown the haematoprotective effect of T $\beta$ 4 against AraC toxicity, suggesting a potentially similar role for this 5 kD



**Figure 1.** Effect of T $\beta$ 4 on the entry of HPP-CFC into cycle. Normal murine bone marrow cells were incubated with control medium (NBMC), with stimulating medium alone (Stim) or in the presence of  $2 \times 10^{-9}$  M T $\beta$ 4 (Stim+T $\beta$ 4) or  $2 \times 10^{-9}$  M AcSDKP (Stim+AcSDKP). AraC or medium was added and cells were then plated. Colonies were counted following 14 culture days and the percentage of HPP-CFC in S phase was calculated from quadruplicate cultures. Results are presented as the % of HPP-CFC in S phase for the control (Stim), and as the decrease of % of HPP-CFC in S phase compared with the control (Stim) (mean  $\pm$  SEM of four experiments). \* $P$ <0.05, \*\* $P$ <0.01 in the comparison to (Stim) and according to Dunnett's *t*-test.

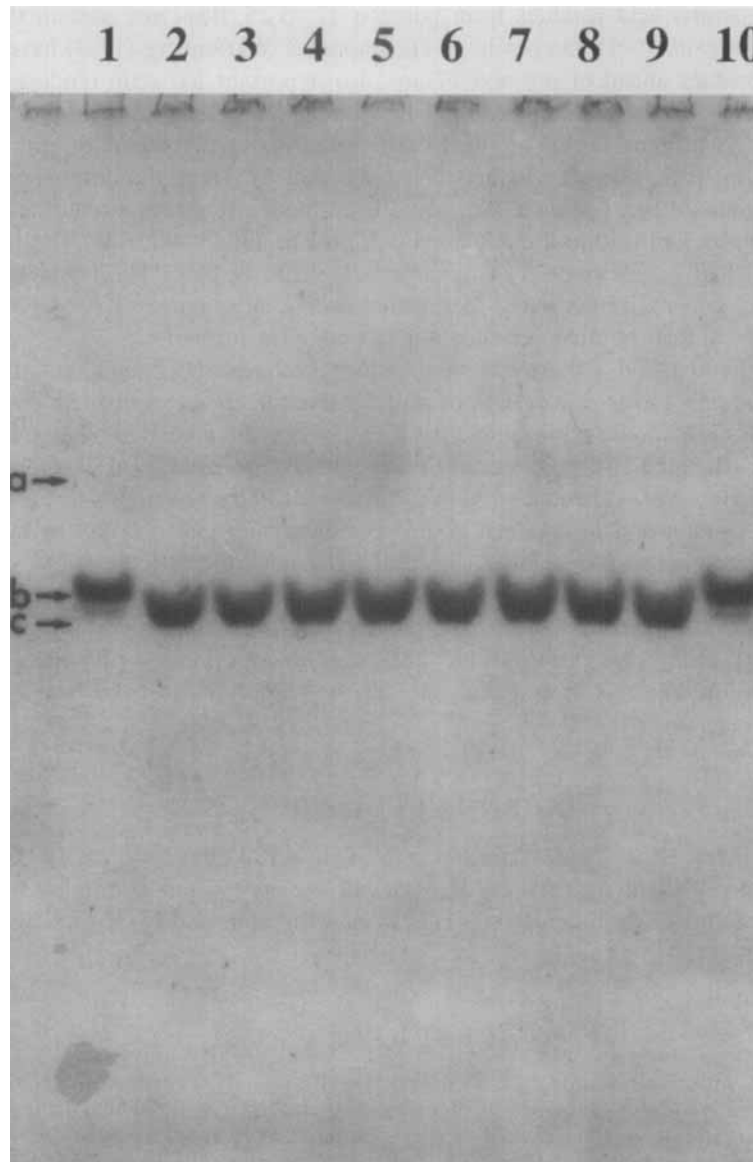
ubiquitous polypeptide (Moscinski *et al.* 1993). An HPP-CFC proliferation assay, used previously to demonstrate the activity of AcSDKP (Robinson *et al.* 1992, Robinson *et al.* 1993), was developed to evaluate the biological activity of T $\beta$ 4. Here we report the ability of T $\beta$ 4 to decrease the proportion of stimulated HPP-CFC in S phase in a manner similar to that observed for AcSDKP and at the same nanomolar concentrations.

It is well established that T $\beta$ 4 expression is cell-cycle regulated. A transient induction of T $\beta$ 4 mRNA was demonstrated either in S phase alone or in S phase and then at G<sub>2</sub>/M phase in thymocytes (Schöbitz *et al.* 1990, Schöbitz, Hannappel & Brand 1991, Schöbitz *et al.* 1991, Otero *et al.* 1993), splenic cells and hepatocytes (Otero *et al.* 1993) triggered into cell cycle



**Figure 2.** Polymerization time course of G-actin in the presence of T $\beta$ 4 and AcSDKP. (a) MgATP-G-actin (10  $\mu$ M) was polymerized spontaneously in the absence (1) or in the presence of 10  $\mu$ M T $\beta$ 4 (4) or 10  $\mu$ M or 50  $\mu$ M AcSDKP (2 and 3); 0.1 M KCl and 1 mM MgCl<sub>2</sub> were added at the time indicated by the arrow; (b) MgATP-G-actin (10  $\mu$ M) was polymerized spontaneously in the absence (curve 1) or in the presence of the following additions: 5  $\mu$ M T $\beta$ 4 (curve 2); 10  $\mu$ M T $\beta$ 4 (curve 3); 10  $\mu$ M T $\beta$ 4 and 10  $\mu$ M AcSDKP (curve 4); 10  $\mu$ M T $\beta$ 4 and 100  $\mu$ M AcSDKP (curve 5).

*in vitro* or *in vivo*. Recently, Zalvide *et al.* (1995) also evidenced a pronounced increase in T $\beta$ 4 mRNA levels at the G<sub>1</sub>/S transition of NIH 3T3 cells induced to proliferate after serum restoration. These variations of T $\beta$ 4 transcripts are observed throughout the cell cycle, and together with the ability of T $\beta$ 4 to bind to G-actin at an equilibrium dissociation constant in the micromolar range (Weber *et al.* 1992), suggest a possible involvement of this polypeptide in intracellular actin redistribution and in the changes in the cytoskeleton which occur during



**Figure 3.** Identification of G-actin/T $\beta$ 4 complex formation by non-denaturing acrylamide gel electrophoresis. 4  $\mu$ M actin alone (lanes 1 and 10); 4  $\mu$ M actin + 8  $\mu$ M T $\beta$ 4 (lanes 2, 5 and 8); 4  $\mu$ M actin + 8  $\mu$ M T $\beta$ 4 + 0.5  $\mu$ M (lane 3), 1  $\mu$ M (lane 4), 4  $\mu$ M (lane 6), 8  $\mu$ M (lane 7) or 40  $\mu$ M AcSDKP (lane 9); (a) T $\beta$ 4, (b) actin, (c) actin/T $\beta$ 4 complex.



the cytokinesis. The intracellular presence of both T $\beta$ 4 and AcSDKP at micromolar concentrations (Pradelles *et al.* 1990, Pradelles *et al.* 1991) and the fact that AcSDKP sequence is present as an N-terminal part of T $\beta$ 4 prompted us to evaluate the possible effect of AcSDKP on actin polymerization.

The possibility of a direct effect of the tetrapeptide in this process was examined. No G-actin binding was observed in the presence of a fivefold excess of AcSDKP. A further series of experiments was carried out in order to determine the competitive effect of AcSDKP on actin/T $\beta$ 4 complex formation. Although, the actin-binding sequence of T $\beta$ 4, including the amino acid residues from position 17 to 25, does not contain the AcSDKP fragment (Safer *et al.* 1991), the results of Hannappel & Wartenberg (1993) have shown that N-terminal residues ahead of position 17 are also important for actin binding. The 13–43 fragment of T $\beta$ 4 failed to inhibit actin polymerization. Therefore some additional structural features in the N-terminal region are likely to be necessary for the biological function of T $\beta$ 4. It was of interest to investigate whether the presence of AcSDKP, the tetrapeptide sequence at the N-terminus of the T $\beta$ 4 molecule, could itself induce some conformational changes in T $\beta$ 4/actin complex and lead to a modulation of T $\beta$ 4/actin binding kinetics. The results reveal that AcSDKP had no effect on T $\beta$ 4 binding to G-actin. Native PAGE analysis of formed complexes and polymerization assays performed over a large range of AcSDKP concentrations showed no effect of AcSDKP on actin/T $\beta$ 4 complex formation.

Although the detailed pathway through which both AcSDKP and T $\beta$ 4 regulate cell proliferation at nanomolar concentrations remains unclear, these results help to rule out the possibility that their similar antiproliferative activities may be mediated by an effect on actin assembly. AcSDKP, the N-terminal amino acid sequence of T $\beta$ 4, appeared to play no role in this process. However, micromolar concentrations of intracellular T $\beta$ 4 are required to interact with G-actin and to create a pool of its monomeric form useful to mediate actin assembly in response to extracellular signalling. The inhibitory effect of T $\beta$ 4 on stem cell proliferation reported here at nanomolar concentrations is potentially mediated by an independent extracellular mechanism. The present results do not exclude the possibility that exogenous T $\beta$ 4, at low concentrations ( $10^{-9}$  M), may act as a precursor for the generation of AcSDKP (Grillon *et al.* 1990) and that this may be responsible for the haemoregulatory activity attributed to T $\beta$ 4.

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