PhD thesis

Isoform-dependent effects of tropomyosins on actin dynamics

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I. Introduction

I.1. Actin

The microfilament system orchestrates a great number of diverse cellular processes, including the establishment of cell geometry, cell motility, intracellular transport, contractility, cytokinesis and transformation. Structurally, the basic building blocks of microfilaments are actin and tropomyosin (TM) (Vindin and Gunning 2013). Actin was discovered by Brunó Straub, in the laboratory of Szent-Györgyi in 1942.

Actin molecular weight is 42.3 kDa and it is composed of 375 amino acids. In cells there are two forms of actin, the monomer or globular (G-actin), and the polymer or filament (F-actin) forms. The process during the monomeric form assembles into filaments entitled polymerization. It is also possible that the monomer actin dissociates from the end of a filament which process is termed depolimerization. The polymerization of the actin has three main phases. The first phase is the nucleation where two or three monomers bind to form a nucleus. The next phase of the polymerization is the diffusion-controlled elongation where monomers incorporate into the forming filament. In the last stage a dynamic equlibrum, the so-called „treadmilling" is dominant. In this phase there is association and dissociation on both ends of a filament but the kinetics at each end are different. On one end of the filament monomers mainly incorporate to the filament, while ont he other end they rather detach from the filament. The promoters at the growing end bind ATP, while the promoters in the shortening end bind ADP because of the hydrolysis of ATP.

I.2. Actin-binding proteins

Tropomyosin

Tropomyosins are elongated, rod-shaped molecules with extensive a-helical conformation, forming parallel-chained coiled-coil dimers. The individual dimers assemble into helical polymers in a head-to-tail manner, cooperatively associating along the longitudinal axis of the actin filament on both sides (Coulton et al. 2008; Flicker, Phillips, and Cohen 1982). In animal and fungal cells, most microfilaments are decorated with tropomyosins (Tpm) that, in addition to conferring actin isoform diversity, substantially contribute to the formation of the individual

filament subcompartments (Gateva et al. 2017; Vindin and Gunning 2013). In mammals, four Tpm genes were demonstrated to produce >40 mRNA variants and ~25 isoforms at the protein level (Gunning et al. 2005). Tropomyosins are always present as polar coiled-coil dimers that cooperatively polymerize in a head-to-tail manner and bind along the groove of the actin filament (Flicker et al. 1982). Traditionally, tropomyosin isoforms involved in the contractile apparatus of muscle cells are referred to as muscle isoforms, while isoforms associated to the cell cytoskeleton are referred to as nonmuscle or cytoskeletal tropomyosins. The expression and localization of tropomyosin isoforms are strictly regulated at both tissue and subcellular level, and often depend on the developmental state as well (Gunning et al. 2005; Gunning, O'neill, and Hardeman 2008). Since the first discovery of TMs in the nervous system several neuronal isoforms have been described (Fine et al. 1973).

Tpm1.11 and Tpm1.12 are short, while Tpm1.10 is a long isoform, as characterized by the use of exon 1a and 2b or exon 1b at the N-terminus, respectively. The γ-Tm gene also encodes tropomyosin isoforms present in neurons, like Tpm3.1 and Tpm3.2, both of them are short isoforms, carrying exon 1b. These isoforms exhibit different subcellular distributions and developmental profiles, as well, suggesting their functional specialization for different neuronal processes.

Gelsolin

Gelsolin belongs to a superfamily of structurally related actin-binding proteins (Nag et al. 2013). Gelsolin contains 6 gelsolin homology domains, named from terminal N to terminal C, in the order GH1-GH6. Gelsolin is found both in subcellular and in extracellular (Koya et al. 2000). Gelsolin is actually a multifunctional regulator of actin dynamics. One gelsolin molecule can bind two actin monomers (McGough et al. 2003; Silacci et al. 2004). In the cytoplasm, gelsolin generally exists as a single isoform. In vitro, gelsolin is able to both nucleate and sever actin filaments, and it also caps the actin-filament barbed ends (Finidori et al. 1992; Harris and Weeds 1984; Yin et al. 1981).

Gelsolin was discovered as a factor inhibiting the sol-gel transition of the cortical actin cytoskeleton in macrophages (Yin and Stossel 1979). These activities require the binding of Ca^{2+} to several conserved sites of the protein characterized by different affinities (Nag et al. 2013).

Calcium binding unlatches the compact globular structure of gelsolin (Burtnick et al. 1997), allowing it to extend into a conformation with active binding sites for G-actin and F-actin on gelsolin-homology domains 1, 4, and 2–3 (Burtnick et al. 1997; McLaughlin et al. 1993; Robinson et al. 1999).

II. Main objectives

Binding of tropomyosin isoforms to actin filaments can exhibit a high degree of cooperativity, in which the formation of so called head-to-tail interactions between the N-, and C-termini of neighboring tropomyosin molecules are crucial. Minor modifications in the amino acid sequence can affect actin binding and consequently the function of tropomyosins. To better understand the function of the Tpm1.12 and Tpm3.1 isoforms within the cell, their interaction with actin wanted to study the biochemical and biophysical approaches.

Our objectives briefly classified into the following headings:

1) We produced the Tpm1.12 and TPM3.1 tropomyosin isoforms as recombinant proteins in sufficient amount for in vitro characterization of their interactions with actin.
2) To test the ability of recombinant Tpm1.12 and TPM3.1 to bind actin filaments (F-actin) cosedimentation assays we designed cosimentation assays.
3) The effect of the Tpm isoforms on the formation of actin filament was investigated by polymerization tests.
4) The aim of our research was to get acquainted with the effect of Tpm1.12 and Tpm3.1 isoforms on the actin polymerization catalyzed by VCA-Arp2/3 complex.
5) To find out how these tropomyosins stabilize the actin filament we wanted to carry out depolymerization tests.
6) We were curious whether Tpm1.12 and Tpm3.1 could bind to gelsolin.
7) We wanted to investigate whether these tropomyosins protect the actin filament against the depolymerizing effect of gelsolin.
8) Furthermore, we were curious as to how the activity of gelsolin changes when complexed with tropomyosin. Does the dynamics, polymerization and depolymerization of the actin filament ends affect if the gelsolin complexes with tropomyosin?

III. Experimental methods

III.1. Purification of proteins

III.1.1. Preparation of actin

Actin was prepared from rabbit hind leg muscle (Spudich and Watt 1971) and gel filtered on a Superdex G75 (GE Healthcare, Little Chalfont, UK) column in buffer A (4 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 0.005% NaN_3 (pH 7.8)). G-actin was stored on ice in buffer A. For the fluorescence measurements, actin was labeled with pyrenyl-iodoacetamide as described previously (pyrene; Invitrogen, Carlsbad, CA) (Kouyama and Mihashi 1981).

III.1.2. Molecular cloning and purification of Tpm1.12 and Tpm3.1

Tpm1.12 and Tpm3.1 isoforms were cloned into a pET28a expression plasmid, and Tpm expression in Escherichia coli BL21 (DE3) cells, and purification was carried out as described previously, with slight modifications. The lysis buffer for the resuspended bacterial pellets additionally contained 10 mM imidazole, 1 mM β-mercaptoethanol, 1% Triton-X 100, and 2 mM $CaCl_2$, and the lysate was centrifuged at 440,000 x g for 1 h at 4 °C. The protein concentration was measured using a BCA protein assay kit (Sigma, St. Louis, MO), and the protein preparations were stored at 0°C in 10 mM Tris, 10 mM KCl, and 1 mM dithiothreitol (pH 7.8).

III.1.3. Purification of skTM

skTM was purified from the remnants of the actin acetone powder [Smillie, 1982], then applied to hydroxyapatite chromatography, and stored frozen in 5 mM Tris pH 7.8 and 1 mM DTT. For the fluorescent measurements actin was labeled with pyrenyl-iodoacetamide as described previously (pyrene, Invitrogen) (Kouyama and Mihashi 1981). The protein concentrations were measured photometrically using ε_{280}=1.11 $ml*mg^{-1}*cm^{-1}$ and ε_{290}=0.63 $ml*mg^{-1}*cm^{-1}$ extinction coefficients for actin and ε_{280}=0.3 $ml*mg^{-1}*cm^{-1}$ for skTM.

III.1.4. Purification of VCA-Arp2/3

Arp2/3 complex from bovine brain and recombinant GST-tagged human VCA were purified as described previously (Egile et al. 1999).

III.1.5. Preparation of gelsolin

For the preparation of gelsolin, a His-tagged full-length sequence in a pET21d(+) vector was used (Nag et al. 2009). Plasmid DNA was transformed into E. coli BL21 (DE3) cells. The protein concentration was measured by spectrophotometry (ε_{280}=1.29 ml*mg^{-1}*cm^{-1}).

III.1.6. Preparation of TEV protease

TEV protease was purified using a 6x His-tag pET24 (TEV-235) plasmid construction (a kind gift of Hüseyin Besir, EMBL) in E.coli BL21 DE3 cells. The protein concentration was determined photometrically using ε_{280}= 51.19 ml*mg^{-1}*cm^{-1}. The final glycerol content was adjusted to 50% and the samples were stored at -20°C.

III.2. Experimental Methods

III.2.1. Co-Sedimentation Assays

Twenty micromolar Mg-G-actin in buffer A was polymerized with 2 mM $MgCl_2$ and 100 mM KCl, then the F-actin solution was diluted to 5 mM in the absence or presence of different concentrations of tropomyosin in polymerization buffer [buffer A supplemented with 2 mM $MgCl_2$ and 100 mM KCl (final concentrations)]. Samples (60 µl) were incubated over night at 4°C, then pelleted (440,000 x g for 30 min at 4°C, Beckman-Coulter, TLA-100 rotor). Pellets and supernatants were separated and analyzed by SDS-PAGE [Laemmli, 1970]. The protein bands on the gel were quantified using densitometry (GeneTools) after Coomassie staining. Control experiments showed that Coomassie stains actin and these tropomyosin isoforms equally (data not shown). The TM:actin ratio in the pellet was calculated and plotted as a function of tropomyosin concentration. The data were fit using the following equation:

$$S = \frac{1}{2} * \left[\left([TM]_0 + [A]_0 + K_d \right) * \frac{S_{max}}{[A]_0} - \sqrt{\left(\left([TM]_0 + [A]_0 + K_d \right) * \frac{S_{max}}{[A]_0} \right)^2 - 4 * \frac{S^2_{max}}{[A]_0} * [TM]_0} \right]$$

where S is the TM: actin ratio in the pellet, S_{max} is the maximum value of S measured at saturating TM concentration, $[TM]_0$ and $[A]_0$ are the total concentration of TM and actin in the samples, respectively and K_d is the dissociation equilibrium constant of the TM-F-actin complex.

The co-sedimentation of gelsolin with actin and Tpms was studied in two complementary experiments. 1) First, 2 mM gelsolin was added to 25 mM F-actin and incubated for 1 h; then, the samples were diluted to 10 mM actin concentration and 0.8 mM gelsolin with different Tpm isoforms (40 mM Tpm1.12, 40 mM Tpm3.1, or 10 mM skTM), with a control sample diluted in polymerization buffer only, and let stand for 2 h. 2) Alternatively, 10 mM F-actin was incubated for 2 h with or without the same concentrations of Tpms as in protocol 1; then, the samples were treated with 0.8 mM gelsolin for 1 h. All measurements were performed using polymerization buffer containing 0.1 mM $CaCl_2$. Samples (100 mL) were pelleted by ultracentrifugation (Beckman-Coulter, TLA-100, 440,000 g for 30 min at 20C); then, pellets and supernatants were separated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were stained with Coomassie Blue and images were madewith ultraviolet illumination (SyngeneBioimaging System,Haryana,India). Theproteinbands on the gels were quantified using densitometry (software by GeneTools, Philomath, OR). The relative amounts of actin in the pellets were calculated by dividing the actin content of the samples by the actin content of the control sample containing only actin, which was prepared under the same conditions. The relative amounts of gelsolin in the pellets were derived by dividing the gelsolin content of the samples by the gelsolin content of the control sample containing only actin and gelsolin, which was prepared under the same conditions. Data from three independent measurements are given as the mean ± SE

III.2.2. Fluorescence Measurements

Fluorescence experiments were performed using a PerkinElmer or a Jobin Yvon spectrofluorimeter (Horiba Scientific). Average rates from at least three independent measurements were calculated. Data are given as the mean ± SE throughout.

III.2.2.1. Polymerization Assays

Five micromolar Mg-G-actin in buffer A (containing 5% pyrene labeled actin) was polymerized in the presence of 2 mM $MgCl_2$, 100 mM KCl (final concentrations) and different amounts of tropomyosin. Polymerization kinetics was followed by measuring the changes in pyrene fluorescence in time. The fluorescence transients were normalized and the polymerization rates were determined by fitting the linear part (0.45–0.55) of the data. Average rates from at least three independent measurements were calculated.

III.2.2.2. Depolymerization Assays

Ten micromolar Mg-G-actin in buffer A (containing 50% pyrene labeled actin) was polymerized over night by adding 2 mM MgCl2 and 100 mM KCl (final concentrations) in the absence or presence of tropomyosin. The F-actin solution was diluted to 50 nM into polymerization buffer. Depolymerization kinetics was followed by measuring the decrease in pyrene fluorescence in time. Depolymerization rates were estimated by linear fitting the first 50 sec of the pyrene transients. Average rates from at least three independent measurements were calculated. Normalized depolymerization rates were calculated as the ratio of the depolymerization rate in the absence of tropomyosin to the depolymerization rate in the presence of tropomyosin.

III.2.3. Surface plasmon resonance

The interactions of Tpms with gelsolin were analyzed by surface-Plasmonresonance (SPR)-based binding technique using the Biacore 3000 instrument (Biacore, GE Healthcare). The Tpm isoforms were directly immobilized onto the sensor chip (CMD500L; XanTec Bioanalytics, Düsseldorf, Germany) via primary amine groups of the proteins using the amine coupling method as recommended by the manufacturer. The surface was first activated by an injection of 35 µ N-ethyl-N' (dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (Biacore, GE Healthcare) solution (200 mM N-ethyl-N' (dimethylaminopropyl) carbodiimide and 50 mM N-hydroxysuccinimide); then, the Tpm was diluted to 30 µg/ml in the immobilization buffer (10 mM Na-acetate (pH 3.5)) and injected over the surface for 7 min at a 10 µl/min flow rate. Excess reactive sites were subsequently blocked by injection of 1 M ethanolamine (pH 8.5) (Biacore, GE Healthcare) for 7 min at a flow rate of 5 µl/min. The control

surface was activated and then blocked with ethanolamine. After the immobilization of the Tpms, gelsolin was diluted in actin polymerization buffer (4 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM -mercaptoethanol, 0.005% NaN_3, 2 mM $MgCl_2$, and 100 mM KCl (pH 7.8)) and injected over the surfaces at various concentrations (0.5, 1, 2, 3, 4, 5, and 7.5 mM) at a flow rate of 10 µl/min. The association phases of the interactions between gelsolin and the Tpm were monitored for 7 min and the dissociation phases in polymerization buffer without the gelsolin were monitored for 6 min to determine the kinetic parameters of association and dissociation for the interactions. The sensor chips were regenerated after each binding assay by a brief injection of 10 mM glycine-HCl (pH 2.1). The binding of gelsolin to the immobilized proteins was monitored as a sensorgram where the resonance unit values were plotted against time. The resonance unit measured at the control surface was subtracted from the data obtained for the protein surfaces. Kinetic parameters were evaluated by the BIAevaluation 3.1 software (Biacore, GE Healthcare) assuming a 1:1 gelsolin/Tpm dimer interaction between the proteins.

IV. Results and discussion

IV.1. Expression and Purification of Tpm1.12 and Tpm3.1

The full length coding sequences of the corresponding mouse tropomyosin isoforms were used for protein production. The average yield of the purified protein was 3–5 mg/L of E. coli culture. The recombinant Tpm1.12 and Tpm3.1 proteins produced a band on Coomassie-stained SDS-PAGE gel with an apparent molecular mass of approximately 37 and 31 kDa. The molecular mass of Tpm1.12 and Tpm3.1 estimated from the sequence were 28.313 and 28.948 kDa, respectively (Protparam). Mass spectrometry gave very similar results to the sequence analysis [28.301 and 28.949 kDa for Tpm1.12 and Tpm3.1].

We produced Tpm1.12 and Tpm3.1 as recombinant proteins in a nontagged form, without any excess amino acids to the native sequence, because any extension at the polypeptide ends may cause unpredictable, even deleterious effects (Bharadwaj et al. 2004). We successfully purified these tropomyosin isoforms in sufficient amount for in vitro characterization of their interactions with actin.

IV.2. Binding of Tpm1.12 and Tpm3.1 to Actin Filaments

The SDS-PAGE analysis revealed that the amount of these tropomyosin isoforms cosedimented with F-actin was increased in a concentration dependent manner, suggesting that both Tpm1.12 and Tpm3.1 bound to actin filaments. As controls, Tpm1.12 and Tpm3.1 at different concentrations were also centrifuged in the absence of F-actin. We found that a small amount of tropomyosin appeared in the pellet even without F-actin. The amounts of the pelleted tropomyosin in the absence of actin were quantified at each concentration and used for the correction of the Tpm1.12 and Tpm3.1 bands obtained with the actin-containing samples. The corrected TM:F-actin band intensity ratios were plotted as a function of tropomyosin concentration (Fig.1.).

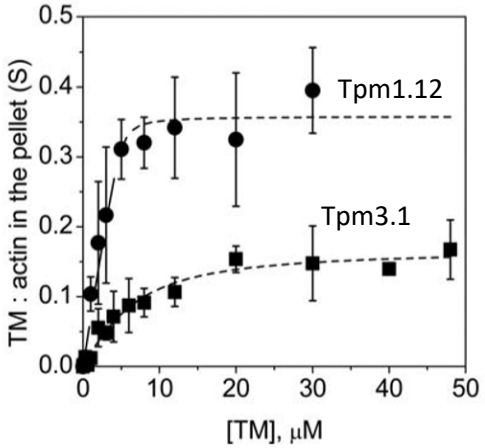


Fig. 1.: Stoichiometric ratio of tropomyosin to actin in the pellet

We found that both Tpm1.12 and Tpm3.1 bind actin filaments, however, their affinities for actin differ (Fig. 2). Tpm1.12 binds F-actin with approximately seven-fold lower affinity (Kd=3.48±0.92 μM) than skTM (Kd=0.5 μM) (Boussouf and Geeves 2007), while Tpm3.1 has some what tighter affinity (Kd=0.10±0.16 μM) than skTM. These differences in the binding strength are consistent with previous results, showing that the affinity of tropomyosins to actin strongly depends on the specific combination of exons encoding the N-, and C-terminus of tropomyosins (Moraczewska, Nicholson-Flynn, and Hitchcock-DeGregori 1999). For the tropomyosin isoforms studied in this paper, the 1b1d exon pair is expected to endow the Tpm3.1 isoform with the highest affinity, exons 1a9a with acetylation (for tissue purified skTM) results in somewhat lower affinity, while the 1b9c exon combination in Tpm1.12 results in poor binding to actin filaments. Our results support the view that the N- and Ctermini of tropomyosins are important determinants of actin affinity by establishing the ability of

tropomyosin isoforms to form end-to-end polymers along the actin filament. The lower affinity of Tpm1.12 binding to actin filaments is also consistent with previous findings in showing that brain isoforms isolated from tissue bind actin with approximately 10-fold lower affinity than muscle isoforms (Broschat and Burgess 1986).

IV.3. Effects of Tpm1.12 and Tpm3.1 on Actin Filament Assembly

To study the effect of Tpm1.12 and TPM3.1 on the kinetics of actin assembly, polymerization assays were performed. To quantify our observations the polymerization rates were derived by linear fitting to the 0.45–0.55 range of the normalized pyrene traces. In agreement with previous observations, we found that 6 μM skeletal muscle tropomyosin (skTM) slowed down the assembly of 5 μM actin by ~60% (Fig. 2). Note that considering the affinity of skTM to actin (Kd=0.5 μM) the 6 μM skTM saturated the binding sites on actin in equilibrium. 40 μM Tpm1.12 and 17 μM Tpm3.1 (more than 10-fold greater than the corresponding Kd values) had only minor effect on actin polymerization (Fig. 2). Similarly to skTM, Tpm1.12 reduced the rate of actin assembly. While Tpm3.1 slightly enhanced actin assembly rate.

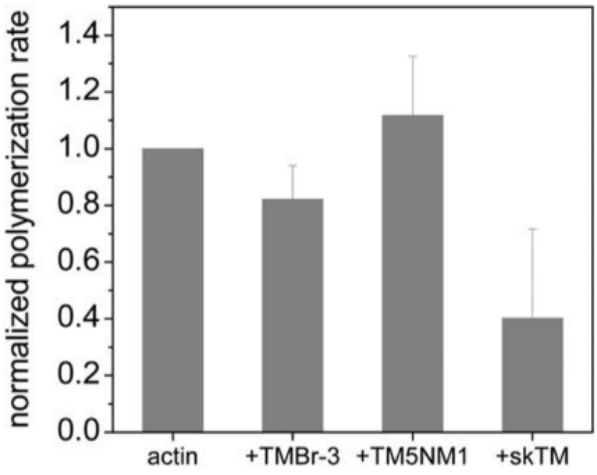


Fig.2.: Normalized rate of actin polymerization 5 μM G-actin (5% pyrene labeled) in the absence and presence of different tropomyosin isoforms, as indicated.

IV.4.: Effects of Tpm1.12 and Tpm3.1 on the Arp2/3 Complex-Catalyzed Actin Assembly

Previous biochemical studies revealed that some tropomyosin isoforms inhibit the Arp2/3 complex-catalyzed actin assembly (Blanchoin et al. 2001; Bugyi, Didry, and Carlier 2010). Cell biological studies revealed that Tpm1.12 and Tpm3.1 had opposite effect on lamellipodia formation and cell migration (Bryce et al. 2003). To study the effect of Tpm1.12 and Tpm3.1 on Arp2/3 complex activity polymerization assays were performed. We found that similarly to skTM, Tpm3.1 inhibited the VCA-Arp2/3 complex-catalyzed actin assembly in a concentrationdependent manner. At the highest Tpm3.1 concentration we applied the inhibition was ~25%. Interestingly, we found that Tpm1.12 had no effect on Arp2/3 complex-catalyzed actin assembly (Fig. 3).

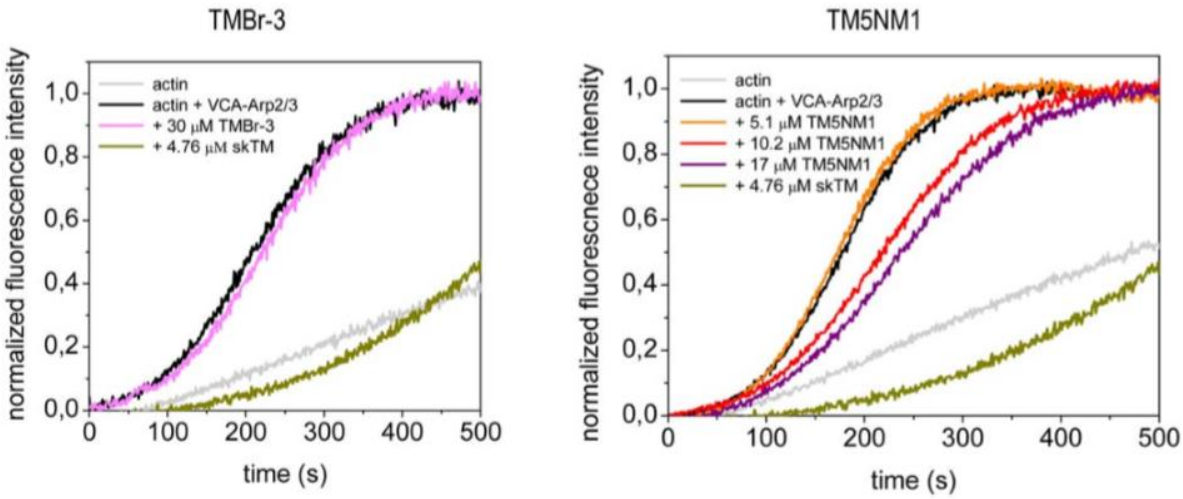


Fig.3.: Time courses of VCA-Arp2/3 complex catalyzed actin assembly monitored by the change in pyrene fluorescence in the absence (black line) and presence of different tropomyosin isoforms, as indicated.

IV.5.: Effects of Tpm1.12 and Tpm3.1 on Actin Filament Disassembly

To study depolymerization, actin (2 μM, containing 50% pyrene labeled actin) was first polymerized and then diluted into polymerization buffer to reach final concentration of 50 nM. This strategy ensures that after dilution the actin concentration is less than the critical concentration, which results in actin filament disassembly dominated by barbed end dynamics. The depolymerization kinetics of actin filaments was followed by monitoring pyrene fluorescence. In the absence of tropomyosin the transients showed single decreasing phase (Fig. 4A upper panel; black curve). When filaments were preincubated with either 10 μM skTM, 40

μM Tpm1.12, or 40 μM Tpm3.1 two phases were observed (Fig. 4A upper panel). The first phase (for approximately 50 s) showed slower depolymerization than in the absence of tropomyosin. Then a second, faster phase appeared. In these experiments the buffer to which actin was diluted did not contain tropomyosin. Consequently, after the dilution (40x) the tropomyosin concentration has decreased substantially. Due to the alteration of the conditions a new equilibrium was established and most of the tropomyosin dissociated from the filaments. We attribute the first phases of the transients to the depolymerization of actin filaments in the actin–tropomyosin complex, while the second observed phase corresponded to the depolymerization after the dissociation of tropomyosin. To confirm this explanation we repeated the experiments by diluting the actin filaments into buffers containing tropomyosin at the same concentration as in the actin samples (10 μM skTM or 40 μM Tpm1.12). The transients obtained under these conditions (Fig. 4A lower panel) were single phased corroborating our conclusions regarding the effect of dissociation of tropomyosin from actin. These observations showed that all the investigated tropomyosins slowed down the depolymerization of actin filaments, and also suggested that dissociation of tropomyosin was slow and occurred on the few tens of seconds time scale. The depolymerization experiments were repeated at various tropomyosin concentrations. The first 50 sec of the transients were fitted with linear functions and the slopes were used to estimate the depolymerization rates. The data showed that these tropomyosin isoforms decreased the depolymerization rate in a concentration dependent manner (Fig. 4B). However, skTM and Tpm3.1 had an approximately two-fold greater effect on actin depolymerization than Tpm1.12, in correlation with the different affinities of these tropomyosins.

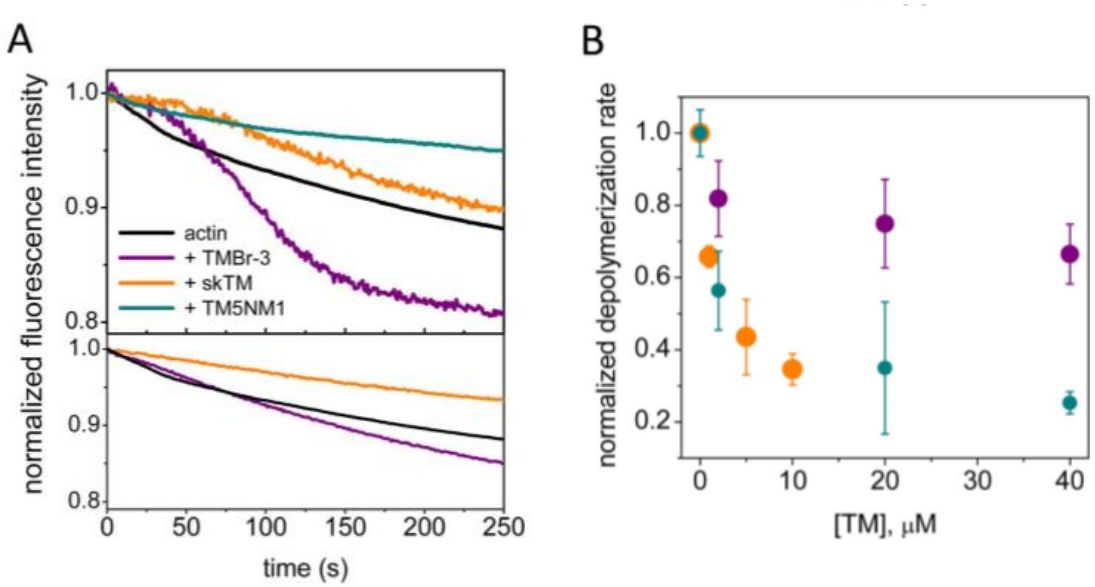


Fig.4.: (A) Depolymerization kinetics of 50 nM F-actin in the absence (black line) or presence of Tpm1.12 (40 mM; purple line), Tpm3.1 (40 mM; dark cyan line) or skTM (10 mM; orange line). The upper panel shows the results obtained when actin was diluted to buffer containing no tropomyosin. The lower panel shows examples of data from experiments in which the dilution was carried out into tropomyosin containing buffer. (B) Normalized depolymerization rates of actin filaments (derived from data similar to those presented in Fig. 3A) as a function of tropomyosin concentration ([TM]). The results are shown for Tpm1.12 (purple circles), Tpm3.1 (dark cyan circles) and skTM (orange circles).

We have carried out SPR-based binding experiments to determine the binding affinity of gelsolin for skeletal muscle and non-muscle Tpm isoforms. Tpm isoforms were immobilized by amine coupling on the surface of sensor chips, and then gelsolin was run over the surfaces at different concentrations. The sensorgrams were fitted with single-exponential functions to determine the corresponding second-order association (k_a) and first-order dissociation rate constants (k_d), and the ratios of these parameters (k_d/k_a) were used to calculate the corresponding dissociation constants (K_{DS}) for the interaction of gelsolin with skTM (A: K_D=1.9 ± 1.4 μM), Tpm1.12 (B; K_D=0.7 ±0.2 μM), and Tpm3.1 (C; K_D=0.3± 0.2 μM). These K_D values indicate relatively tight affinities between gelsolin and the Tpms, which fall within the range one would expect for physiologically significant protein-protein interactions, indicating that the binding of gelsolin to Tpms probably has functional consequences.

IV.7.: Gelsolin accelerates actin polymerization in vitro

Next, we tested whether the binding of Tpms to gelsolin affects the corresponding activities of these proteins. First, we characterized the recombinant gelsolin in a nucleation assay. Polymerization of Ca-actin was carried out in the absence or presence of gelsolin at different concentrations (2–500 nM) (Fig. 2 A). Salt-induced actin polymerization is described by the initial slow nucleation step (1–2 min), an ascending elongation phase, and a steady-state phase, where the addition and dissociation of actin monomers are inequilibrium. Addition of nanomolar concentrations of gelsolin increased the initial rate of actin assembly, as reflected by the increasing slope of the curves (Fig. 2, B and C). The time required for the slow lag phase, corresponding to the nucleationstep, became shorter within creasing gelsolin concentrations. This observation was consistent with the known nucleating activity of gelsolin (Yin et al. 1981). At higher gelsolin concentrations (>200 nM), the polymerization curves displayed an overshoot, which may be explained by the severing and monomer-sequestering activities of gelsolin. When the proportion of gelsolin relative to actin was low these effects were not dominant, since most of the gelsolin was consumed for nucleation, resulting in capped filaments. Increasing the gelsolin/actin ratio possibly leaves more free gelsolin to sever the elongating filaments, rendering them shorter. The height of the steady-state plateau phase decreased proportionally to the amount of gelsolin added (Fig. 2 A). This likely reflects gelsolin sequestration of actin monomers, preventing their incorporation into filaments and resulting in lower fluorescence intensity. All these observations indicate that the recombinant gelsolin behaves as expected based on previous reports.

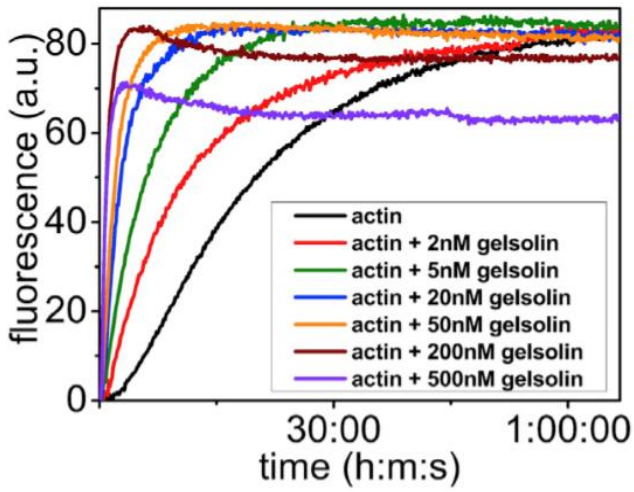


Fig.5.: Gelsolin increases the rate of actin polymerization. Gelsolin was added to 3 µM G-actin at different final concentrations and polymerization was followed by pyrene fluorescence.

IV.8.: Tpms enhance the effect of gelsolin on actin polymerization

We investigated whether other Tpm isoforms can influence the effects of gelsolin on actin polymerization. Actin was polymerized in the presence of gelsolin or gelsolin-Tpm complexes. In the latter cases, gelsolin and Tpm were pre-incubated for 30 min in the presence of 100 mM Ca 2þ. The rate of gelsolin-mediated actin polymerization was increased by all Tpm isoforms (Fig. 6A). To quantify the effects of Tpms, we calculated the elongation rates from the initial 5–25% segments of the transients relative to the maximal fluorescence intensity. The smallest effect on actin assembly was observed with Tpm1.12, a medium effect with Tpm3.1 (57%), and the largest with skTM (76%). In control experiments, we found that in the absence of gelsolin, Tpms at the applied concentrations did not influence the rate of actin polymerization (Fig. 6B). One possibility for the faster polymerization kinetics is that Tpms may promote the nucleating activity of gelsolin. Another possible mechanism by which Tpms may increase the rate of actin polymerization is to enhance the severing activity of gelsolin that is expected to produce more free filament ends for the monomer association.

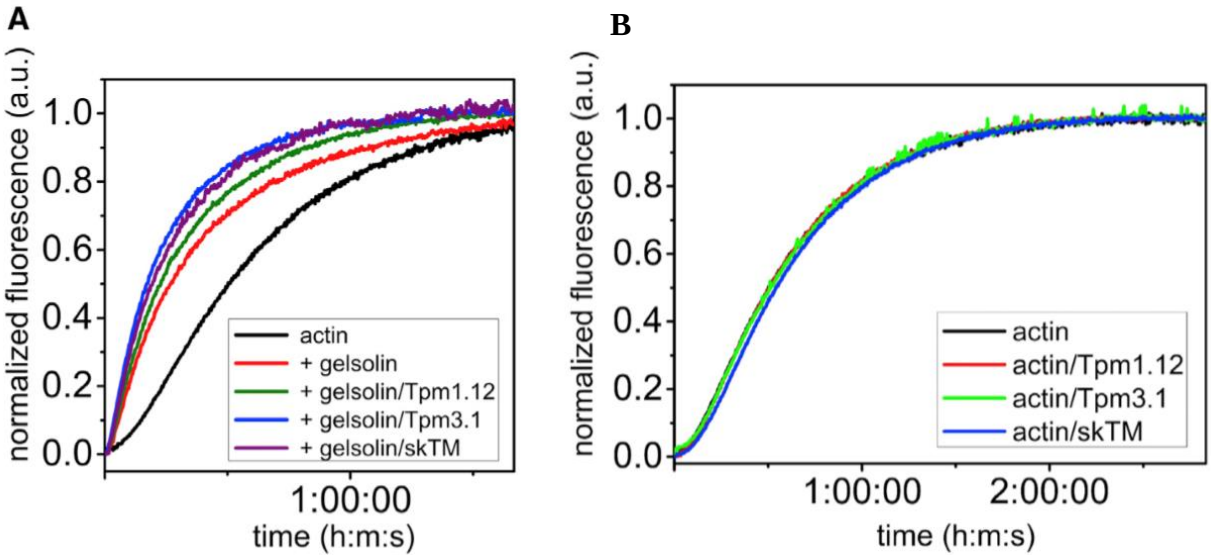


Fig.6.: Tropomyosins enhance the effect of gelsolin on actin polymerization. (A) In these assays, 2 mM actin was polymerized alone or in the presence of either 4.5 nM gelsolin or 4.5 nM gelsolin preincubated with different Tpm isoforms. Gelsolin and Tpms were prepared in stock solutions for 30 min after mixing them at 300 nM and 10 mM concentrations, respectively. (B) Polymerization curves for the same amount of actin as in (A) with and without Tpms and without gelsolin. To see this figure in color, go online.

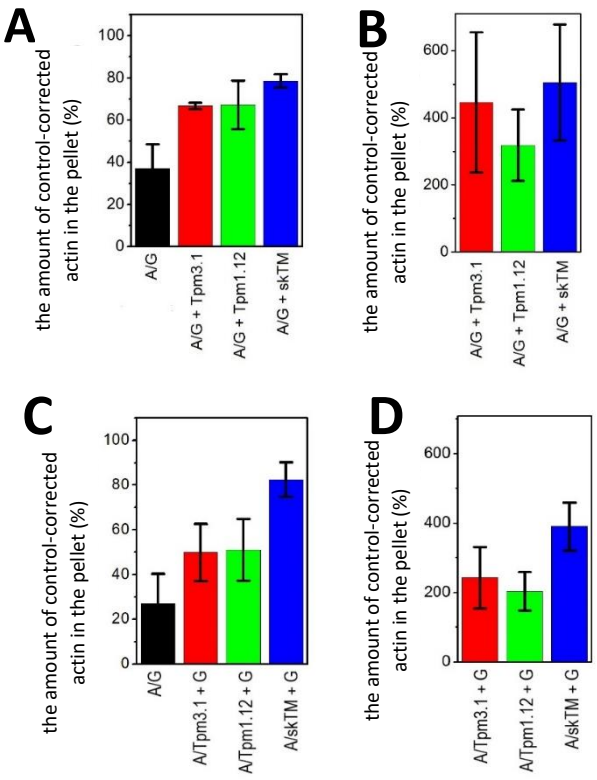
IV.9.: Tpms alter the rate of actin filament disassembly catalyzed by gelsolin

To investigate the effect of Tpms on the severing activity of gelsolin, depolymerization measurements were carried out. We used pre-formed pyrene-labeled actin filaments that were subsequently diluted to concentrations below the critical concentration of the barbed end (~0.12 μM) (Bugyi and Carlier 2010; Fujiwara, Vavylonis, and Pollard 2007). Under these conditions, the incorporation of free actin monomers was negligible, so spontaneous subunit dissociation from the filament ends could be monitored. The spontaneous disassembly kinetics of actin filaments was relatively slow due to the low rate constants for dissociation of actin subunits and also to the low concentration of filament ends. Severing by gelsolin increases the number of pointed ends but caps the barbed ends, resulting in a higher net rate of depolymerization that was consistent with previous observations (Tóth et al. 2016). When pre-incubated gelsolin-Tpm complexes were added to the actin filaments, the rate of decrease in fluorescence was more pronounced than that observed with gelsolin alone, for all three isoforms of Tpm, suggesting higher rates of depolymerization. When Tpms were added to the actin filaments at saturating concentrations in the absence of gelsolin, they did not result in an enhancement of the spontaneous depolymerization rate. Moreover, Tpm1.12 and Tpm3.1 slightly inhibited this process, whereas skTM had a much stronger protective effect. These observations are in correlation with our previous results showing that Tpms decrease the rate of the spontaneous depolymerization of Mg^{2+}-F-actin. Taken together, these observations lead us to conclude that gelsolin in complex with Tpm has a higher activity for severing actin filaments than gelsolin alone.

IV.10.: Protective action of tropomyosin against gelsolin on actin filament

Two approaches were used in the study. First, actin filaments were incubated with gelsolin to allow sufficient time for filament degradation, and then tropomyosin was added to the system. In the second case, actin filaments were incubated with tropomyosin to allow sufficient time for tropomyosin to bind to the filament, and then gelsolin was added. When 0.8 μM gelatin was added to 10μM F-actin, the amount of actin in the pellet decreased to ~ 35-40% compared to control (Fig. 7A). When tropomyosin was added to the samples, the amount of actin in the pellet increased. This suggests that tropomyosins partially protected actin filaments from the depolymerization effect of gelsolin. When comparing tropomyosin-treated samples to those containing gelsolin alone, we found that the amount of gelsolin varied 2-5-fold (Fig. 7B). As a

control, the experiment was performed in the absence of actin. We have found that tropomyosin has no effect on the deposition of gelsolin.



7. ábra: Evaluation of cosedimentation tests

V. Summary

New results:

1) We produced Tpm1.12 and Tpm3.1 as recombinant proteins in a nontagged form, without any excess amino acids to the native sequence.
2) We found that both Tpm1.12 and Tpm3.1 bind actin filaments, however, their affinities for actin differ.
3) We found Tpm1.12 and Tpm3.1 had small effects on the rate of actin polymerization. While Tpm1.12 slightly reduced, Tpm3.1 slightly reduced increased the rate of polymerization.
4) In our depolymerization assays, we have found that both isoforms reduce the rate of filament degradation in concentration dependence. Furthermore, it has been found that dissociation of tropomyosins from actin filament is a slow process of about 10 seconds.
5) Surface Plasmon Resonance (SPR) measurements showed that all three tropomyosines tested bind to gelsolin. The binding affinity for Tpm1.12 is $K_D = 0.7 \pm 0.2$ μM, for Tpm3.1 $K_D = 0.3 \pm 0.2$ μM.
6) Gelsolin is more efficient in disassembling actin filaments when it is in complex with Tpm than in its free form, even if the actin filaments are saturated by Tpms.
7) Skeletal muscle tropomyosin has a protective effect on gelsolin-induced depolymerization.
8) The increase in Ca 2+ concentration in the micromolar range increases the activity of gelsolin and the complex of gelsolin-tropomyosin.

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