

Telokin (kinase-related protein) modulates the oligomeric state of smooth-muscle myosin light-chain kinase and its interaction with myosin filaments

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Telokin, an abundant gizzard protein, inhibited phosphorylation of regulatory light chain when filamentous myosin was used as the substrate but no inhibition was observed with myosin subfragment 1. At physiological telokin-to-myosin molar ratio (1:1), the inhibition amounted to a 3.5-fold reduction in the initial phosphorylation rate whereas at high molar excess of telokin over myosin, we observed an up to 20-fold decrease in this rate. In agreement with previous observations [Shirinsky, Vorotnikov, Birukov, Nanaev, Collinge, Lukas, Sellers and Watterson (1993) *J. Biol. Chem.* **268**, 16578–16583], telokin did not inhibit phosphorylation of the isolated regulatory light chain of myosin and only moderately (35%) inhibited that of heavy meromyosin. To gain a better understanding of the mechanism of this inhibition, we investigated the effects of telokin on the recently described [Babiyhuk, Babiyhuk and Sobieszek (1995)

Biochemistry **34**, 6366–6372] oligomeric properties of smooth-muscle myosin light-chain kinase (MLCK). We showed, on the one hand, that telokin rapidly solubilized the large kinase oligomers formed at low ionic strength. With soluble kinase, on the other hand, telokin acted to increase the relative concentration of MLCK dimers and to decrease that of the hexamers and octamers. This, in turn, resulted in a reduction in the amount of MLCK bound to myosin because filamentous myosin appeared to exhibit a higher affinity for the hexamers than for the dimers. Telokin by itself was also shown to dimerize and oligomerize in solution and this oligomerization was greatly enhanced in the presence of MLCK. We suggest that telokin affects myosin phosphorylation by modulation of the oligomeric state of MLCK and its interaction with myosin filaments.

INTRODUCTION

Telokin was first isolated as a by-product of calmodulin purification from chicken gizzard by Dabrowska et al. [1]. The concentration of telokin in gizzard tissue was estimated to be in the range 80–90 μM [2]. This is considerably higher than the concentration of smooth-muscle myosin light-chain kinase (MLCK), estimated to be approx. 5 μM [3,4]. Telokin has also been detected in other smooth-muscle-containing tissues such as trachea, ileum and uterus [5]. It has been established that telokin is also expressed in other tissues such as chicken heart, lung and skeletal muscle, although the levels of expression are much lower than those in gizzard [6]. The finding that telokin is present in lung tissue is probably due to the presence of smooth muscle in the bronchi and bronchioles.

Telokin is an acidic protein with a pI of 4.5 and an apparent molecular mass of 24 kDa as measured by SDS/PAGE, although sequence data give a lower value of 18 kDa [7]. The structure of telokin at 2.8 Å (0.28 nm) resolution has been determined by Holden et al. [8] from X-ray-diffraction data. The overall molecular folding of telokin consists of seven strands of anti-parallel β -pleated sheets that wrap around to form a barrel. There is also an extended tail of eight amino acid residues at the N-terminus of the polypeptide chain.

Telokin is also called kinase-related protein because its amino acid sequence is identical with that of the 154-residue C-terminal fragment of smooth-muscle MLCK [5,7,9,10]. This fragment is adjacent to the C-terminal site of the calmodulin-binding domain

but is not present in the skeletal-muscle enzyme [11,12]. The function of this part of the kinase molecule is not known.

It has been shown that telokin is expressed as an independent protein, since telokin and MLCK mRNAs are induced by two different promoters [5,6]. Telokin mRNA arises from a promoter within an intron of the MLCK gene [5,6]. Telokin and the C-terminal domain of MLCK show amino acid sequence similarity to several quite different muscle proteins such as titin, C-protein and twitchin [13–15]. All these proteins are characterized by the presence of a number of repeating sequence motifs referred to as type-I and type-II titin-like motifs. It has been suggested that these motifs play a role in the interaction of muscle proteins with myosin [13] and a similar role has been suggested for the C-terminal domain of the kinase [16]. Telokin contains the type-II titin-like sequence motif [13].

It was recently suggested [2] that telokin may play a role in the stabilization of unphosphorylated smooth-muscle myosin minifilaments. Telokin binds to the heavy meromyosin (HMM) fragment of unphosphorylated myosin but not to the myosin rod or myosin fragments lacking the hinge region and light chains. The binding affinity is, however, relatively low [2]. The published data indicate that telokin does not affect the activity of MLCK when the regulatory light chain (ReLC) or its peptide analogue are used as substrate [9,10]. It does, however, reduce the phosphorylation rate when HMM is used as substrate [2].

To gain a better understanding of the role of telokin in smooth muscle, we investigated its effects on the oligomeric properties of the kinase. Our results indicate for the first time that telokin

Abbreviations used: EDC, 1-ethyl-3-(dimethylaminopropyl)carbodi-imide hydrochloride; HMM, heavy meromyosin; MLCK, myosin light-chain kinase; oPDM, *N,N*-(1,2-phenylene)dimalleinimide; PKA, protein kinase A; ReLC, smooth-muscle myosin regulatory light chain; SF1, myosin subfragment 1; DTE, dithioerythritol.

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influences the oligomeric state of MLCK and in this way modulates the interaction between the kinase and myosin filaments.

MATERIALS AND METHODS

Chemicals and protein preparations

1-Ethyl-3-(dimethylaminopropyl)carbodi-imide hydrochloride (EDC) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and *N,N*-(1,2-phenylene)dimaldimide (oPDM) was from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other chemicals used were of analytical grade and were purchased from either Merck (Darmstadt, Germany) or Fluka Chemie AG (Buchs, Switzerland). The gel-filtration molecular-mass standards were obtained from Pharmacia (Uppsala, Sweden).

Purification of turkey gizzard MLCK [17,18], calmodulin [18], myosin [19] and regulatory myosin light chain [20] as well as preparation of HMM and myosin subfragment 1 (SF1) [19] was as described in the corresponding references. The MLCK 64 kDa fragment was obtained by trypsin digestion of the purified enzyme (20 μ M) at 1:50 (w/w) ratio for 20 min. After termination of the trypsin treatment with a 2-fold molar excess of soyabean trypsin inhibitor, the digest was purified on a DEAE-Sepharose 6B-Cl column eluted with 100–350 mM NaCl gradient. The catalytic subunit of protein kinase A (PKA) was a gift from Professor E. Krebs (University of Washington, Seattle, WA, U.S.A.).

Kinase extracts obtained from gizzard myofibrils [21] were used as the starting material for telokin preparation. With no assay for telokin available, we used urea-glycerol-gel electrophoresis [22] for its detection. This approach was successful because of some similarities in migration between telokin and myosin light chains.

Purification of telokin

Myofibrils made from 800 g of tissue were extracted with 3 litres of a kinase extraction solution [17]. The extract was fractionated between 40 and 55% $(\text{NH}_4)_2\text{SO}_4$ saturation for MLCK and between 55 and 75% for telokin. After centrifugation (12000 g; 30 min), the 55–75%-satd. pellet was dissolved in AA buffer [60 mM KCl, 2 mM MgCl_2 , 0.5 mM dithioerythritol (DTE), 10 mM imidazole, pH 7.5] containing 0.25 mM PMSF and kept frozen (-30°C). Normally, four such pellets were used for one telokin preparation. The dissolved pellets were dialysed extensively against the same buffer and clarified (12000 g; 30 min). Before anion-exchange chromatography, tropomyosin was removed by isoelectric precipitation at pH 4.8. The pH adjustment was critical. A deviation of 0.3 of a pH unit resulted in considerable loss of telokin because of its co-precipitation with tropomyosin.

After centrifugation (12000 g; 20 min), the supernatant was promptly adjusted to neutral pH and applied to a DEAE-Sepharose 6B-CL (Pharmacia, Uppsala, Sweden) column (30 cm \times 2.5 cm) equilibrated with AA buffer. After washing of the column with the same buffer, telokin was eluted with a linear 150–350 mM NaCl gradient (2 \times 750 ml) in AA buffer containing, in addition, 0.5 mM EGTA. Tubes containing telokin (eluted at about 280 mM NaCl) were identified by urea-gel electrophoresis and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 75% saturation. After solubilization of the pellet in AA buffer containing 0.5 mM EGTA and dialysis against the same buffer, telokin was further purified on an AcA54 gel-filtration column (92 cm \times 3.2 cm). The major peak eluted from the column represented a homogeneous preparation and was concentrated

by $(\text{NH}_4)_2\text{SO}_4$ precipitation. After solubilization and dialysis (as above) the stock solution was kept frozen at -30°C until use.

Telokin was also purified from a whole-muscle extract by a modification of the myofibril method which bears some similarities to the method used by Ito et al. [7]. Telokin preparations from gizzard myofibrils and from whole muscle extracts exhibited identical properties.

Experimental procedures

Measurements of the rate of phosphorylation of myosin (30 μ M), which contained endogenous MLCK and calmodulin [23], were carried out as previously described [18] at 15°C in BW buffer (40 mM KCl, 10 mM imidazole, 0.5 mM DTE, 10 mM Bistris, pH 6.6) containing 0.1 mM CaCl_2 and 150 nM microcystin-LR. The reaction was initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol; NEN, Boston, MA, U.S.A.) diluted as previously described [16] with a final ATP concentration of 0.3–0.7 mM. Phosphorylation of the isolated ReLC (290 μ M), HMM (40 μ M) and SF1 (90 μ M) by MLCK (50, 200 and 1500 nM respectively) was carried out for 30 s at 25°C in AA buffer which contained 0.1 mM CaCl_2 and 1.5-fold molar excess of calmodulin over kinase.

Labelling of telokin (250 μ M) with ^{32}P was carried out by adding the catalytic subunit of PKA (0.9 μ M) in the presence of 0.4 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, at 25°C for 2 h. The ^{32}P -labelled telokin was subjected to gel filtration on an AcA54 column (83 cm \times 1.5 cm) equilibrated with AA buffer.

Binding of MLCK to myosin in the presence of increasing concentrations of telokin was performed in BW buffer. After centrifugation of the mixtures (30 min at 10000 g), the pellets were resuspended in the initial volume and, together with the supernatants, they were analysed by SDS/PAGE (see below).

Liberation of endogenous MLCK from myosin by telokin was carried out in a somewhat similar way. Before an experiment, filamentous myosin was additionally rinsed by resuspending it in a 10-fold larger volume of the BW buffer and pelleting it by centrifugation. The pellet was resuspended again in the same buffer at the required concentration, usually 50 μ M. Increasing amounts of telokin were added to centrifuge tubes containing 1.5 ml aliquots of this myosin and, after mixing, these were centrifuged for 30 min at 30000 g (18000 rev./min). Protein composition of the supernatants was then analysed by SDS/PAGE after 30-fold concentration of the samples with 5% trichloroacetic acid.

Cross-linking with oPDM and EDC was used to analyse MLCK oligomerization and to identify the protein-protein interaction. AA buffer (containing no DTE) was used and the reaction was initiated by adding 200 μ M oPDM or 5 mM EDC. After 15–30 min incubation at 25°C , cross-linking was terminated by adding a 2-fold molar excess of DTE.

The extent of MLCK oligomerization was also analysed by high-speed centrifugation with a Beckman Airfuge. A 10 μ M MLCK solution in AA buffer was first clarified (14000 g for 30 min) and then, after the addition of increasing amounts of telokin, centrifuged at 170000 g, for 30 min. Supernatants and pellets were analysed by SDS/PAGE.

Other procedures

SDS/PAGE was carried out in minislab gels, essentially as described by Matsudaria and Burgess [24]. We used a 3% stacking gel and a 9–18% gradient separating gel with modifications and improvements as described by Sobieszek [25].

All protein concentrations were measured by the biuret method [26] except for MLCK and calmodulin which were

determined from their absorbance at 278 nm using the absorption coefficient $A^{0.1\%} = 1.1$ for the kinase [27] and $A^{0.1\%} = 0.18$ for calmodulin [28] with the respective molecular masses of 107.5 and 16.7 kDa [29].

RESULTS AND DISCUSSION

Effects of telokin on myosin phosphorylation and activity of MLCK

It has been recently demonstrated by Shirinsky et al. [2] that telokin attenuates the initial phosphorylation rate of ReLC when

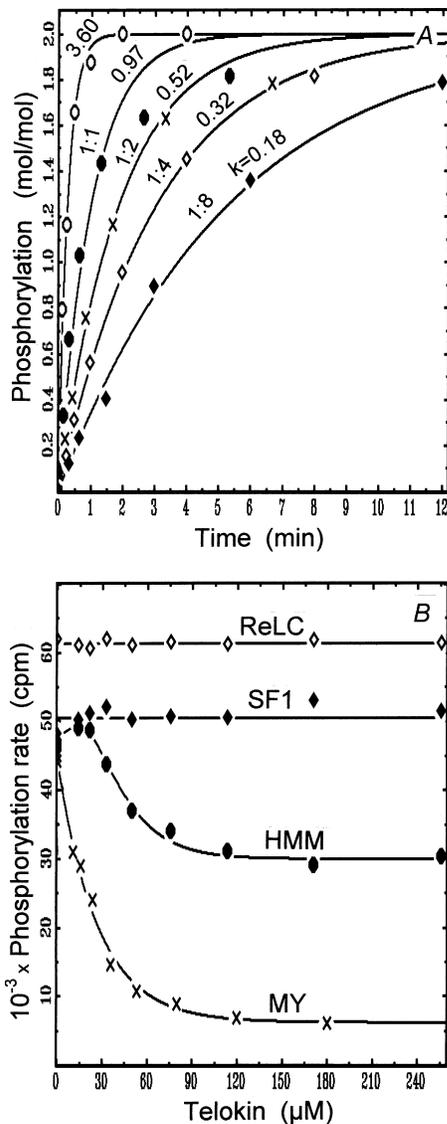


Figure 1 Effects of telokin on phosphorylation of filamentous myosin (A) and other MLCK substrates (B)

(A) Inhibition of the myosin phosphorylation by telokin. Phosphorylation curves were obtained in the presence of increasing concentrations of telokin with myosin/telokin molar ratios given on each curve (the first curve represents a control with no telokin added). Filamentous myosin (30 μM) which contained endogenous MLCK and calmodulin was used in these experiments. Phosphorylation is expressed as mol of phosphate incorporated/mol of myosin. Note that telokin decreased the apparent first-order rate constant (k) of phosphorylation and not the maximal phosphorylation level. In (B) initial phosphorylation rates were measured for isolated ReLC (\diamond), SF1 (\blacklozenge), HMM (\bullet) and myosin (MY; \times) in the presence of increasing concentrations of telokin. Note that no inhibition was observed for the ReLC and SF1.

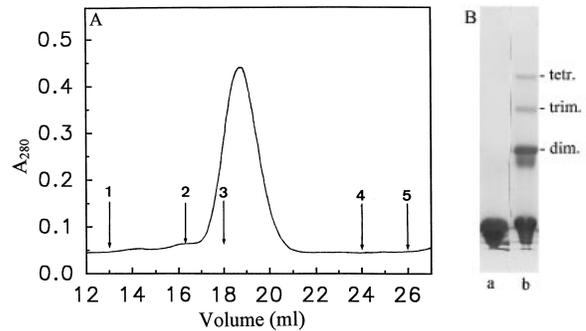


Figure 2 Telokin dimerization

(A) Gel filtration of purified telokin on an AcA54 column (0.6 cm \times 100 cm) equilibrated with AA buffer. Elution positions of molecular-mass standards are labelled with arrows: 1, Blue Dextran (2000 kDa); 2, BSA (67 kDa); 3, ovalbumin (43 kDa); 4, chymotrypsinogen A (25 kDa); 5, ribonuclease (13.7 kDa). Note that telokin was eluted just behind ovalbumin, i.e. as a protein of approx. 40 kDa. (B) SDS/PAGE of covalent cross-linked telokin. Telokin (80 μM) was cross-linked by oPDM for 60 min (lane b). An untreated telokin control is in lane a. Note the formation of cross-linked products corresponding to telokin dimer (dim.; 54 kDa), trimer (trim.; 85 kDa) and tetramer (tetr.; 125 kDa).

HMM is used as the substrate. In agreement with this observation we found that telokin strongly decreased the initial rate of ReLC phosphorylation when intact myosin was used as the substrate of MLCK. Figure 1(A) shows the phosphorylation curves of filamentous gizzard myosin obtained at increasing telokin/myosin molar ratios together with the corresponding apparent first-order rate constants (k). These constants characterize, or are equal to, the initial phosphorylation rates. At the physiological telokin/myosin ratio (1:1), the inhibition amounted to a 3.5-fold reduction in the initial phosphorylation rate, whereas at an 8-fold molar excess of telokin over myosin, we observed an approx. 20-fold decrease in this rate (Figure 1A; compare the k values). The myosin used in these experiments contained relatively high levels of an endogenous MLCK and calmodulin but its contamination by an endogenous myosin light-chain phosphatase was very low [23]. The latter was nevertheless inhibited by addition of 150 nM Microcystin-LR.

We also confirmed previous observations of Shoemaker et al. [9] and Collinge et al. [10] that telokin had no effect on the MLCK activity when isolated ReLC was used as substrate for the kinase (Figure 1B). More significant, however, was the observation that phosphorylation of SF1 was also not affected by telokin (Figure 1B). Thus this soluble single-headed subfragment behaved, as substrate, exactly as the isolated (soluble) ReLC. Phosphorylation of the two-headed (also soluble) HMM was only moderately (about 30%) inhibited by telokin in comparison with that of filamentous myosin. These results indicate that the observed reduction in phosphorylation rate by telokin probably resulted from a modification of the interaction between MLCK and myosin filaments and that the neck region of the myosin molecule played a role in this interaction.

Telokin dimerization

During gel filtration on the AcA54 column we noticed that telokin was eluted sooner than would be expected from its 18 kDa molecular mass. Its elution position was therefore carefully determined and compared with that of the other standard proteins used in molecular-mass evaluation. As shown in Figure 2(A), telokin migrated as a protein of nearly 40 kDa. This indicates that, under the conditions used, telokin was

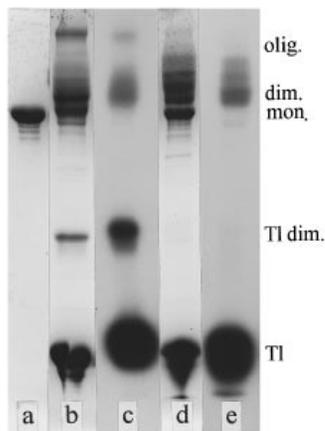


Figure 3 Interaction of telokin with MLCK dimeric and oligomeric species as revealed by covalent cross-linking

An untreated kinase control is in lane a. The kinase ($5 \mu\text{M}$) was cross-linked with ^{32}P -labelled telokin ($80 \mu\text{M}$) by oPDM (lane b, SDS/PAGE; lane c, autoradiogram) or EDC (lane d, SDS/PAGE; lane e, autoradiogram). The positions of the MLCK monomer (mon.), dimer (dim.) and oligomers (olig.) as well as the telokin dimer (TI dim.) are labelled. Note the presence of radioactivity in bands migrating slightly more slowly than the dimeric and oligomeric kinase species.

present in the form of a dimer. This conclusion was independently confirmed in our cross-linking experiments (Figure 2B). Using the standard cross-linker oPDM [30] and SDS/PAGE, we demonstrated formation of a polypeptide of 54 kDa in a homogeneous telokin preparation (Figure 2B, lane b). Thus the telokin dimer, like the monomer of MLCK itself, migrates much more slowly than would be expected from its molecular mass (36 kDa). As the difference is so large, however, we cannot exclude the possibility that the 54 kDa band corresponds to a telokin trimer, i.e. $3 \times 18 \text{ kDa}$.

Interaction of telokin with MLCK molecules

Interaction of telokin with MLCK was also apparent from oPDM and EDC cross-linking experiments in which ^{32}P -labelled telokin (phosphorylated by PKA) was used. EDC is known to form zero-length covalent bonds between amino and carboxyl groups in the contact area between two protein molecules [31], whereas oPDM cross-links thiol groups only [30]. Autoradiograms (Figure 3) demonstrate that telokin formed cross-linked products not only with monomeric MLCK but also with its dimeric and higher oligomeric forms. As indicated above, in the presence of oPDM (but not EDC) a 54 kDa cross-linked product was also formed and this corresponded to the telokin dimer (Figure 3; compare lanes b and d). There was no difference in the cross-linking of unphosphorylated telokin and that phosphorylated by PKA (results not shown).

We have previously concluded [32] that a short 3 kDa difference peptide (between the 64 kDa inactive and the 61 kDa constitutively active MLCK large fragment; see [33]) was responsible for the kinase oligomerization. Thus the 64 kDa fragment should interact with telokin in a manner similar to that of the intact kinase. To test this hypothesis, we purified this fragment and analysed its interaction with telokin by oPDM and EDC cross-linkage and SDS/PAGE.

As shown in Figure 4, telokin interacted not only with the 64 kDa fragment but also with its oligomeric aggregates (155 kDa band). When this interaction was revealed by means of oPDM,

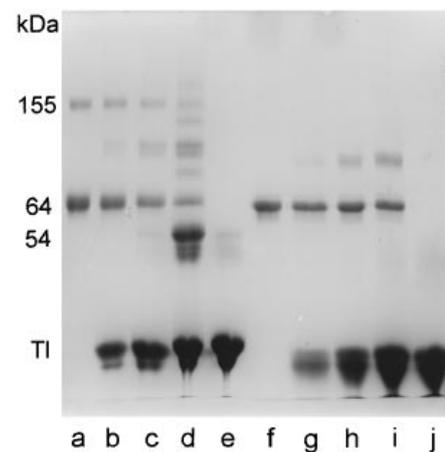


Figure 4 Interaction of telokin with the minimal 64 kDa kinase fragment still capable of oligomerization

Lanes a–e show cross-linking by oPDM, and lanes f–j show that of EDC. Lanes a and f correspond to controls with no telokin added whereas for lanes e and j no 64 kDa fragment was present. Telokin concentrations were: lanes b and g, $20 \mu\text{M}$; lanes c and h, $40 \mu\text{M}$; lanes d, i, e and j, $80 \mu\text{M}$. Note the decrease in the intensity of the 155 kDa tetramer and the 64 kDa monomer bands with increasing amounts of telokin added. For more details see the text.

the intensity of both the bands rapidly decreased as the concentration of telokin decreased (Figure 4, lanes a–d). With the EDC, however, no oligomeric aggregates were visible and, as a result, only the interaction of telokin with the 64 kDa ‘monomer’ was observed (Figure 4, lanes f–i). This at first glance unexpected observation became clear when one compares the oPDM and EDC cross-linkage patterns for intact kinase. As can be seen in Figure 5(A) (lane b) and Figure 5(B) (lane b) oPDM appeared to show only kinase hexamers and octamers but not the dimers (Figure 5A, lane b) whereas for the EDC the dimers were most readily ‘visualized’ (Figure 5B). Correspondingly, the observed

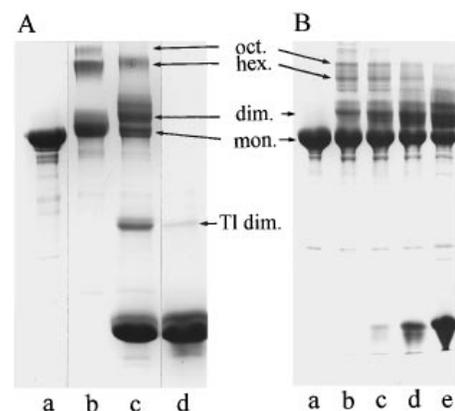


Figure 5 Effects of telokin on the oligomeric equilibria of MLCK

In (A), the kinase ($5 \mu\text{M}$) was cross-linked with telokin ($80 \mu\text{M}$) by oPDM (lane c). Lane a shows untreated kinase, lane b shows cross-linked kinase alone and lane d shows cross-linked telokin alone. In (B), the kinase ($10 \mu\text{M}$) was cross-linked in the presence of: 0 (lane b), 10 (lane c), 40 (lane d) or 160 (lane e) μM telokin by EDC. The untreated kinase used as a control is shown in lane a. Note the decrease in intensity of the bands corresponding to kinase hexamers (hex.) and octamers (oct.) with increasing telokin concentration. dim., dimer; mon., monomer; TI, telokin.

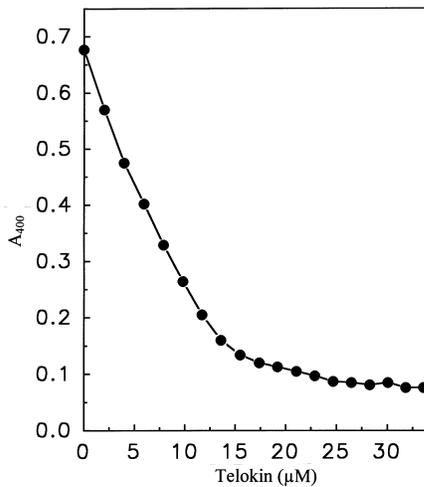
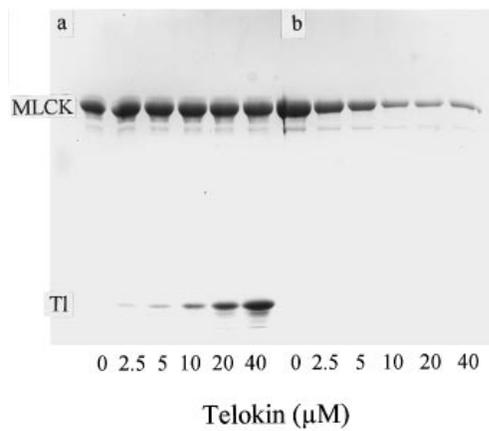


Figure 6 Telokin-induced solubilization of MLCK large oligomers demonstrated by low-speed centrifugation (top) and absorbance measurements (bottom)

Top, increasing amounts of telokin were added to the kinase suspension (10 μM), and after low-speed centrifugation, the resulting supernatants (a) and pellets (b) were analysed by SDS/PAGE. The pellets were resuspended in half the volume of the supernatants. Then 12 μl of the pellets and 6 μl of the supernatants were loaded on the gel. Note that telokin decreased the amount of the kinase in the pellets. TI, telokin. Bottom, 2 μl aliquots of 1800 μM telokin were added to 500 μl of the low-ionic-strength MLCK large oligomer suspension and A_{400} was measured. The MLCK concentration was 20 μM . The BW buffer contained only 20 mM KCl. Note the clearing of the solution at a molar ratio of MLCK to telokin of approx. 1:1.

155 kDa band was related or corresponded to the kinase hexamer, and its intensity decrease is consistent with the suggested telokin-induced monomerization or dimerization of the kinase. Figure 4 also illustrates that the kinase affected the oligomeric state of telokin. Its addition to telokin resulted in the opposite effect, i.e. kinase-induced oligomerization of telokin (compare Figure 4, lanes c and d). The mechanism of this intriguing ‘oligomeric exchange’ remains to be elucidated.

Analogous effects of telokin were observed for the oligomeric forms of intact MLCK. In the case of oPDM cross-linkage, adding telokin resulted in the disappearance of the band corresponding to the octamers and a decrease in the intensity of the hexamer band (Figure 5A). In some patterns of EDC cross-linkage we could clearly observe the decrease in the intensity of these hexamer and octamer bands paralleling the amount of telokin added. At the same time the intensity of the dimer band increased (Figure 5B).

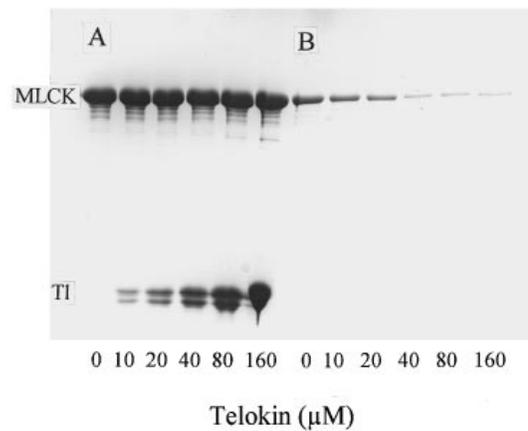


Figure 7 Dissociation of the MLCK oligomers by telokin

Increasing concentrations of telokin were added to clarified kinase solutions (10 μM), and after centrifugation at high speed the supernatants (A) and pellets (B) were analysed by SDS/PAGE. Pellets were resuspended in half the volume of the supernatants and the same amounts were loaded on the gels. Note that telokin decreased the amount of kinase in the pellets. TI, telokin.

Effects of telokin on the oligomeric state of MLCK

As noted earlier [16], at low ionic strength, MLCK forms large insoluble oligomers which could be pelleted by a low-speed centrifugation (10000 g ; 30 min). The addition of increasing amounts of telokin to suspensions of these oligomers solubilized the kinase and decreased the amount of kinase that could be pelleted (Figure 6, top). Thus telokin disassembled these oligomers and induced their solubilization. This solubilization was more clearly demonstrated by decreased absorbance of the kinase suspensions at 400 nm (Figure 6, bottom). This is a standard method for measuring the degree of protein aggregation.

It is apparent from our oPDM and EDC cross-linking experiments (e.g. Figure 5) that telokin increased the relative concentration of MLCK dimers. The observed increase in MLCK dimer formation was accompanied by disassembly of MLCK hexamers and its higher oligomeric forms. This effect was also demonstrated by high-speed centrifugation (170000 g) of the soluble MLCK (supernatant resulting from low-speed centrifugation of MLCK). At this centrifugal force, the high-molecular-mass oligomers (e.g. hexamers and octamers) were pelleted, but not the monomers or dimers. Figure 7 clearly shows that telokin decreased the amount of the pelleted MLCK.

Telokin and MLCK binding to myosin filaments

Consistent with the observations of Shirinsky et al. [2], we demonstrated that telokin reduced the binding of MLCK to filamentous myosin (Figure 8). This reduction was clearly shown in our sedimentation experiments in which increasing amounts of telokin were added to constant amounts of filamentous myosin and MLCK. Telokin decreased the amount of kinase bound to the myosin component and increased the kinase concentration in the supernatant. In an analogous way, addition of telokin to myosin filaments containing endogenous MLCK resulted in dissociation of the kinase. The kinase content of the supernatants obtained after pelleting of the filaments increased proportionally to the amount of telokin added (Figure 8C).

In a separate study (E. B. Babychuk and A. Sobieszek, unpublished work) we concluded that dimeric forms of kinase were bound to filamentous myosin at considerably lower affinity

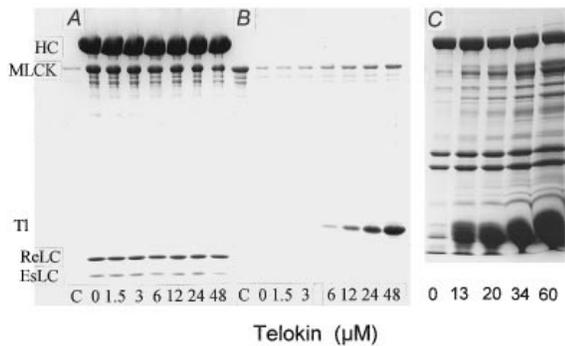


Figure 8 Reduction of MLCK binding to myosin filaments by telokin

SDS/PAGE of pellets (A) and supernatants (B) of centrifuged myosin filament ($3 \mu\text{M}$) suspensions together with added MLCK ($3 \mu\text{M}$) are shown as a function of added telokin. In (C) telokin was added to filamentous myosin ($50 \mu\text{M}$) containing endogenous MLCK and calmodulin [23]; the supernatants could only be analysed by SDS/PAGE after 30-fold concentration of the samples. The positions of myosin heavy chain (HC), kinase (MLCK), telokin (TI), as well as ReLC and essential light chain (EsLC) are labelled. Concentrations of telokin are given under the corresponding lanes. Lanes C in (A) and (B) are the controls of MLCK alone, from which it can be seen that the extent of MLCK aggregation was relatively low, i.e. about 80% of kinase remained in the supernatant in the absence of myosin. Note that telokin increased the amount of kinase in the supernatants.

than the kinase hexamers. This is consistent with previous reports [23,34] in which calmodulin weakened the affinity of MLCK for myosin. It has been shown independently [34] that this weakening of the affinity results from enhancement of kinase dimer formation induced by calmodulin and a parallel reduction of the relative hexamer concentration.

The present data support the recent conclusion of Sobieszek et al. [35] and Babychuk et al. [32], who showed that, in solution, MLCK dimers are in equilibrium with monomeric and oligomeric forms of the kinase. Telokin appears to shift this equilibrium towards kinase dimers, reducing at the same time the relative concentration of the higher oligomeric forms of the kinase. The relative concentrations of these three species have been determined by a light-scattering method [36]. At physiological concentrations of the kinase ($5 \mu\text{M}$) there is about 53% of the dimers and 45% of the monomers whereas the oligomer content is low (2%). The relative affinities of these species for filamentous myosin are not known, but from the $7.1 \mu\text{M}$ value of the K_m constant obtained for filamentous myosin at catalytic concentrations of the kinase (A. Sobieszek, unpublished work), it is clear that the monomers and dimers exhibit relatively low affinity for myosin. In contrast, from the very high binding affinity of the kinase for myosin during their co-precipitation and the very tight association of the endogenous kinase with the myosin filaments [23], it appears that the oligomers exhibit a very high affinity for these filaments ($K_d \approx 20\text{--}50 \text{ nM}$) because only the less soluble oligomers can readily be incorporated within the filament architecture. Thus the telokin-induced dimerization or monomerization should result in about a 100-fold reduction of the apparent affinity of the kinase for filamentous myosin. This reduction of the affinity is not expected for phosphorylation of the soluble isolated ReLC or SF1 subfragment because, under our experimental conditions, at catalytic concentrations of MLCK its oligomer content would be negligible. The relatively low inhibitory effect of telokin on phosphorylation of HMM is consistent with this interpretation because this myosin subfragment does not form filaments. HMM, however, contains the

neck region of the myosin molecule, which is responsible for the binding of its ReLC. Thus, as expected, the myosin neck must play a role in the binding of MLCK to myosin filaments. We postulate that the role of telokin *in vivo* is modulation, or control, of the phosphorylation rate of the myosin filament via regulation of the oligomeric state of the kinase.

There are, no doubt, additional factors that affect interaction of telokin with the kinase. One of these is a very slow phosphorylation of telokin by MLCK, requiring, in addition, relatively high (non-catalytic) concentrations of the kinase (A. Sobieszek and K. Nieznanski, unpublished work). This phosphorylation demonstrates further similarities between telokin and the ReLC and indicates a possible mechanism of telokin action. Being such a slow and inefficient substrate for the kinase, telokin can under our experimental conditions very effectively compete with the ReLC for its catalytic centre. This is because ReLCs are immobilized within the filament whereas telokin is free in solution. [A very efficient phosphorylation of the isolated ReLCs added to myosin filaments containing endogenous MLCK and calmodulin fully supports this conclusion (A. Sobieszek, unpublished work).] Thus the mechanism of telokin action *in vivo* could consist of a long half-life 'transitory' occupation of the kinase catalytic centre by this modulator, the release being controlled by its own phosphorylation.

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