Kinase-related protein (telokin) is phosphorylated by smooth-muscle myosin light-chain kinase and modulates the kinase activity

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Telokin is an abundant smooth-muscle protein with an amino acid sequence identical with that of the C-terminal region of smooth-muscle myosin light-chain kinase (MLCK), although it is expressed as a separate protein [Gallagher and Herring (1991) *J. Biol. Chem.* **266**, 23945–23952]. Here we demonstrate that telokin is also similar to smooth-muscle myosin regulatory light chain (ReLC) not only in its gross physical properties but also as an MLCK substrate. Telokin was slowly phosphorylated by MLCK in the presence of Ca²⁺ and calmodulin and could be readily dephosphorylated by myosin light-chain phosphatase. A threonine residue was phosphorylated with up to 0.25 mol/mol stoichiometry. This low stoichiometry, together with the observed dimerization of telokin [Sobieszek and Nieznanski (1997) *Bio-*

chem. J. **322**, 65–71], indicates that the telokin dimer was acting as the substrate with a single protomer being phosphorylated. Our enzyme kinetic analysis of the phosphorylation reaction confirms this interpretation. Because telokin phosphorylation also required micromolar concentrations of MLCK, which also facilitates the formation of kinase oligomers, we concluded that the oligomers are interacting with telokin. Thus it seems that telokin modulates the phosphorylation rate of myosin filaments by a mechanism that includes the direct or indirect inhibition of the kinase active site by the telokin dimer, and that removal of the inhibition is controlled by slow phosphorylation of the telokin dimer, which results in MLCK dimerization.

INTRODUCTION

Telokin is an abundant acidic protein first discovered by Dabrowska et al. [1] in gizzard smooth muscle. Its amino acid sequence is identical with that of the C-terminal fragment of smooth-muscle myosin light-chain kinase (MLCK), which is adjacent to the calmodulin-binding domain of this kinase [2–5]. The functions of this fragment and of telokin are unknown, although the structure of telokin has been recently determined at 2.8 Å resolution by Holden et al. [6] from X-ray diffraction results.

Telokin is expressed as an independent protein because telokin and MLCK are transcribed from two different promoters [2,7]. Telokin transcripts are initiated by a promoter within an intron of the MLCK gene. Telokin and the C-terminal domain of MLCK (here termed as telokin-like domain) show amino acid sequence similarity to several quite unrelated muscle proteins such as titin, C-protein and twitchin [8–10]. 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. Telokin contains the type II titin-like sequence motif. It has been suggested that these motifs have a role in the interaction of muscle proteins with myosin [8], and a similar role has been suggested for the telokin-like domain of the kinase [11].

Shirinsky et al. [12] suggested that telokin might have a role in the stabilization of unphosphorylated smooth-muscle myosin mini-filaments. Telokin was also shown to inhibit the phosphorylation of myosin filaments while having no effect on phosphorylation of the isolated smooth-muscle myosin regulatory light chain (ReLC) [12] or of the myosin subfragment 1 (SF1) [13]. This selective inhibition of filamentous myosin was recently attributed to a telokin-induced dimerization (or monomerization) of MLCK [13]. In agreement with our earlier

suggestions [14], it seems that the kinase large oligomers are bound to the myosin filament and that their monomerization (or dissociation into dimers) results in liberation of the kinase from the filaments, which are subsequently phosphorylated at a much lower rate. Partial inhibition of the phosphorylation of heavy meromyosin indicates that the myosin neck region has a role in the interaction of MLCK with myosin because this subfragment does not form filaments.

Here we demonstrate that telokin is also similar to the ReLC of myosin. When telokin was phosphorylated by MLCK, the telokin-induced inhibition of myosin phosphorylation was removed, which indicates the existence of a telokin-dependent modulatory pathway in smooth-muscle regulation.

MATERIALS AND METHODS

Telokin and MLCK were extracted from a smooth-muscle myofibril-like preparation [15] by the same kinase extraction solution [16]. The telokin purification procedure was described recently [13]. The purification of turkey gizzard MLCK [16], calmodulin (CM) [17], myosin [18] and the ReLC [19] as well as the preparation of heavy meromyosin and myosin SF1 [18] was done as described in the corresponding papers. The catalytic subunit of cAMP-dependent protein kinase (cAMP kinase) was a gift from Professor E. Krebs (University of Washington, Seattle, WA, U.S.A.) or was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Phosphorylation of telokin and autophosphorylation of MLCK was performed in our 'AA' buffer, which has the following composition (in mM): KCl 60, imidazole 10, dithioerythritol 0.5, imidazole 10 with pH adjusted to 7.4 at 4 °C. Other details were as described previously [11,13]. Unless stated

Abbreviations used: cAMP kinase, cAMP-dependent protein kinase; CM, calmodulin; MLCK, smooth-muscle myosin light-chain kinase; MLCP, myosin light-chain phosphatase; ReLC, smooth-muscle myosin regulatory light chain; SF1, myosin subfragment 1.

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otherwise, telokin phosphorylation experiments were performed in the presence of a 4-fold molar excess of CM (20 μ M) above that of MLCK (5 μ M) and with 0.1 mM CaCl_2 and 250–500 nM of Microcystin-LR added.

SDS/PAGE was performed in minislab gels, essentially as described by Matsudaria and Burgess [20]. We used a 3 % (w/v) stacking gel and a 9–18 % (w/v) gradient separating gel with modifications and improvements as described by Sobieszek [21].

All protein concentrations were measured by the biuret method [22] except for MLCK and CM, which were determined from their absorption at 278 nm by using $A^{0.1\%} = 1.1$ for the kinase [23] and $A^{0.1\%} = 0.18$ for CM [24] with respective molecular masses of 107.5 and 16.7 kDa [25].

RESULTS

During the development of our purification procedure we noticed some similarities between the physical properties of telokin and the ReLC. Telokin behaved almost like the ReLC during gel filtration and ion-exchange chromatography, as well as during urea and SDS/PAGE. These similarities prompted us to investigate whether telokin, like the ReLC, could be phosphorylated by MLCK. As shown in Figure 1, telokin was indeed phosphorylated by this specific kinase. However, the phosphorylation rates of telokin were approx. 1/200 those of the ReLC. Also relatively low were the maximal phosphorylation levels because they ranged from 0.1 to 0.3 mol/mol. Although telokin phosphorylation was enhanced by the presence of CM and Ca^{2+} (Figure 1), there was also considerable and variable phosphorylation in the absence of these ligands (see below).

In parallel experiments we established the optimum CM-to-MLCK ratio for telokin phosphorylation. Unexpectedly, a 2–4-fold molar excess of CM was required for the maximal incorporation or the highest initial rate (Figure 2; see also below). Phosphate incorporation was one-fifth to one-third less in the absence of CM or when Ca^{2+} was removed by EGTA. As shown

Figure 1 Phosphorylation of telokin by MLCK in the presence ($\diamond$) and the absence ($\bullet$) of Ca^{2+} and CM

For comparison, phosphorylation of the C-terminal telokin-like fragment of MLCK ($\times$) and phosphorylation of telokin by cAMP kinase ($\bullet$) are also shown. Concentrations were (in μ M): telokin 100, MLCK 5, CM 20, cAMP kinase 0.8.

Figure 2 Phosphorylation progress curves of telokin at different CM concentrations

MLCK-to-CM molar ratios are given on each curve. Note that the rates and the maximal incorporations were optimal at an approx. 1:4 molar ratio of MLCK to CM. Unless stated otherwise, this ratio was used in all following figures. MLCK and telokin concentrations were 5 and 100 μ M respectively in this and the other figures.

Figure 3 Telokin phosphorylation and MLCK autophosphorylation by autoradiography (lanes b, d and f) with corresponding SDS/PAGE gels (lanes a, c and e)

Note that under conditions optimal for intermolecular autophosphorylation [11], ^{32}P incorporation into the kinase and telokin bands took place (lane b). In contrast, when an excess of CM was present, autophosphorylation was inhibited (lane d). In the absence of CM (lane f), the phosphorylation was restricted to a very weak band representing a proteolytically modified telokin.

in Figure 3 (lanes c–f), this CM-independent ^{32}P incorporation seemed to be associated with a proteolytically modified fragment of telokin. However, the possibility of a telokin-dependent autophosphorylation of a similar MLCK fragment cannot be excluded. This phosphorylation varied greatly not only from one telokin preparation to another but was also variable for the same telokin when different MLCK preparations were used.

Figure 4 Identification of the residues involved in the phosphorylation of telokin

Note that threonine was the residue that was phosphorylated by MLCK in the presence of CM (lane b), whereas relatively low phosphorylation of a serine residue was also observed in the absence of CM (lane c). As established previously (see [1]), a serine residue is phosphorylated by cAMP kinase (lane a).

For these reasons CM-independent phosphorylation was not investigated further.

It has been established previously that a serine residue in telokin is phosphorylated by cAMP kinase [1]. In agreement with these observations, we established that the catalytic subunit of this non-specific kinase phosphorylated telokin up to approx. 0.7 mol/mol (Figure 1), consistent with a single phosphorylation site, and that serine was the residue phosphorylated (Figure 4, lane a). In contrast, MLCK phosphorylated telokin at a threonine residue (Figure 4, lane b). The relatively small amount of radioactivity co-migrating with serine was shown to arise from CM-independent phosphorylation (Figure 4, lane c). We therefore conclude that the phosphorylation sites of telokin for these two kinases were different. This difference was also apparent from the specificity of dephosphorylation of these two sites by smooth-muscle myosin light-chain phosphatase (MLCP). In phosphorylation experiments done in the presence or the absence of Microcystin-LR, telokin labelled with ^{32}P by the catalytic subunit of cAMP kinase was not readily dephosphorylated by MLCP, but telokin phosphorylated by MLCK was dephosphorylated by this phosphatase (see below). The same result occurred with CM-independent phosphorylation. Thus the two serine residues phosphorylated by MLCK and cAMP kinase seemed to be different.

The myofibrillar type of MLCP used in these experiments is closely associated with MLCK [26] and CM [27]. It was therefore important to determine the effect of this endogenous MLCK-MLCP on telokin phosphorylation. As shown in Figure 5, for a standard MLCK preparation containing relatively high levels of this phosphatase, relatively low phosphorylation of telokin was observed. However, when Microcystin-LR was added at 0.25 μM or more to inactivate the phosphatase, the rate and maximal level

Figure 5 Phosphorylation of telokin by MLCK in the presence (◆) and the absence (◇) of Microcystin-LR

Note that standard MLCK preparations contain endogenous MLCP, which resulted in a decrease in phosphorylation to one-half to one-third (see also [26,27]).

Figure 6 Telokin phosphorylation at different MLCK concentrations

The rate and extent of phosphorylation strongly depend on relatively high (non-catalytic) concentrations of MLCK.

of incorporation increased 2–3-fold. A characteristic feature of this type of phosphorylation curve was the biphasic shape, which could be greatly modified by different relative contents of the endogenous phosphatase.

Because of the very low phosphorylation rates observed, it was necessary to use relatively high MLCK concentrations in addition to the 4-fold molar excess of CM, which was added to inhibit MLCK autophosphorylation. Significant levels of ^{32}P incorporation were obtained only above 2.5 μM kinase (Figure 6)

Figure 7 Double-reciprocal relationship between telokin phosphorylation rate and concentration

Although the relationship was not linear, the apparent affinities at the lowest (K_m approx. 90 μM ; $\diamond$) and the highest (K_m approx. 1700 μM ; $\times$) range of telokin concentrations could be estimated. For the highest telokin concentration, the coordinate scales are extended 10-fold. The MLCK and CM concentrations were 5 and 20 μM respectively.

unless very high concentrations of telokin were present. Higher rates and maximal phosphorylation levels were obtained at 20 μM MLCK, the highest concentration tested. At this kinase concentration and with 100–250 μM telokin added, there was no significant increase in the phosphorylation stoichiometry (Figure 6). It is therefore possible that formation of the enzyme ternary complex or the phosphorylation reaction itself resulted in blocking or inhibition of MLCK so that the kinase could not phosphorylate additional telokin molecules.

In a classical enzyme kinetics experiment, we attempted to characterize the telokin phosphorylation reaction. The double-reciprocal plots of the rate against telokin concentration were not linear and did not show saturation (Figure 7), although the highest concentrations used were approaching 2 mM. At low concentrations, the slopes of the plots decreased instead of increasing as would be expected from the relatively high enzyme concentration present. Thus the system exhibited a high degree of negative co-operativity. The estimated co-operativity coefficient (h) was 0.8, and this value is underestimated because of the non-saturating conditions under which it was obtained. Because the plots of the rate against MLCK concentration at a constant telokin level were linear (results not shown), we concluded that affinity of MLCK for a telokin dimer was much higher than for a monomer and that a single protomer (within the dimer) was phosphorylated. This explains not only the relatively low phosphorylation stoichiometry but also the high degree of negative co-operativity; it is consistent with the effects of telokin on MLCK oligomerization [13].

The phosphorylation rates of telokin were comparable to the rates observed during CM-dependent autophosphorylation of MLCK, which also requires relatively high concentrations of this enzyme [11]. It was therefore necessary to evaluate the possible contribution of MLCK autophosphorylation during our telokin phosphorylation measurements when their concentrations were

Figure 8 Dependence of the phosphorylation ($\diamond$) and the autophosphorylation ($\times$) rates (A) on CM concentrations, with the corresponding stained SDS/PAGE gel (B) and autoradiograph (C)

Note that biphasic phosphorylation in the presence of telokin ($\diamond$) resulted from the biphasic dependence of the kinase autophosphorylation rate on CM ($\times$; see also [11]). Note also that at the highest CM concentrations the incorporation observed was due only to telokin phosphorylation.

comparable. We investigated this by varying the CM-to-MLCK ratio by using not only the ^{32}P -incorporation measurements but also autoradiography. The use of autoradiography allowed us to discriminate between phosphorylation of telokin and that of MLCK. As illustrated in Figure 8, optimum conditions for phosphorylation were those under which no autophosphorylation of MLCK was observed, i.e. a 4-fold molar excess of CM

Figure 9 Effect of telokin phosphorylation on inhibition of myosin phosphorylation rate

(A) Inhibition of the unphosphorylated telokin (●) in comparison with that of telokin phosphorylated by MLCK in the presence of ATP (◆) and adenosine 5' [thio]triphosphate (×). (B) Comparison of the inhibition of telokin phosphorylated by MLCK (◆) with that phosphorylated by cAMP kinase (◇), both in the presence of adenosine 5' [thio]triphosphate; (●), control. For more details see the text. The myosin (50 μM) used in these experiments was in filamentous form and contained endogenous MLCK and CM (see [29]).

over that of the kinase (Figure 8C, lanes 18, 27 and 40). At these high CM-to-MLCK ratios there was no significant contribution of CM-independent autophosphorylation of the kinase because this reaction is inhibited by high CM [11,28].

We have previously shown that telokin markedly inhibited phosphorylation rates when filamentous myosin was used as a substrate, but had no effect on phosphorylation of the soluble SF1 [13]. It was therefore important to see how the phosphorylation of telokin affected its inhibitory properties. As shown in Figure 9(A), the inhibition of myosin phosphorylation by phosphorylated telokin (phosphorylated up to approx. 25%) was much lower, especially at lower telokin concentrations, than that

observed for non-phosphorylated telokin. The removal of inhibition by thiophosphorylated telokin was even more pronounced and did not require the addition of Microcystin-LR. (Protein phosphatases are not active toward the thiophosphate groups.) Surprisingly, a telokin phosphorylated by cAMP kinase behaved similarly (Figure 9B), although its ability to overcome the inhibition was one-half to one-third that of MLCK-phosphorylated telokin. It should be emphasized, however, that approx. 3-fold higher levels of telokin phosphorylation are produced by cAMP kinase. Thus telokin phosphorylation in general has the same physiological effect, but its inhibitory action was most effectively overcome if telokin was phosphorylated by MLCK at the threonine residue.

DISCUSSION

The sequence identity between telokin and telokin-like domain of MLCK indicates that telokin could be involved in modulating the kinase activity. Initial experiments done to substantiate this conclusion were, however, inconclusive. Shirinsky et al. [12] demonstrated that telokin inhibited the phosphorylation of both intact myosin and heavy meromyosin but had no effect on the phosphorylation of the isolated ReLC. Because the same kinase was used for these three substrates there was no simple explanation for these observations. Furthermore the stabilizing effect of telokin on myosin mini-filaments in the presence of ATP [12] does not fit these observations. This latter effect was nevertheless interpreted as indicating that telokin might have an important role in maintaining the stability of non-phosphorylated myosin filaments.

Recently we presented results that clarify to some extent the role of telokin in smooth muscle. First, it is important to realize that MLCK is an oligomeric enzyme [17] and not, as previously assumed, a monomeric one. In solution MLCK forms dimers and oligomers that are in equilibrium with the kinase monomers [14]. Secondly, we have shown that the addition of telokin to a MLCK solution shifted this equilibrium in the direction of dimers and monomers. Because only the oligomers seem to be tightly bound to myosin filaments [13,29], such a shift results in a decrease in the amount of kinase bound. Consequently, myosin phosphorylation rates are markedly decreased [13]. In contrast, when MLCK is in solution and acts on the soluble substrate (the isolated ReLC), no effect is observed. Significantly, this lack of inhibition has also been demonstrated for the soluble SF1 [13]. The previously reported decrease in heavy meromyosin phosphorylation [12] was shown to be relatively moderate [13] and indicates that the neck portion of the myosin molecule is involved in its association with MLCK.

More direct evidence to support our hypothesis came from experiments in which telokin was added to native-like filamentous myosin preparations that contained endogenous MLCK and CM (see [29]). This addition resulted not only in a decrease to one-tenth (or less) in the phosphorylation rates (A. Sobieszek, unpublished work) but also in the release of the kinase from the filaments [13]. The amount of the kinase in the supernatant (after pelleting the filaments) was proportional to the concentration of telokin added with a saturation of the effect at a physiologically relevant 1:2 molar ratio of telokin to myosin.

In the present paper we provide additional results that are consistent with the hypothesis that telokin modulates MLCK activity *in vivo*. Our results elaborate on the similarities between telokin and the ReLC. We have shown that telokin acted as a substrate for MLCK and was phosphorylated by this regulatory enzyme. However, the phosphorylation rates *in vitro* were relatively low and were comparable to those observed for MLCK

intramolecular [28] and intermolecular [11] autophosphorylation. Because the major site of the intermolecular autophosphorylation has been localized within the telokin-like domain of the kinase [11], which has an identical amino acid sequence to that of telokin [2,7], it is likely that analogous phosphorylation sites might be involved. These results are also consistent with the proposed modulatory role of telokin because the changes in binding of the kinase to myosin filaments are expected to be relatively slow.

More significant, however, was the observation that this slow phosphorylation of telokin could reverse its initial inhibition of myosin phosphorylation. The removal of the inhibition, demonstrated in the present paper, was not complete but it was consistent with the relatively low (10–30%) phosphorylation levels of telokin that we observed. We expect that under the conditions *in vivo*, telokin is not free in the cytoplasm but is functionally localized in oligomeric form (most probably on myosin filaments) where it can be phosphorylated to much higher levels. The resulting removal of the inhibition could therefore be complete. Consistent with this interpretation is a recent report that demonstrated that telokin is phosphorylated *in situ* [30].

It was recently shown that MLCK also phosphorylates CM at a slower rate and to a lesser extent [31]. Perhaps the very slow phosphorylation (by MLCK) of substrates other than the ReLC is included in the modulatory mechanism(s) that influence not only the oligomeric state of the kinase but also affect its low-affinity CM-binding sites. The role of these sites in MLCK regulation has not been considered so far, although it has been known for some time that their saturation with CM results in an inhibition of kinase autophosphorylation [11,32].

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