Purification and Characterization of a Smooth Muscle Myosin Light Chain Kinase-Phosphatase Complex

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We show that a myofibrillar form of smooth muscle myosin light chain phosphatase (MLCPase) forms a multienzyme complex with myosin light chain kinase (MLCKase). The stability of the complex was indicated by the copurification of MLCKase and MLCPase activities through multiple steps that included myofibril preparation, gel filtration chromatography, cation (SP-Sepharose BB) and anion (Q-Sepharose FF) exchange chromatography, and affinity purification on calmodulin and on thiophosphorylated regulatory light chain columns. In addition, the purified complex eluted as a single peak from a final gel filtration column in the presence of calmodulin (CaM). Because a similar MLCPase is present in varying amounts in standard preparations of both MLCKase and myosin filaments, we have named it a kinase- and myosin-associated protein phosphatase (KAMPPase).

The KAMPPase multienzyme complex was composed of a 37-kDa catalytic (PC) subunit, a 67-kDa targeting (PT) subunit, and MLCKase with or without CaM. The approximate molar ratio of the PC and PT subunits was 1:2 with a variable and usually higher molar content of MLCKase. The targeting role of the PT subunit was directly demonstrated in binding experiments in which the PT subunit bound to both the kinase and to CaM. Its binding to CaM was, however, Ca$^{2+}$-independent. MLCKase and the PT subunit potentiated activity of the PC subunit when intact myosin was used as the substrate. These data indicated that there is a Ca$^{2+}$-independent interaction among the MLCPase, MLCKase, and CaM that are involved in the regulation of phosphatase activity.

Phosphorylation of myosin by myosin light chain kinase (MLCKase) represents the key activation step leading to contraction of smooth muscle (for reviews, see Refs. 1–4). Relaxation or inactivation of myosin is accomplished by a myosin light chain phosphatase (MLCPase) that has a controversial identification and subunit composition (see Ref. 5). In numerous previous studies (for references, see Ref. 6), many types of cytosolic MLCPases have been purified exhibiting different specific activities toward phosphorylated myosin or isolated myosin regulatory light chain (ReLC). A common feature of all of these phosphatases seems to be the presence of not only a catalytic (PC) subunit of about 35–38 kDa but also another subunit in the range of 55–72 kDa. The function of the latter subunit has not been established. Initial attempts to classify these serine/threonine phosphatases were not very successful (7), and it appears that smooth muscle MLCPases could be either the P1 or the P2A type.

Several years after our initial report on the first myofibrillar MLCPase (8), we and others again turned our attention to MLCPases from smooth muscle. In our new approach, the phosphatase was purified by CaM affinity chromatography and was shown to be composed of 37-kDa catalytic and 67-kDa targeting subunits (9). The only other myofibrillar phosphatase known so far was purified and partly characterized by Alessi et al. (Ref. 10; see also Refs. 11 and 12). It is composed of three subunits: a 37-kDa catalytic subunit and two regulatory subunits of 130 and 20 kDa. Although the sequence of all three subunits has been determined, the role of the regulatory subunits is not understood (see Ref. 5). In this report, we describe further results on our myofibrillar smooth muscle protein phosphatase, which is closely associated with MLCKase and myosin filaments and is called, therefore, a kinase- and myosin-associated protein phosphatase (KAMPPase). We show for the first time that this association results in a functional multienzyme complex between these two key regulatory enzymes of smooth muscle.

MATERIALS AND METHODS

Chemicals and Protein Preparations—[γ-32P]ATP was purchased from DuPont NEN and diluted with cold ATP of special grade (Boehringer Mannheim) to the required specific activity and concentration (13, 14). DEAE-Sepharose 6B-CL, Q-Sepharose FF, SP-Sepharose BB, Sephacryl S-200, and CNBr-activated Sepharose-4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Ectrophoresis reagents were purchased from Bio-Rad. The other chemicals were of analytical grade and were purchased from E. Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Inhibitors of protein phosphatases such as microcystin-LR and okadaic acid were purchased from LC Service Corp. (Woburn, MA).

Turkey gizzard MLCKase (8), myosin (3), and calmodulin (13), as well as phosphorylated ReLC and unphosphorylated ReLC of myosin (15), were purified as described previously. A 32P-labeled isolated ReLC or intact myosin was prepared as described (9).

The buffer (AA) used throughout this study had the following composition: 60 mM KCl, 2 mM MgCl$_2$, 0.5 mM dithiothreitol, and 10 mM imidazole with pH adjusted to 7.5 at 4 °C. It was used, with the necessary additions, during protein purification, activity measurements, binding experiments, and all other procedures.

Phosphatase and Kinase Activity Assays—Phosphatase activity was
measured by the release of $^{32}$P from labeled ReLC (or myosin) as described previously (8). The assays were carried out at 25 °C in AA buffer. The concentration of $^{32}$P-ReLC light chain was in the range of 50–150 μM. Bovine serum albumin (0.1%) was added as an inert protein carrier.

MLCKase assays were carried out as described previously (14) with intact myosin (or its isolated ReLC) chain as the substrate together with radioactive ATP. More specific details of individual assays are given in the corresponding figure legends.

The phosphatase activities were also measured by a simple modification of our MLCKase activity assays (14). This method was particularly suitable for the activity measurements of the kinase-rich multienzyme complexes. In this case, the $^{32}$P-labeling of myosin (or other substrates) only required the addition of radioactive ATP, and the dephosphorylation reaction was initiated by removal of Ca$^{2+}$ with EGTA. The first time point aliquots (spotted onto 3MM chromatographic paper pieces and dropped into 5% trichloroacetic acid solution) were used for estimation of the MLCKase activity or of the $^{32}$P labeling level. From the subsequent time points (after removal of Ca$^{2+}$), the phosphatase activity was evaluated.

Binding to Immobilized CaM and Other Procedures—An affinity gel centrifugation method was used for identification of the targeting subunit. Approximately 200 μl of CaM affinity gel were placed into 0.45-μm Ultrafree-MC filter units (Millipore, Bedford, MA). Equilibrations, sample applications, washings, and elutions were done by layering 350 μl of a given solution on top of the gel. After a 15-min equilibration, the gels were centrifuged for 15–30 s at 1,000 rpm in a small table top centrifuge. The washes and the eluates were analyzed by SDS-PAGE after 7–10-fold concentration of the samples with 5% trichloroacetic acid precipitation. To aid the precipitation and localization of the tiny pellet, 100 μg (10 μl) of the purified ReLC or essential light chain were added as an inert protein carrier.

Coupling of ReLC to CNBr-activated Sepharose 4B and thiophosphorylation of ReLC affinity columns as well as their elutions were performed as described (9). SDS-PAGE was performed in 7.5–15 or 9–18% gradient acrylamide minislab gels by the procedure of Matsudaira and Burgess (16) in the buffer system of Laemmli (17) with some improvement (8). The eluates from ReLC affinity columns as well as their elutions were performed as described (9). SDS-PAGE was performed in 7.5–15 or 9–18% gradient acrylamide minislab gels by the procedure of Matsudaira and Burgess (16) in the buffer system of Laemmli (17) with some improvement (8).

RESULTS

Purification of the MLCKase and MLCPase Complex—To obtain fraction that contained solely the high molecular weight MLCPase, the 40–55% ammonium sulfate fraction obtained from the myofibrillar MLCKase and MLCPase extract was first subjected to a gel filtration step on an AcA34 column (5.0 × 95 cm) in AA buffer. The fractions containing MLCKase and MLCPase activities eluted together as a relatively wide peak of about 350 kDa. These fractions were further purified on Q-Sepharose FF or DEAE-Sepharose 6B-CL columns as described in the preceding paper (9). Although this purification step made it possible to identify the SDS-PAGE bands corresponding to MLCPase catalytic (PC) and targeting (PT) subunits, a satisfactory separation of the kinase and the phosphatase was not obtained.

Because of the high affinity of the kinase for CaM and the expected very low affinity of the phosphatase for CaM, a CaM affinity column was used but also failed to separate the kinase from the phosphatase. In our hands, the MLCKase always, and to a varied extent, copurified with the phosphatase. However, the phosphatase, which was not bound to the CaM affinity column, was usually free of the kinase activity. The fraction that did not bind to and eluted from CaM affinity columns in the presence of Ca$^{2+}$ is dealt with in the preceding paper (9). The MLCKase preparations eluted from CaM affinity column in the absence of Ca$^{2+}$ are heavily contaminated by the phosphatase, even when the columns are washed with 0.36 M salt. These MLCKase-MLCPase complexes are the subject of the present report.

Better separation of MLCKase and a kinase-phosphatase complex was obtained by application of the 350-kDa kinase-phosphatase fraction to a pair of chromatography columns (strong cation and anion exchanger) connected in tandem. After extensive washing and separate elution of these two columns, we found that most of the kinase was bound by the first column (Fig. 1A), and the reverse was true for the phosphatase. Practically all of the phosphatase activity passed through the first column and was bound by the second column (Fig. 1B). The level of KAMPPase was so high that, despite the addition of 0.5 μM microcystin-LR, the kinase activity was suppressed everywhere except in the fractions from the “ascending” and “descending” tubes outside of the phosphatase peak (Fig. 1B). On SDS-PAGE (Fig. 2B), this type of KAMPPase preparation showed the PT and PC subunits and variable amounts of the kinase (130-kDa band). In addition, CaM, tropomyosin (TM), calcineurin (Calc), and other weak bands were also present.

When the MLCKase-MLCPase complex eluted from the second anion exchange column was applied to a CaM affinity
column, all of the KAMPPase activity was bound by the column, provided that the kinase content was not too low and the losses of the PT subunit resulting from proteolytic degradation were not too high. In the presence of 0.1 mM Ca\(^{2+}\), the activity remained bound even at 360 mM salt but could be eluted together with the kinase with a buffer containing 2 mM EGTA. The four major bands in the eluted complex corresponded to the subunits described previously (Fig. 3, lane \(i\)). The four subunits were seemingly tightly associated. No dissociation was observed even after a 3.5-h preincubation in 3.5 M LiBr (in the absence of Ca\(^{2+}\)) followed by passage of the complex through a Sephacryl S-200 column in buffer containing 2 mM EGTA (results not shown). Based on this observation, we believe that MLCKase and MLCPase form a multienzyme complex of the type described for other enzyme systems (21). A similar multienzyme complex was formed after dialysis of the concentrated KAMPPase fraction obtained from the DEAE- or Q-Sepharose columns (Fig. 3, lane \(k\)). Both complexes are only partly soluble at physiological salt concentrations and are bound to myosin filaments with relatively high affinity when sedimented through a 5–50% sucrose gradient (results not shown).

Contamination of MLCKase by KAMPPase—Consistent with the suggested tight binding of KAMPPase to MLCKase, KAMPPase activity was present in all of the MLCKase preparations, and the bands corresponding to the two phosphatase subunits could be identified on heavily loaded SDS-PAGE gels (Fig. 3; compare lanes \(a\) and \(b\)). The presence of this endogenous KAMPPase activity in MLCKase preparations depended not only on the integrity of the PT subunit but also on the presence of CaM. In addition to the kinase, CaM was also present in the KAMPPase activity peak of the Q-Sepharose column (see Fig. 2B), but it was lost during the next ammonium sulfate concentration step when 0.5 mM EGTA was present in the elution buffer.

The presence of phosphatase activity in apparently homogeneous MLCKase preparations also was shown in another type of experiment. The kinase undergoes a slow autophosphorylation (22). However, the rate and the maximal level of this autocatalytic reaction are strongly affected by the presence of this endogenous phosphatase. As shown in Fig. 4A, the autophosphorylation (rate and maximal level) could be increased 3–10-fold by adding either okadaic acid or microcystin-LR, two of the most potent protein phosphatase inhibitors (5). For standard MLCKase preparation purified from the kinase peak of the DEAE-Sepharose but not passed through CaM affinity col-
The endogenous KAMPPase present may completely inhibit the autophosphorylation. In this case, the addition of 2.5 mM okadaic acid results in a 10-fold increase in the autophosphorylation rate (Fig. 4A). The autophosphorylation also could be decreased by adding the purified KAMPPase or its PC subunit (see Ref. 9). This indicates that autophosphorylated MLCKase was a good substrate for the KAMPPase.

Because of the presence of endogenous KAMPPase in purified MLCKase preparations, it is apparent that these preparations should also be considered as containing MLCKase-MLCPase multienzyme complexes. These types of preparations, having relatively low phosphatase content, exhibit a characteristic autophosphorylation rate change when the CaM:kinase ratio is optimal for autophosphorylation. The steep initial rise in the CaM-dependent autophosphorylation is followed by a decline (Fig. 4B). The system should, however, approach steady state at which the

**Fig. 4.** Tight association of MLCPase activity with purified MLCKase (A and B) and its possible release by telokin (C). A, the presence of an endogenous MLCKase-phosphatase was apparent from an increase in the kinase autophosphorylation after adding phosphatase inhibitors (○, ○), controls (●, ●), DEAE-purified MLCKase (○, ○), or MLCKase preparation purified on CaM affinity and gel filtration columns (○, ○). B, effects of this endogenous phosphatase on the autophosphorylation at different CaM concentrations (inset shows that this activity could also be stimulated by CaM.) Note that at the optimal CaM concentration (10 μM), the phosphatase activity did not remain constant but increased because the autophosphorylation level of the kinase (15 μM) declined. C, effect of telokin on KAMPPase activity of the multienzyme complex (○, ○), the PC subunit (○, ○), and PTC holoenzyme (●, ●). Dephosphorylation rates were measured with 32P-RsLC as the substrate. For more details see “Materials and Methods.”
autophosphorylation level remains constant (e.g. Fig. 4B; CM = 0 μM). The decrease in autophosphorylation indicates that there was an increase in the KAMPPase activity that also depended on CaM. We suggest that as a result of autophosphorylation, the PC subunit may be activated or simply dissociates from the kinase. A similar or related 2-fold increase in the endogenous KAMPPase activity was observed after the addition of CaM to the kinase (Fig. 4B; inset).

We have recently shown that the addition of telokin to MLCKase increases the relative concentration of MLCKase dimers (or monomers) and decreases that of the oligomers (23). Since evidence given in the following section indicates that oligomeric species are responsible for association of MLCKase and MLCPase, telokin may facilitate dissociation of the phosphatase or its subunit from MLCKase oligomers. As shown in Fig. 4C, the addition of increasing amounts of telokin to the purified complex resulted in progressive increases in its MLCPase activity. At the same time, telokin had no effect on the phosphatase activity of the purified PC subunit or the PTC holoenzyme (Fig. 4C). We interpret this observation as telokin-induced dissociation of the KAMPPase or its PC subunit from the kinase-phosphatase complex.

Identification of the Targeting Subunit—The kinase-phosphatase complex was subjected to further purification steps, which included hydroxylapatite, gel filtration, and commonly used short (5-cm) γSP-ReLC affinity columns. None of these columns produced any substantial separation of the kinase from the already identified KAMPPase subunits, although several different ionic strengths were used. However, use of a long (0.9 × 60-cm) γSP-ReLC affinity column together with elution by a linear 120–360 mM NaCl gradient resulted in separation of the kinase-free PTC holoenzyme and the kinase-containing multienzyme complex (Fig. 4; see also Fig. 3, lanes e and j). The presence of the PT and PC subunits in both complexes indicates again that the PT subunit acted as the PC targeting subunit for the kinase. The long affinity columns amplified the small differences in the affinity of the complex for the γSP-ReLC light chain by introducing gel filtration effects.

Fractionation of the multienzyme complex or its components observed during simple gel filtration chromatography is shown in Fig. 6. In the absence of CaM and at 360 mM salt, some separation between the kinase and the PTC holoenzyme was...
observed, but not between the PT and PC subunits (Fig. 6A). This indicates that affinity of the PT subunit for the kinase was lower than that for the PC subunit. A similar separation was observed for a MLCKase preparation containing endogenous KAMPPase (Fig. 6B). The elution position of the phosphorylation peak corresponded to a molecular mass of about 150 kDa. Under similar conditions, the purified PC subunit eluted as a protein of 65 kDa. In contrast, in the presence of unsaturating CaM levels, there was close comigration of the KAMPPase activity and the absorption of the multienzyme complex (Fig. 6C). The elution position of the purified KAMPPase was not affected by the addition of CaM, indicating that CaM and Ca\(^{2+}\) were not absolutely needed for association of the PC and PT subunits (Fig. 6C), although Ca\(^{2+}\) was definitely needed for CaM binding to the kinase. Thus, the 67-kDa subunit appeared to be targeting not only the kinase but also CaM and could, therefore, act as an anchor for other CaM-binding enzymes.

The targeting role of the PT subunit for CaM and MLCKase was also demonstrated in direct binding experiments with our miniature CaM affinity columns (see “Materials and Methods”). The purified C subunit was not bound by CaM (Fig. 7A), but in the presence of the PT subunit most of the PC subunit was bound to the column and eluted at high ionic strength (Fig. 7, B and C). The elution was preceded by washing the miniature columns with the AA buffer containing 0.1 mM Ca\(^{2+}\). Although not all of the PT subunit applied was bound by the miniature columns, the presence of intact kinase enhanced this binding (Fig. 7D). This indicates again that both the kinase and CaM were the targets for the PT subunit. The incomplete binding of the PC subunit in the presence of the PT subunit (Fig. 7, B and C) also could result from the limiting amount of the PT subunit present in the purified preparation loaded onto the CaM affinity columns. Degradation or modification of the PT subunit results in a doublet detected on SDS-PAGE. It is clear from the figures that only the upper band of this doublet binds to the columns.

Possible Localization of the Complex on Myosin—We have previously shown (24) that MLCKase and CaM are very tightly bound to filamentous smooth muscle myosin. As a result, the addition of Ca\(^{2+}\) and ATP to purified myosin leads to a very rapid phosphorylation of the regulatory light chain. In similar experiments, we showed that the MLCPase was also associated with the same type of myosin preparations. As shown in Fig. 8, after initial rapid phosphorylation, myosin becomes dephosphorylated by this endogenous phosphatase. Both enzymes are simultaneously active, and the resulting phosphorylation rate and extent depended only on the kinase and the phosphatase activities of the myosin. However, as soon as the ATP concentration becomes relatively low, the kinase no longer acts, and the phosphatase activity prevails. This results in biphasic phosphorylation progress curves (Fig. 8). Correspondingly, the duration of the high levels of phosphorylation could be extended by adding microcystin-LR (Fig. 8). At the myosin concentration used (30–50 \(\mu\)M), the consumption of ATP resulting from the parallel action of the two enzymes was very low. Filamentous myosin ATPase was responsible for the rapid deactivation of ATP.

That the purified MLCKase-MLCPase complex or its PC subunit is very active toward phosphorylated filamentous myosin supports the hypothesis that a similar complex is responsible for the dephosphorylation of myosin filaments in vivo. This was shown in our experiments comparing these activities for the three relevant substrates, namely isolated ReLC, filamentous myosin, and actomyosin. In this case, the endogenous MLCKase or MLCPase was removed from the myosin (see Ref. 24) and actomyosin (see Ref. 25), and the complex (added before the initiation of the assay) was the only source of both the activities. As shown in Fig. 9, the complex phosphorylated all three substrates up to the maximal levels of 0.7–1.0 mol/mol and at approximately the same high rate. In contrast, the dephosphorylation rates, measured after the addition of EGTA, were very different. Under approximately the same conditions, the filamentous myosin became fully dephosphorylated within the 2–3 min following removal of Ca\(^{2+}\), while the isolated light chain was hardly dephosphorylated at all during the same time interval.
subunits of the same $M_\text{r}$, and are from gizzard muscle, they must be at least closely related.

The presence of the 130-kDa MLCKase band in our multienzyme complex, on the one hand, and of a 130-kDa regulatory subunit in the MLCPase preparation of Alessi et al. (10), on the other hand, raises questions. A plausible explanation would be the presence of a 130-kDa subunit comigrating with the kinase on SDS-PAGE gels. Such a subunit would have to bind CaM (as the kinase does) and have some properties similar to this enzyme but be free of MLCKase activity. We consider this possibility as unlikely because the presence of the 130-kDa band in our KAMPPase preparations was always accompanied by high levels of kinase activity. Even a lack of such activity does not necessarily mean that the kinase is completely absent. In such situations, demonstration of the kinase activity required the addition of microcystin-LR to the assay medium and high dilution of the preparations. Further studies are needed to clarify this possible controversy.

**Kinase-Phosphatase Complex—**One of the novel aspects of the present study is our suggestion that the two key regulatory enzymes of smooth muscle form a multienzyme complex. Our observations, including those of the preceding paper (9), together with those from other groups (references given below when applicable) can be summarized as follows: 1) presence of endogenous MLCPase activity in virtually all apparently homogeneous MLCKase preparations (27); 2) copurification of the MLCKase activity with MLCPase during almost all of the purification steps (see also Ref. 28); 3) formation of a kinase-phosphatase complex that bound to the CaM affinity column in a $\text{Ca}^{2+}$/CaM-dependent manner and which could not be eluted even at high salt concentrations; 4) binding of this complex to filamentous myosin; 5) presence of endogenous MLCKase and MLCPase activities on myosin filaments (3, 29); 6) common presence of a 67-kDa subunit (for references see the Introduction), which was identified here as a CaM- and MLCKase-targeting subunit.

Consistent with this hypothesis is the recent discovery that protein kinase A and protein phosphatase 2B associate together with a common targeting protein of 79 kDa (30). In addition, our recently published observations on cross-linking of MLCKase (31) and the results of light scattering measurements (32) point to properties of smooth muscle MLCKase that are particularly relevant to complex formation. These results indicate that, in solution, the kinase is oligomeric. The kinase dimers are in equilibrium with monomers and oligomers such that, under approximately physiological conditions (5 $\mu\text{M}$ of MLCKase in AA buffer containing 120 mM NaCl), there are approximately 53% dimers, 45% monomers, and 2% oligomers.

The very low binding stoichiometry indicates that the phosphatase may bind predominantly to the kinase oligomers. This conclusion is consistent not only with the data showing the effect of telokin or CaM on the multienzyme complex, but also with our light scattering measurements (32). The determinations of a relative molecular weight by light scattering demonstrates that during elution from the strong cation exchange columns (the conditions facilitating separation of the phosphatase), the kinase is monomeric. This contrasts with the kinase (or the complex) eluted from the CaM affinity column that exhibited the highest relative molecular weight, i.e. the highest oligomer content (16%) (32). The CaM-dependent targeting of the PC subunit to the MLCKase oligomers is consistent with the previously observed (13) high affinity binding (or trapping) of $^{125}$I-CaM by these oligomers during gel filtration experiments. In addition, the binding of MLCKase by both cation exchange and anion exchange gels demonstrated in the present study indicates that this enzyme possesses two strong and
oppositely charged domains that could be responsible for the observed oligomerization. One of these domains, not involved in the binding to an anion exchanger gel, could bind the PT subunit and, in this way, be responsible for formation of the complex.

Possible Regulatory Pathways—The regulatory potential was demonstrated by the 10–20-fold stimulation of KAMPPase activity by preincubation with micromolar concentrations of CoCl₂ (9). Significantly, this activation was not observed for the subunit but also that the interaction between the subunits indicates not only a great regulatory potential of the targeting multienzyme complex or for the PTC holoenzyme. This indicates a role for CoCl₂ (9). This activation was not observed for the subunit and, in this way, be responsible for formation of the complex. One would expect that the association of the PC subunit within the complex should have more significant regulatory consequences than the commonly observed modification of its specific activity. We suggest that, in general, formation of the complex plays a role in the localization of the MLCPase on the myosin filaments. A possible consequence of the association of the complex with myosin filaments could be a direct, first step inhibition of the kinase by the MLCPase as suggested recently by Johnson and Snyder (4). Although we favor this suggestion, it is clear that this would be difficult to distinguish experimentally from an indirect inhibition resulting from dephosphorylation catalysis by the phosphatase. A CaM-dependent inhibition of the kinase activity at low CaM:MLCKase molar ratios has already been demonstrated (34). However, we concluded previously that the inhibition does not result from dephosphorylation catalysis by the endogenous KAMPPase. This endogenous kinase-phosphatase was, however, responsible for the observed, apparently CaM-dependent, decline of the CaM-dependent autophosphorylation levels of the kinase. Therefore, we conclude that there is a regulatory step that initially permits significant levels of autophosphorylation despite presence of the phosphatase. Elucidation of this initial inactivating step should help in understanding the regulation of the kinase and the phosphatase activities of the multienzyme complex described in the present study.

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