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Functional and structural similarities between the inhibitory region of troponin I coded by exon VII and the calmodulin-binding regulatory region of the catalytic subunit of phosphorylase kinase

(actin/troponin C/actomyosin ATPase/skeletal muscle)

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ABSTRACT **A sequence homology has been noted between the carboxyl quarter of the catalytic γ subunit of phosphorylase kinase and the region of troponin I coded by exon VII. Because this portion of troponin I contains the inhibitory region that interacts with actin and troponin C, we have examined whether the γ subunit of phosphorylase kinase can functionally mimic troponin I by also interacting with actin and troponin C. We have found that troponin C not only activates the isolated γ subunit of phosphorylase kinase but also binds with approximately the same affinity as calmodulin. Although actin had no effect on the activity of the γ subunit alone, it did inhibit the activity of γ-calmodulin and γ-troponin C complexes. Conversely, the γ subunit was able to inhibit actomyosin ATPase in a process that could be overcome by calmodulin. These results suggest that actin and calmodulin (or troponin C) compete for binding to the γ subunit. Moreover, the structural and functional similarities between the γ subunit and troponin I suggest that the γ subunit of phosphorylase kinase may have evolved from the fusion of a protein kinase protogene with a progenitor of exon VII of troponin I.**

Phosphorylase kinase is a Ca^{2+}-dependent regulatory enzyme in the cascade activation of glycogenolysis (1, 2). Its requirement for Ca^{2+} ions is thought to couple muscle contraction with energy production (3). The enzyme is a hexadecameric oligomer with the subunit stoichiometry $(\alpha_2\beta_2\gamma_2\delta_2)_2$ (4, 5). The α and β subunits are inhibitory (6, 7), the γ subunit is catalytic (8, 9), and the δ subunit is calmodulin (CaM) (4). This integral CaM subunit inhibits an intrinsic Ca^{2+}-independent activity of the catalytic subunit (10), stimulates its overall phosphotransferase activity while making it Ca^{2+}-sensitive (11), and may limit the number of promoters that associate (12).

Reimann *et al.* (9) first suggested that the 386-residue catalytic γ subunit may interact with CaM through some domain contained within its carboxyl-terminal 110 amino acids because in this region the homology between the γ subunit and other protein kinases completely disappears. Since then this region has been evaluated by sequence comparisons with other known CaM-binding proteins, by a computer algorithm designed to screen for basic amphipathic helices, and by synthetic peptides. These approaches have targeted about three-fourths of the 110 residues as being potentially involved in the binding of CaM (13–17). Within this region of the γ subunit, a limited sequence homology with troponin I (TnI) also has been noted (16, 17). Because this homologous region in TnI has been implicated in the binding of both troponin C (TnC) and actin, we have examined whether these latter two proteins influence the properties of the isolated γ subunit of phosphorylase kinase and whether the γ subunit is capable of mimicking inhibition of actomyosin ATPase by TnI.

MATERIALS AND METHODS

Enzymes, Proteins, and Tetradecapeptide. Phosphorylase kinase was isolated from white skeletal muscle of New Zealand White rabbits through the DEAE-cellulose chromatography step as described (6, 18). The catalytic γ subunit was isolated in 8 M urea/0.1 M H_3PO_4/1 mM EDTA/1 mM dithiothreitol, pH 3.3, as described (6). Dilution buffer for the isolated γ subunit was 50 mM Tris·HCl/50 mM β-glycerophosphate/0.1 mM EDTA/0.1 mM dithiothreitol, pH 6.8. Phosphorylase *b* was isolated as described (19) and residual AMP was removed by treatment with cocoanut charcoal. Bovine brain CaM, rabbit muscle actin and myosin, and bovine serum albumin were from Sigma. TnC from rabbit skeletal muscle was generously provided by James D. Potter of the University of Miami School of Medicine and was homogenous by SDS/PAGE. The synthetic tetradecapeptide substrate (SDQEKRKQISVRGL), corresponding to the phosphorylation site of phosphorylase *b* (20), was kindly provided by Thomas J. Fitzgerald of St. Jude Children's Research Hospital, Memphis, TN.

Protein Concentrations. The concentrations of phosphorylase kinase and phosphorylase *b* were determined spectrophotometrically by using published absorbance indices (18, 21). Solutions of CaM were prepared based on its dry weight. The concentration of TnC was determined by the Bio-Rad protein assay using CaM as the standard. When the effects of TnC and CaM were being compared, their concentrations were carefully matched by using the Bio-Rad protein assay. The concentration of the γ subunit was determined as described (6). All other protein concentrations were determined by the Bio-Rad assay using bovine serum albumin as the standard.

Activity Assays. The phosphorylation of phosphorylase *b* and tetradecapeptide was followed using a filter paper assay (22) and a phosphocellulose strip assay (23), respectively. The standard assays were performed at 30°C and pH 6.8 and contained (at final concentrations) 50 mM Tris·HCl, 50 mM β-glycerophosphate, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.3 mM $CaCl_2$, 10 mM $Mg(CH_3CO_2)_2$, 1.5 mM [γ-^{32}P]ATP (ICN), and phosphorylase *b* (3 mg/ml) or 50 μM tetradecapeptide. The assays were initiated by addition of MgATP and samples were removed at 15-min intervals for determination of ^{32}P incorporation into substrate.

The actomyosin ATPase activity was determined essentially by the method of Pollard (24). When isolated γ subunit was included in these assays, it was renatured by diluting 1:100 with 15 mM Hepes/15 mM KCl/50 μM dithiothreitol/

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Abbreviations: CaM, calmodulin; TnI, troponin I; TnC, troponin C.
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50 μM EDTA/0.3 mM $CaCl_2$, pH 6.8, and concentrated using an Amicon microconcentrator. Solutions of myosin, actin, CaM, and bovine serum albumin were prepared in the same buffer. Assay components were mixed in the proportions described in the figure legends and incubated for 10 min prior to assay. The assays were performed at 30°C and pH 6.8 and were initiated by addition of 10 μl of MgATP to 100 μl of assay mixture. Final concentrations in the standard assays were 14 mM Hepes, 14 mM KCl, 45 μM dithiothreitol, 45 μM EDTA, 0.27 mM $CaCl_2$, 58 mM urea, 2 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, actin (18 μg/ml), and myosin (30 μg/ml). Any additional components are listed in the figure legends. At appropriate intervals 20 μl was removed from the assay mixtures and pipetted into vials containing 20 μl of an acid mixture [1.4 M sulfuric and 4.3% (wt/vol) silicotungstic] and 100 μl of organic solvent [isobutanol/benzene, 1:1 (vol/vol)]. After vigorously mixing for 2 sec, 20 μl of 10% (wt/vol) ammonium molybdate was added and the sample was mixed on a vortex mixer for 10 sec. Then 20 μl was removed from the upper organic phase and spotted on filter paper, and radioactivity was measured in a liquid scintillation counter for the amount of [^{32}P]P_i liberated. Control tubes containing all components of the assay mixture except myosin were used for generating background blanks.

RESULTS

A prominent sequence homology between the carboxyl terminus of the γ subunit of phosphorylase kinase and TnI, from rabbit fast skeletal muscle, is evident between residues 100 and 115 of TnI and residues 298 and 325 of the γ subunit (Fig. 1). Maximal alignment of the sequences requires a 12-residue loop in the γ subunit. This homologous region of TnI (10 of 16 residues identical) is contained entirely within its so-called "inhibitory peptide" (residues 96–116, Fig. 1, top line), a peptide produced by CNBr-cleavage that interacts with both actin and TnC and is nearly as effective as TnI in inhibiting actomyosin ATPase (26–28). We thus set out to determine whether this structural homology has functional manifestations; i.e., could the γ subunit of phosphorylase kinase mimic TnI by interacting with TnC and actin?

Isolated γ subunit has only a low phosphotransferase activity (6) but is dramatically stimulated by CaM (6, 11). We have found, in fact, that TnC can substitute for CaM in activating the γ subunit (Fig. 2). Although the maximal stimulation observed with CaM was 10-fold greater than with TnC, the two proteins bound to the γ subunit with equivalent affinities (0.31 and 0.36 μM, respectively, for half-maximal activation) (Fig. 2 *Inset*).

Although actin was found to neither stimulate nor inhibit the phosphotransferase activity of the γ subunit alone (data not shown), we did find that actin inhibited both γ–CaM and γ–TnC with either phosphorylase *b* or tetradecapeptide as acceptor substrates for phosphoryl transfer (Fig. 3). These findings suggest that actin prevents the interaction between the γ subunit and CaM or TnC. The tetradecapeptide was used as an alternative substrate to eliminate any possible steric effects that might be caused by the large phosphorylase *b* dimer.

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          96                    111                  112  116
Troponin I: NQKLFDLRGKFKRPPL----------RRVRM
              :  |   | | | | |    |          | | | :
γ Subunit:  EVRHFSPRGKFKVI CLTVLASVRIYYQYRRVKP
          294                   309                  322  326

              143                  146  150
Troponin I:   VKK----------EDTEK
              | | |              |  |  |
γ Subunit:    VKKGQQQNRAALFENTPK
              358                       375
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FIG. 1. Alignment of sequences of TnI (25) and the γ subunit of phosphorylase kinase (9) from rabbit fast skeletal muscle. The upper line for TnI represents the entire "inhibitory peptide" derived from CNBr cleavage (26, 27). Optimal alignment of these two noncontiguous sequences requires gaps of 12 and 10 residues in the TnI sequence.

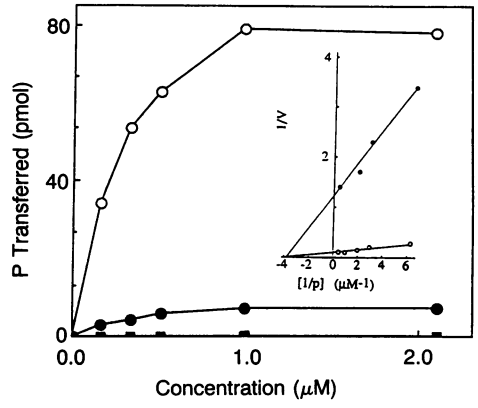

FIG. 2. Stimulation by CaM and TnC of the phosphorylase conversion activity of the isolated γ subunit. Prior to assay the isolated γ subunit was diluted 1:10 with dilution buffer and 10 μl of CaM or TnC was added to 40 μl of the diluted γ subunit. A 10-μl sample was removed from this γ–CaM or γ–TnC mixture and added to 50 μl of an assay mixture containing all components except MgATP. After 15 min the reactions were initiated with 10 μl of MgATP. In addition to the standard components the phosphorylation assays contained γ subunit (1.1 μg/ml), 0.1 M urea, and the indicated concentrations of CaM or TnC. The ordinate is product formed per 20 μl in 15 min. ○, CaM; ●, TnC; ■, γ subunit alone. (*Inset*) Double reciprocal plot of the data [1/p] refers to the reciprocal concentrations of CaM or TnC. The apparent values for maximal velocity are 10.6 and 1.1 mol of phosphate transferred per min per mol of γ subunit and for half-maximal activation are 0.31 and 0.36 μM for CaM and TnC, respectively. The maximal velocity (V) for the reconstituted γ–CaM complex corresponds to about 1% of the maximal activity calculated for the γ subunits within the holoenzyme assayed at pH 8.2.

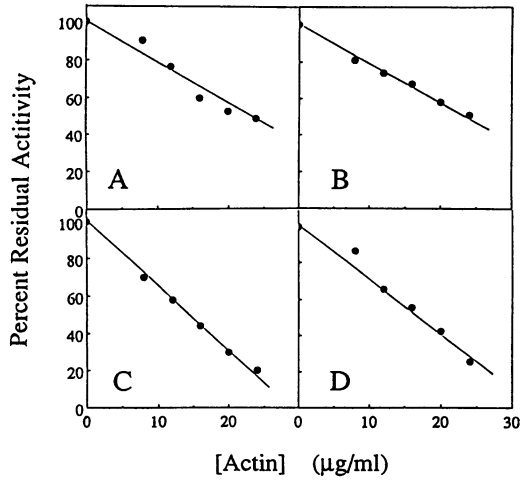

FIG. 3. Inhibition by actin of the phosphorylation activity of γ–CaM and γ–TnC. To achieve the indicated concentrations of actin, the isolated γ subunit was diluted 1:5 with dilution buffer containing various concentrations of actin and CaM (40 μg/ml) or TnC (40 μg/ml). From these dilution mixtures, 10 μl was removed and added to 80 μl of an assay mixture containing all components except MgATP. After 10 min the reactions were initiated with 10 μl of MgATP. In addition to the standard components, the phosphorylation assays contained γ subunit (4 μg/ml), 0.16 M urea, the indicated concentrations of actin, and CaM (4 μg/ml) or TnC (4 μg/ml). (*A*) γ–CaM and phosphorylase *b*. (*B*) γ–TnC and phosphorylase *b*. (*C*) γ–CaM and tetradecapeptide. (*D*) γ–TnC and tetradecapeptide.

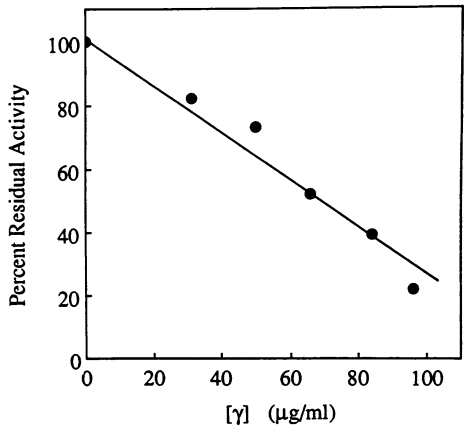

FIG. 4. Inhibition of actomyosin ATPase activity by the γ subunit. The actomyosin ATPase was assayed in the presence of the indicated concentrations of the γ subunit. Samples were removed at 30-min intervals for determination of $[^{32}P]P_i$ liberation.

The inhibition by actin in Fig. 3 could theoretically be caused either by the binding of actin to the γ subunit resulting in blockage of the binding of CaM and TnC to the γ subunit or alternatively by the binding of actin to CaM and TnC also resulting in blockage of their binding to the γ subunit. It should be noted, however, that it has been established that actin does not bind to TnC (29). To gain evidence supporting the direct interaction between the γ subunit and actin, we asked whether the γ subunit, like TnI, could inhibit actomyosin ATPase. As shown in Fig. 4, increasing the concentration of the γ subunit in the assay caused a progressive decrease in the actomyosin ATPase activity. When myosin alone was assayed in the same manner, the γ subunit did not inhibit (data not shown). These results suggest that the γ subunit binds to actin and prevents it from stimulating the ATPase activity of myosin.

The data in Figs. 2–4 plus the sequence homology of the γ subunit with the inhibitory peptide of TnI are consistent with the exclusive binding of either actin or CaM to the γ subunit. If actin and CaM do compete for binding to the γ subunit, then one would predict that the inhibition of actomyosin ATPase by the γ subunit would be less in the presence of CaM than in the absence. In fact, the inhibitory effect of the γ subunit

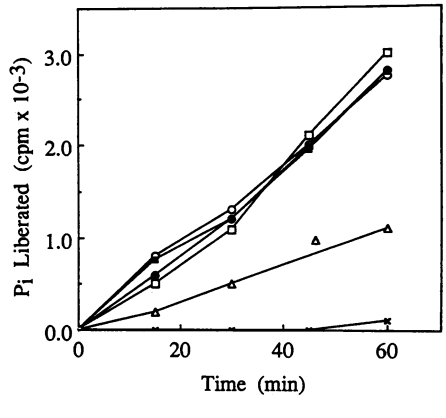

FIG. 5. Ability of CaM to overcome the inhibition of actomyosin ATPase activity by the γ subunit. The standard actomyosin ATPase assay was followed with various other proteins added. Where indicated CaM and bovine serum albumin were present at a final concentration of 83 μg/ml and the γ subunit was at 54 μg/ml. Samples were removed at 15-min intervals for determination of $[^{32}P]P_i$ release. ○, Actomyosin; ■, actomyosin plus bovine serum albumin; ●, actomyosin plus γ subunit and CaM; □, actomyosin plus CaM; △, actomyosin plus γ subunit; X, γ subunit plus CaM.

is completely abolished when excess CaM is present in the ATPase assay (Fig. 5).

DISCUSSION

Although TnC and CaM have more than 50% sequence homology (30), they are not necessarily interchangeable. For instance, TnC cannot substitute for CaM in activating myosin light chain kinase (31) and only poorly activates the erythrocyte (Ca^{2+} + Mg^{2+}) ATPase (32); on the other hand, it effectively replaces CaM in activating microtubule depolymerization (33) and cyclic nucleotide phosphodiesterase (34). Likewise, CaM can substitute for TnC in neutralizing the inhibition of actomyosin ATPase by TnI (35, 36). The sites at which TnI interacts with TnC have been localized to a region near the amino terminus of TnI and to a second site located within the CNBr-derived inhibitory peptide depicted in the top line of Fig. 1 (26, 27). Within this inhibitory peptide, the lysine, leucine, and phenylalanine residues are perturbed when TnC binds to TnI (27). Six of these 8 residues are identical or conserved in the γ subunit of phosphorylase kinase (Fig. 1), which is consistent with our finding that TnC can replace CaM in stimulating the catalytic activity of the γ subunit (Fig. 2). A shorter sequence of TnI from residue 104 to residue 115 has been reported to be nearly as effective as the longer CNBr peptide (residues 96–116) in inhibiting actomyosin ATPase (37, 38). Through studies with synthetic peptides, 7 of the 12 residues within this shorter peptide have been implicated as being very important in interacting with TnC in the presence of Ca^{2+} (positions 106, 108, and 111–115) (38); 6 of these 7 residues are identical or conserved in the γ subunit of phosphorylase kinase (Fig. 1). Another study has implicated Gly-104 of TnI, also conserved in the γ subunit, as being involved in TnC binding (39). Finally, chemical crosslinking has directly shown that TnC interacts with this region of TnI; Lys-105 and -107, both conserved in the γ subunit, were the most highly crosslinked residues (28). Based on the known function of this region of TnI in interacting with TnC, on its sequence homology with the γ subunit of phosphorylase kinase, and on the ability of TnC to substitute for CaM in activating the γ subunit (Fig. 2), it is highly probable that the stretch of residues from approximately position 296 to position 325 in the γ subunit contains a, or the, major determinant for the binding of TnC. Through use of synthetic overlapping peptides Dasgupta *et al.* (17) have identified two domains (termed N and C) in the γ subunit of phosphorylase kinase that have the ability to bind CaM with high affinity. The first of these, domain N, spans residues 287–331 and contains the sequence homologous to the inhibitory peptide of TnI. As was noted by Dasgupta *et al.* (17), domain N, like the inhibitory peptide of TnI, does not contain a tryptophan, although this residue is commonly found in CaM-binding domains. One cannot help but wonder whether this region of the γ subunit might have evolved more for the binding of TnC than for the binding of CaM. Along this line, it has been noted (17) that a rat isoform of the γ subunit that is enriched in testes but is not found in muscle does, in fact, contain two tryptophan residues in this region (40).

In addition to binding TnC, the inhibitory peptide of TnI also binds specifically to actin–tropomyosin, indicating that the Ca^{2+}-dependent switch between muscle relaxation and contraction involves a switch of the inhibitory region of TnI between actin–tropomyosin and TnC. For the interaction between TnI and actin–tropomyosin, Lys-107, Leu-111, Arg-112, Val-114, and Arg-115 of TnI are the most important residues (38). All of these residues are identical or conserved in the γ subunit of phosphorylase kinase (Fig. 1), which is consistent with our observation that actin appears to bind to the γ subunit and prevent TnC or CaM from binding (Figs. 3–5). Such a competition between actin and CaM would be

expected if the two proteins recognized relatively short overlapping binding domains in a common target protein. In fact, this idea of a competition between actin and CaM for a common target protein has been developed as the "flip–flop mechanism" for the regulation of cytoskeletal proteins (41).

Sequence homology between TnI and the γ subunit of phosphorylase kinase is not apparent outside of the two regions shown in Fig. 1, nor is there homology between the respective intervening stretches (residues 117–142 of TnI and residues 327–357 of the γ subunit). The homologous region between the two proteins shown at the bottom of Fig. 1 does not have as clearly a defined function in TnI as the inhibitory peptide shown above it, although in the presence of Ca^{2+} this region of TnI, specifically Cys-133, is reportedly found closer to TnC (25) and further from actin (42). The homology in these two regions of the γ subunit and TnI becomes more suggestive when one notes that every identical residue in the sequences shown in Fig. 1 starting with Arg-103 of TnI (Arg-301 of the γ subunit) and ending with Lys-150 of TnI (Lys-375 of the γ subunit) is identical in TnIs from rabbit fast, slow, and cardiac muscle as well as from chicken fast muscle (43). The fact that the sequence homology ends at position 150 of TnI is especially intriguing because that is the exact boundary of exon VII in the TnI gene from chicken and quail fast skeletal muscle (44, 45), the only tissues in which the organization of the TnI gene has been analyzed. Exon VII codes for residues 92–150 of TnI, which includes the entire inhibitory peptide and both regions of homology shown in Fig. 1. This suggests that the γ subunit of phosphorylase kinase may have evolved from the fusion of a protein kinase protogene with a progenitor of exon VII of TnI. If true, this would mean that a single exon coding for a well-defined regulatory function genetically relates the enzyme responsible for conferring Ca^{2+} sensitivity to carbohydrate metabolism and energy production in muscle (phosphorylase kinase) to a key protein involved in conferring Ca^{2+} sensitivity to muscle contraction (TnI). If it is discovered that a single exon codes for a region of the γ subunit that includes residues 298–375 and that at least one intron exists between this domain and residue 276, where the homology with other protein kinases ceases (9), then this hypothesis would be strengthened.

This study has emphasized the structural and functional similarities between the carboxyl-terminal quarter of the γ subunit of phosphorylase kinase and the inhibitory domain of TnI; however, it must be remembered that in phosphorylase kinase the γ subunit is part of an $(\alpha_2\beta_2\gamma_2\delta_2)_2$ hexadecamer. It is not yet known if the region of the γ subunit that is homologous to TnI actually resides on the surface of the phosphorylase kinase oligomer or if it is masked by intersubunit contacts. There is evidence, however, that the oligomeric phosphorylase kinase holoenzyme, like its isolated γ subunit, in fact, does interact with TnC and actin. Both of these proteins have been reported to stimulate the activity of the holoenzyme (46, 47) as have the troponin complex and artificial thin filaments (48). In addition, actin copurifies with phosphorylase kinase from dogfish skeletal muscle (49) and chicken smooth muscle (50). All of these observations suggest a physical, and perhaps regulatory, association of phosphorylase kinase with the contractile apparatus.

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