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Characterization of the Regulatory Domain of the γ-Subunit of Phosphorylase Kinase

THE TWO NONCONTIGUOUS CALMODULIN-BINDING SUBDOMAINS ARE ALSO AUTOINHIBITORY*

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Phosphorylase kinase is a multimeric protein kinase ($\alpha_4\beta_4\gamma_4\delta_4$) whose enzymatic activity is conferred by its γ-subunit. A library of 18 overlapping synthetic peptides spanning residues 277–386 of the γ-subunit has been prepared to use in identifying important regulatory structures in the protein. In the present study, the library was screened to identify regions that might function as autoinhibitory domains. Peptides from two distinct regions were found to inhibit the Ca^{2+}-activated holoenzyme. The same regions were previously found to bind calmodulin (*i.e.* the δ-subunit; Dasgupta, M. Honeycutt, T., and Blumenthal, D. K. (1989) *J. Biol. Chem. 264*, 17156–17163). The most potent substrate antagonist peptides were PhK13 (residues 302–326; K_i = 300 nM) and PhK5 (residues 342–366; K_i = 20 μM). Both peptides inhibited the holoenzyme competitively with respect to phosphorylase *b* and noncompetitively with respect to Mg·ATP. When the pattern of inhibition with both peptides present was analyzed, inhibition was observed to be synergistic and modestly cooperative indicating that the two peptides can simultaneously occupy the protein substrate-binding site(s). These data are consistent with a model in which the regions of the γ-subunit represented by PhK5 and PhK13 work in concert as regulatory subdomains that transduce Ca^{2+}-induced conformational changes in the δ-subunit to the catalytic γ-subunit through a pseudosubstrate autoinhibitory mechanism.

Phosphorylase kinase is among the largest and most complex of the protein kinase superfamily (reviewed by Pickett-Gies and Walsh (1)). Because of its complex structure, the catalytic activity of phosphorylase kinase is sensitive to a variety of effectors and chemical modifications including pH, divalent metal ions, ionic strength and composition, glycogen, calmodulin, nucleosides, phosphorylation state, limited proteolysis, and substrate conformation. The subunit composition of phosphorylase kinase is $\alpha_4\beta_4\gamma_4\delta_4$, where the α-, β-, and δ-subunits are regulatory and the γ-subunit harbors the catalytic site. The α- and β-subunits can be phosphorylated, and this modification increases the enzyme's catalytic activity, whereas the δ-subunit is identical to calmodulin and confers Ca^{2+} dependence to the enzyme's activity. Although the α-, β-, and δ-subunits are all thought to interact directly with the γ-subunit, the molecular mechanisms by which these regulatory subunits effect changes in catalytic activity is not well understood at present.

A common mechanism by which the catalytic activity of many protein kinases appears to be regulated is through a pseudosubstrate autoinhibitory mechanism (see reviews by Soderling (2) and Kemp and Pearson (3)). This intrasteric form of regulation was first described in the cAMP-dependent protein kinase, but there is now substantial evidence that this mechanism may also be operating in the cGMP-dependent protein kinases, myosin light chain kinases, calmodulin-dependent type II kinases, the protein kinase-Cs, as well as the protein phosphatase calcineurin (2, 3). The simplest mechanistic model for this form of regulation consists of two conformational states: an autoinhibited enzymatically inactive (or less active) state and a deinhibited active (or more active) state. In the autoinhibited state, an autoinhibitory regulatory domain occupies the enzyme's substrate-binding site and prevents access of the protein substrate to the catalytic site. Upon binding of an allosteric activator to the regulatory domain, a conformational change is induced in the autoinhibitory domain that removes it from the substrate-binding site allowing free access of the substrate to the catalytic site.

Typically, the amino acid sequence of an autoinhibitory domain closely resembles the consensus sequence of that enzyme's preferred substrates and is then termed a pseudosubstrate domain. In a number of cases, protein kinase pseudosubstrate domains also contain phosphorylatable residues, and these residues can be autophosphorylated. Thus, identification of putative pseudosubstrate autoinhibitory domains often entails visual inspection of a protein kinase's regulatory domain for sequences that resemble consensus substrate sequences. Candidate pseudosubstrate sequences are then chemically synthesized and tested for their ability to inhibit the enzyme in question. Although the peptide representing an enzyme's pseudosubstrate domain should act as a competitive inhibitor of the protein (or peptide) substrate, this is not always the case (2, 3). Moreover, it is conceivable that selecting a putative pseudosubstrate domain on the basis of similarity to a substrate consensus sequence could result in a false positive identification, particularly if the inhibitory capacity of the putative synthetic pseudosubstrate is low. Thus, positive identification of putative pseudosubstrate domains must be supported by other data including site-directed mutagenesis and x-ray crystallographic structures.

The present investigation is the second phase of a long term effort to characterize the structure-function relationships of the

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regulatory domain of the γ-subunit of phosphorylase kinase. The regulatory domain of the γ-subunit is defined here as the region spanning amino acids 277–386 of this subunit. The first phase of this investigative effort involved identification of putative δ-subunit-binding domains in the regulatory domain by preparing a library of 18 overlapping synthetic peptides spanning residues 277–386 and screening each peptide for its ability to bind calmodulin (4). Two noncontiguous peptides were identified as being likely δ-subunit-binding domains. In the present study, the same synthetic peptide library has been used to screen for possible γ-subunit autoinhibitory domains. Two noncontiguous regions in the γ-subunit regulatory domain have been identified that display the properties expected of pseudosubstrate autoinhibitory domains. These results are being used to direct structural studies to further elucidate the molecular mechanisms underlying the regulation of phosphorylase kinase catalytic activity.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—A series of 18 overlapping 25-residue peptides corresponding to the C-terminal 110 residues (277–386) of the γ-subunit of phosphorylase kinase was synthesized and characterized as described by Dasgupta *et al.* (4). Each peptide was assigned a number based on its position relative to the C terminus of the γ-subunit of phosphorylase kinase (4). The nomenclature and sequence of each peptide are shown in Table I. Shorter analogs of both PhK13 and PhK6 were synthesized as described in Blumenthal and Krebs (5). The nomenclature and sequence of these peptides is described in Table II. All of the peptides in Tables I and II were synthesized with glycine-amide C termini. The composition and sequence of every synthetic peptide was confirmed by quantitative amino acid analysis using a Beckman model 6800 amino acid analyzer following acid hydrolysis and by sequence analysis using an Applied Biosystems Protein Sequencer model 477A.

Protein Preparation—Phosphorylase kinase was purified from rabbit skeletal muscle according to Cohen (12), and phosphorylase *b* was prepared according to Krebs and Fisher (6). Rabbit skeletal muscle myosin light chain kinase was purified as described by Takio *et al.* (7). Calmodulin was prepared from bovine testis by using the following sequence of steps: batchwise chromatography using DEAE-cellulose (DE52, Whatman Ltd.), Ca^{2+}-dependent interaction chromatography (8) using phenyl-Sepharose (Sigma), and gel filtration chromatography (Bio-Gel A-0.5m, Bio-Rad).

Phosphorylase Kinase Assay—The rate of incorporation of ^{32}P into glycogen phosphorylase *b* was used to measure phosphorylase kinase activity. The final reaction mixtures (50 μl) for the inhibition experiments contained 50 mM Tris, 50 mM β-glycerol phosphate, pH 8.2, 1 mM dithiothreitol, 30 μM phosphorylase *b*, 10 mM magnesium acetate, 100 μM calcium chloride, 1 mM [γ-^{32}P]ATP (≈300 counts/min/pmol; DuPont NEN), 1 μg/ml nonactivated phosphorylase kinase, and varying concentrations of synthetic γ-subunit peptides as indicated in the figure legends. Reactions were performed in duplicate at 30 °C, and 20-μl samples of each reaction were spotted on Whatman 3MM filter paper squares (1 × 1 cm) at 5 and 15 min. The squares were immediately immersed in 10% trichloroacetic acid, 4% sodium pyrophosphate and processed as described by Corbin and Reimann (9). All rates of phosphorylation were linear with respect to time for at least 15 min.

Calmodulin-binding Assay—Calmodulin-binding activity was quantitated using a myosin light chain kinase inhibition assay as described previously (4). The reaction mixtures (50 μl final volume) for peptide inhibition experiments contained 50 mM MOPS,[1] pH 7.0, 1 mM dithiothreitol, 130 μM substrate peptide (KKRPQRATSNVFS-amide), 10 mM magnesium acetate, 0.2 mM calcium chloride, 10 nM calmodulin, 1.3 nM skeletal muscle myosin light chain kinase, 1 mM [γ-^{32}P]ATP (≈300 counts/min/pmol; DuPont NEN), and varying concentrations of synthetic γ-subunit peptides as indicated. Reactions were performed in duplicate at 30 °C, and 20-μl samples of the reaction were spotted on Whatman P81 phosphocellulose filter paper squares (1 × 1 cm) at 5 and 15 min. The squares were immediately immersed in 75 mM phosphoric acid and processed as described by Roskoski (10). All rates of ^{32}P incorporation were linear with respect to time for at least 15 min.

Protein Estimation—The concentration of myosin light chain kinase was determined by the method of Bradford (11), using bovine serum albumin as a standard. Other protein concentrations were determined spectrophotometrically using values of $E_{280\ nm,1\%}$ of 12.4 for phosphorylase kinase (12) and 13.2 for phosphorylase *b* (13), respectively, and a value of $E_{276\ nm,1\%}$ of 1.8 for calmodulin (14). Concentrations of phosphorylase *b* are expressed in terms of molar concentrations of monomer.

Kinetic Analysis—The K_i values of the peptides that inhibited phosphorylase kinase activity were determined using the following equation for competitive inhibition (15):

$$i = 1 - \frac{v_i}{v_0} = \frac{[I]}{[I] + K_i\left(1 + \frac{[S]}{K_m}\right)} \qquad \text{(Eq. 1)}$$

where i is the fractional inhibition observed in the presence of peptide inhibitor, [I] is the concentration of peptide inhibitor, [S] is the concentration of phosphorylase *b* (30 μM) used in the reaction mixture, K_m is the Michaelis constant for phosphorylase *b* (33 μM), and v_i and v_0 are the reaction rates in the presence and absence of peptide inhibitor, respectively. K_i values were determined by Marquardt weighted nonlinear least-squares fit estimation using the MathView Professional (v 1.2) program run on a Macintosh computer. The assumption of competitive inhibition used in these calculations was validated by kinetic analysis (see "Results").

RESULTS

The purpose of the present investigation was to determine the possible location of autoinhibitory domain(s) within the C terminus of the γ-subunit. The approach taken was analogous to that previously used to identify δ-subunit (integral calmodulin)-binding domains in the γ-subunit. This approach utilizes a library of eighteen overlapping 25-residue synthetic peptides (the sequences and nomenclature are shown in Table I) based on the C-terminal 110 residues of the γ-subunit. The library was constructed in such a way that every 20-residue segment between residue 277 and the C terminus (residue 386) of the γ-subunit is represented in one of the 18 peptides. Each peptide in the library was assayed for its ability to inhibit the catalytic activity of nonactivated (nonphosphorylated) phosphorylase kinase holoenzyme. The assay reaction was performed at pH 8.2 in the presence of Ca^{2+} with limiting concentrations of the substrate, phosphorylase *b*. Under these conditions, a peptide that can compete with phosphorylase *b* for the substrate-binding site of the enzyme will inhibit enzyme activity. The results of the assay are depicted in Table I, where the K_i value of each peptide is compared with its previously determined K_D for calmodulin (4). The K_i value of each peptide was calculated as described under "Experimental Procedures." Peptides that showed no appreciable inhibition of phosphorylase kinase activity at a concentration of 50 μM are indicated as having K_i values >100,000 μM.

The data in Table I indicate that the γ-subunit regulatory domain peptides which exhibit the most potent inhibitory activity are located in two distinct regions that closely correspond to the regions previously shown to be involved in δ-subunit interactions (4). Moreover, the peptides from each region that most strongly inhibited phosphorylase kinase activity, PhK5 and PhK13 (K_i values of 20 μM and 300 nM, respectively), are the same peptides from each region that were previously found to bind calmodulin with the highest affinity (K_D values of 20 and 6.5 nM, respectively (4)). The K_i values estimated for PhK5 and PhK13 are both within the range of values reported for autoinhibitory domain peptides from other protein kinases (40 nM to 25 μM; see reviews by Soderling (2) and Kemp and Pearson (3)).

To determine the mechanism(s) by which PhK5 and PhK13 inhibit the catalytic activity of phosphorylase kinase, the kinetics of inhibition for each peptide were analyzed as a function of phosphorylase *b* and ATP concentration. The data obtained with PhK13 and PhK5 are plotted in Fig. 1. Fig. 1*A* shows double-reciprocal plots of phosphorylation rates as a function of

[1] The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.

TABLE I

Inhibition of phosphorylase kinase activity by γ-subunit regulatory domain peptides

The nomenclature, residue range, sequence, and K_i value for each synthetic peptide are shown. The K_i values were estimated by non-linear curve-fitting as described under "Experimental Procedures." Dissociation constants for calmodulin binding are also indicated for each peptide (4).

Name	Residues	Sequence	Calmodulin binding	Substrate antagonism
			K_D, nM	K_i, nM
PhK1	362–386	QQQNRAALFENTPKAVLFSLAEDDY	(>50,000)	(>100,000)
PhK2	357–381	WVKKGQQQNRAALFENTPKAVLFSL	4,700	(>100,000)
PhK3	352–376	RIYGHWVKKGQQQNRAALFENTPKA	794	(>100,000)
PhK4	347–371	DAYAFRIYGHWVKKGQQQNRAALFE	190	50,000
PhK5	342–366	LRRLIDAYAFRIYGHWVKKGQQQNR	20	20,000
PhK6	337–361	YALRPLRRLIDAYAFRIYGHWVKKG	36	33,000
PhK7	332–356	VIRDPYALRPLRRLIDAYAFRIYGH	430	(>100,000)
PhK8	327–351	VTREIVIRDPYALRPLRRLIDAYAF	(>50,000)	(>100,000)
PhK9	322–346	RRVKPVTREIVIRDPYALRPLRRLI	(>50,000)	(>100,000)
PhK10	317–341	IYYQYRRVKPVTREIVIRDPYALRP	(>50,000)	(>100,000)
PhK11	312–336	LASVRIYYQYRRVKPVTREIVIRDP	(>50,000)	(>100,000)
PhK12	307–331	ICLTVLASVRIYYQYRRVKPVTREI	14	2,000
PhK13	302–326	GKFKVICLTVLASVRIYYQYRRVKP	6.5	300
PhK14	297–321	HFSPRGKFKVICLTVLASVRIYYQY	35	2,000
PhK15	292–316	VEEVRHFSPRGKFKVICLTVLASVR	56	2,000
PhK16	287–311	FQQYVVEEVRHFSPRGKFKVICLTV	640	10,000
PhK17	282–306	LAHPFFQQYVVEEVRHFSPRGKFKV	(>50,000)	(>100,000)
PhK18	277–301	TAEEALAHPFFQQYVVEEVRHFSPR	(>50,000)	(>100,000)

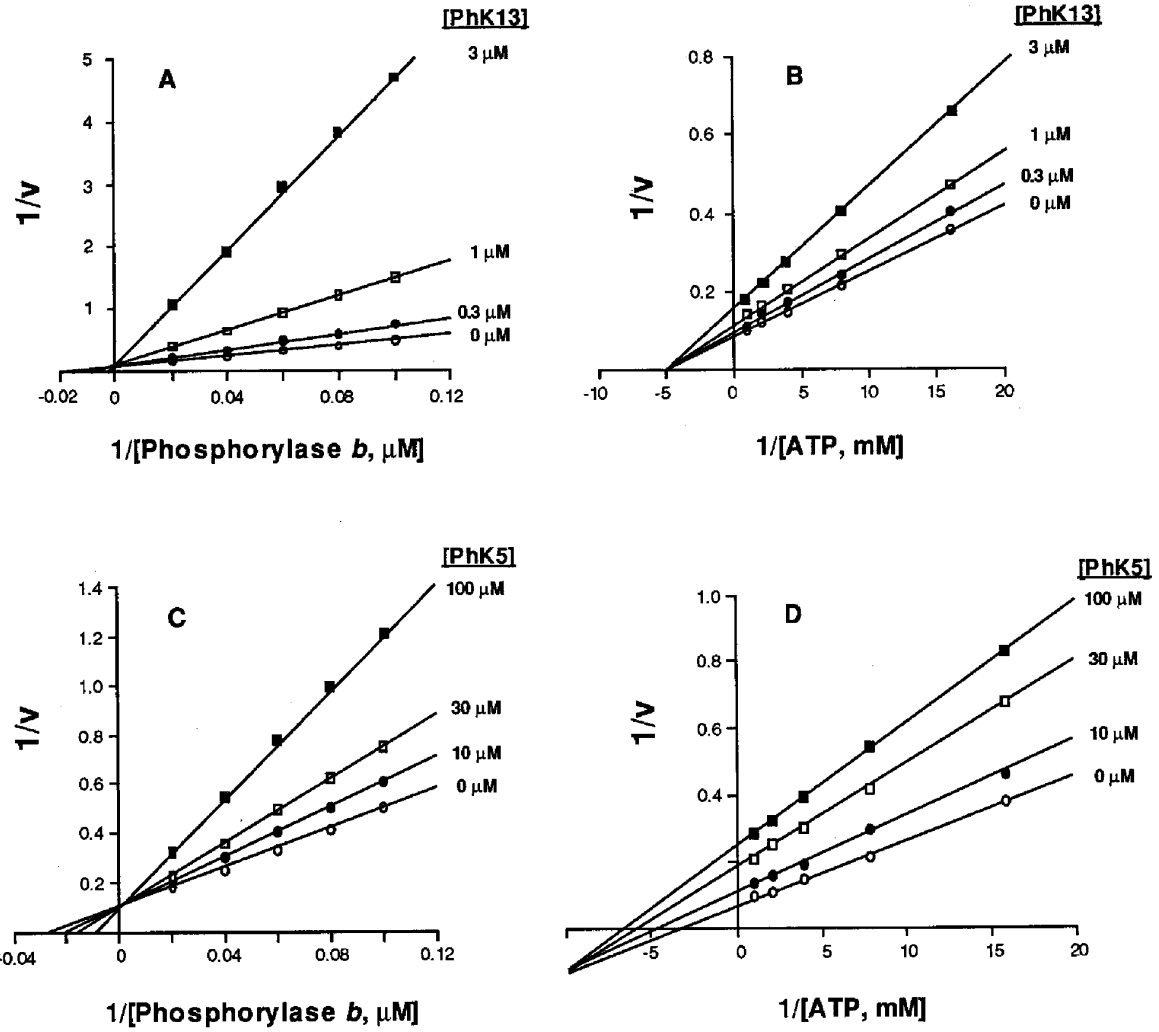

FIG. 1. **Double-reciprocal plots of phosphorylase kinase inhibition by PhK5 and PhK13.** *Panels A* and *B* show double-reciprocal plots of phosphorylase kinase inhibition by the indicated concentrations of PhK13 as a function of phosphorylase *b* and ATP concentration, respectively. *Panels C* and *D* show the corresponding plots for inhibition by PhK5. The concentration of ATP used in *panels A* and *C* was 1 mM. The concentration of phosphorylase *b* used in *panels B* and *D* was 30 μM. Other details of the reaction conditions are given under "Experimental Procedures."

phosphorylase *b* concentration obtained when nonactivated phosphorylase kinase was assayed in the presence of Ca^{2+} (100 μM), a fixed concentration of ATP (1 mM), and various fixed concentrations of PhK13 (0.3–3 μM). The intersection of the double-reciprocal plots for the various concentrations of PhK13 on the (1/*v*)-axis at [phosphorylase *b*]$^{-1}$ = 0 indicates that

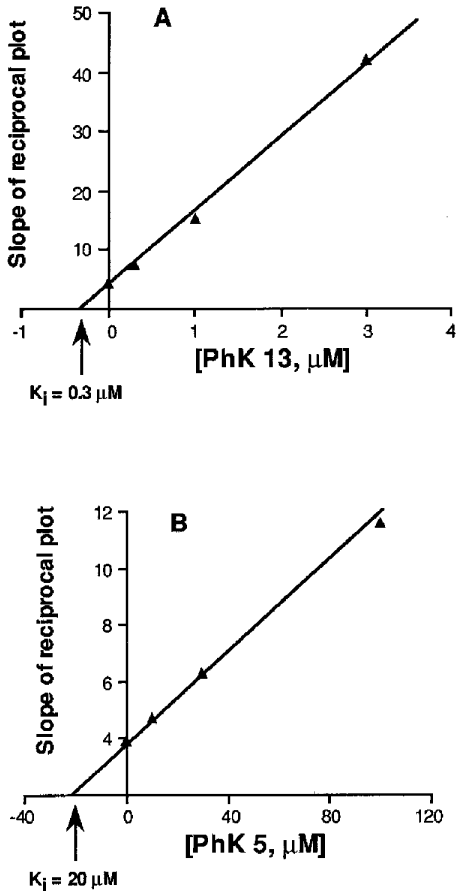

FIG. 2. **Determination of K_i values for PhK13 and PhK5 from replots of slope *versus* peptide concentration.** Slopes of lines determined from plots shown in Fig. 1, *A* and *C*, were replotted as a function of PhK13 and PhK5 concentration, respectively, to determine K_i values for PhK5 and PhK13 with respect to phosphorylase *b*.

PhK13 acts as a competitive inhibitor of phosphorylase *b*. A replot of the slopes in Fig. 1*A* as a function of PhK13 concentration (shown in Fig. 2*A*) yields an estimate of 300 nM for the value of the K_i, the same value as estimated by non-linear curve-fitting procedures (Table I). Double-reciprocal plots of phosphorylase kinase activity as a function of ATP concentration, at various fixed concentrations of PhK13, and at a fixed concentration of phosphorylase *b* (30 μM) are shown in Fig. 1*B*. In this case, the double-reciprocal plots intersect on the $[ATP]^{-1}$-axis indicating that PhK13 acts as a pure noncompetitive inhibitor with respect to ATP (15).

The double-reciprocal plots shown in Fig. 1 indicate that the peptide PhK5 inhibits the catalytic activity of phosphorylase kinase in a manner similar to PhK13. Fig. 1*C* shows a series of plots in double-reciprocal format where the *x* axis represents the reciprocal of the concentration of phosphorylase *b*, the *y* axis represents the reciprocal of the reaction rate, and each line represents data obtained at the indicated concentration of PhK5. As in the case of PhK13, the lines converge on the *y* axis at $[\text{phosphorylase } b]^{-1} = 0$, indicating competitive inhibition by PhK5 with respect to phosphorylase *b*. A replot of the slopes of the lines in Fig. 1*C* as a function of PhK5 concentration (Fig. 2*B*) yields a K_i value for PhK5 of 20 μM, the same value obtained by non-linear curve-fitting procedures (Table I). The double-reciprocal plots shown in Fig. 1*D*, where the rate of phosphorylase kinase activity is plotted as a function of ATP concentration at various fixed concentrations of PhK5, indicate that PhK5 exhibits hyperbolic (partial) mixed-type inhibition with respect to ATP (15). A hyperbolic mixed-type inhibition pattern indicates that PhK5 causes a change in the K_m for ATP, as well as a change in the V_{max}, and is thus acting as a partial noncompetitive inhibitor. Intersection of the double-reciprocal plots below the *x* axis (1/[ATP]) indicates that PhK5 causes a decrease in the K_m for ATP (15). Secondary replots (not shown) of the reciprocal of the *y* axis intercept (1/Δ intercept) as a function of the reciprocal of PhK5 concentration indicate that binding of PhK5 to phosphorylase kinase decreases the K_m for ATP by about 2-fold ($\alpha = 0.47$) and reduces V_{max} by about 5-fold ($\beta = 0.18$). A pure noncompetitive inhibitor would have no effect on K_m ($\alpha = 1$) and would totally block catalysis when bound to the enzyme ($\beta = 0$).

Thus, PhK5 and PhK13 both act as competitive inhibitors of phosphorylase *b*, and both are noncompetitive inhibitors of ATP, although unlike PhK13, PhK5 is not a pure noncompetitive inhibitor of ATP. The pattern of inhibition seen with PhK5 and PhK13 is consistent with the phosphorylation reaction mechanism being a sequential rapid-equilibrium random Bi-Bi mechanism, as previously reported (16–18). The pattern of inhibition seen here is similar to what was reported using synthetic peptides based on the putative autoinhibitory domain of skeletal muscle myosin light chain kinase, an enzyme that also exhibits a rapid-equilibrium random Bi-Bi mechanism (19, 20). Those peptides inhibited myosin light chain kinase competitively with respect to phosphate acceptor substrate and noncompetitively with respect to ATP (21). As in the case of myosin light chain kinase (21), addition of a molar equivalent of calmodulin relative to inhibitor peptide was able to overcome inhibition (data not shown).

The finding that both PhK5 and PhK13 are competitive inhibitors of phosphorylase *b* phosphorylation raises the question as to whether the peptides can act synergistically or whether their inhibition is mutually exclusive. To answer this question, the activity of phosphorylase kinase inhibited by mixtures of PhK5 and PhK13 was analyzed graphically using Dixon plots, as shown in Fig. 3. In Fig. 3*A*, the reciprocal of the reaction rate is plotted as a function of PhK13 concentration at various fixed concentrations of PhK5. The same data are shown in Fig. 3*B*, but in this plot the reciprocal of the reaction rate is plotted as a function of PhK5 concentration, with each line representing a different concentration of PhK13. In Fig. 3, *A* and *B*, the lines converge indicating that inhibition by PhK5 and PhK13 with respect to phosphorylase *b* is synergistic (15). Parallel lines would have indicated that the two peptides inhibited the enzyme in a mutually exclusive manner. The point at which the lines converge in each plot provides information regarding the degree of cooperativity between the two inhibitors. The *x* axis value where the lines converge is equal to $-\alpha K_i$, where K_i is the inhibition constant for the inhibitor that was varied and α is the coefficient of cooperativity. When $\alpha = 1$, it indicates a lack of cooperativity between the inhibitors. When $\alpha > 1$, it indicates that inhibitor binding is negatively cooperative, whereas when $\alpha < 1$, it indicates that inhibitor binding is positively cooperative. The values of α calculated from Fig. 3, *A* and *B*, were 0.35 and 0.67, respectively, based on the previously determined K_i values for PhK13 and PhK5 (0.3 and 20 μM, respectively; *cf.* Table I and Fig. 2). These two estimates for α are in reasonable agreement with each other and indicate that the simultaneous binding of PhK5 and PhK13 to the phosphorylase *b* substrate-binding site shows modest positive cooperativity ($0.3 \leq \alpha \leq 0.7$). These values indicate that the binding of one peptide to the enzyme enhances the binding of the other peptide by about a factor of 2. Synergistic inhibition between PhK5 and PhK13 also indicates that phosphorylase kinase contains a distinct binding site for each peptide. Taken together with the data that both peptides are competitive inhib-

itors of phosphorylase *b* suggests that phosphorylase kinase may have two distinct binding sites for phosphorylase *b*.

To better define the determinants in PhK5 and PhK13 critical for autoinhibitory function *versus* δ-subunit interaction, a series of truncated peptides was prepared and assayed for phosphorylase kinase inhibitory activity and calmodulin binding activity. Because standard solid-phase peptide synthesis proceeds from the carboxyl terminus toward the amino terminus of the peptide, a series of truncation peptides which share a common carboxyl terminus can be easily prepared by removing aliquots of peptide resin at different stages during the synthesis. Table II shows the sequences, calmodulin-binding affinity, estimated K_i for autoinhibition, and nomenclature for the eight peptides that were synthesized. The sequences of PhK5, PhK6, and PhK13 and their respective data are also presented in Table II for purposes of comparison.

Because PhK5 and PhK6 exhibit similar K_i values for autoinhibition and similar affinities for calmodulin, it is reasonable to propose that the minimum essential determinants for autoinhibition and δ-subunit binding function for this regulatory subdomain would be contained in the sequence common to both peptides. This core sequence is represented by the peptide termed PhK6E which has the same amino terminus as PhK5 and the same carboxyl terminus as PhK6, but which lacks the carboxyl-terminal sequence of PhK5 (Gln-Gln-Gln-Asn-Arg) and the amino-terminal sequence of PhK6 (Tyr-Ala-Leu-Arg-Pro). The K_i value for autoinhibition with PhK6E is the same as that for PhK6 and approximately 50% higher than for PhK5, suggesting that the sequence Tyr-Ala-Leu-Arg-Pro (337–341) does not contain determinants essential for autoinhibition, whereas determinants in the sequence Gln-Gln-Gln-Asn-Arg (362–366) contribute modestly to the K_i value. With regard to its affinity for calmodulin, PhK6E binds calmodulin 5-fold weaker than PhK6 and 10-fold weaker than PhK5, suggesting that the sequences Tyr-Ala-Leu-Arg-Pro (337–341) and Gln-Gln-Gln-Asn-Arg (362–366) both contribute significantly to the interactions of the γ-subunit with the δ-subunit. The peptide PhK6D, which is two residues shorter at its amino terminus than PhK6E, had a K_i value for autoinhibition that was only about 30% greater than PhK6E, but bound calmodulin about 8-fold weaker, indicating that Leu-Arg (342–343) contributes significantly to δ-subunit binding, but is only marginally important for autoinhibition. Similar effects on affinity for calmodulin and K_i for autoinhibition were observed when the sequence Arg-Leu-Ile (344–346) was removed on going from PhK6D to PhK6C. The peptide PhK6B, which is three residues shorter than PhK6C, has no detectable autoinhibitory activity at concentrations as high as 50 μM, but still retains the ability to bind calmodulin with low affinity (K_D = 20 μM). The peptide PhK6A, which is two residues shorter than PhK6B, has no detectable autoinhibitory activity or affinity for calmodulin. Thus, it appears that the sequence represented by PhK5 is the shortest sequence in the C-terminal regulatory domain of the γ-subunit with optimal autoinhibitory activity. The differences in affinity for calmodulin observed between PhK6 and PhK6E suggest that adding the sequence Tyr-Ala-Leu-Arg-Pro (337–341) to the amino terminus of PhK5 to form a 30-residue peptide (337–366) might increase the affinity for calmodulin above that seen with PhK5, but this idea has not been tested.

The studies with peptides based on PhK13 included two truncated peptides that were shorter than PhK13 and one peptide that was one residue longer at its amino terminus than PhK13 (Table II). The longer peptide PhK13C (301–326) had Arg^{301} added to its amino terminus and showed a 6.5-fold higher affinity for calmodulin than PhK13 and a K_i for autoinhibition that was 33% lower than for PhK13. These data suggest that this single Arg residue is important for both δ-subunit interactions and autoinhibition. Removing the six residue sequence Arg-Gly-Lys-Phe-Lys-Val (301–306) from the amino terminus of PhK13 resulted in a 10-fold decrease in affinity for calmodulin and less than a 2-fold increase in the K_i for autoinhibition relative to PhK13. Removing an additional five residues, Ile-Cys-Leu-Thr-Val (307–311), resulted in a 15-residue peptide (312–326) that had no detectable affinity for calmodulin and no detectable autoinhibitory activity. These data suggest that the sequence Ile-Cys-Leu-Thr-Val (307–311) is essential for both autoinhibition and δ-subunit interactions. Consistent with this proposal is the finding that all of the PhK peptides that contain this sequence (PhK12 through PhK16) have autoinhibitory and calmodulin binding activity, whereas peptides immediately flanking these peptides are lacking in both functional activities (Table I).

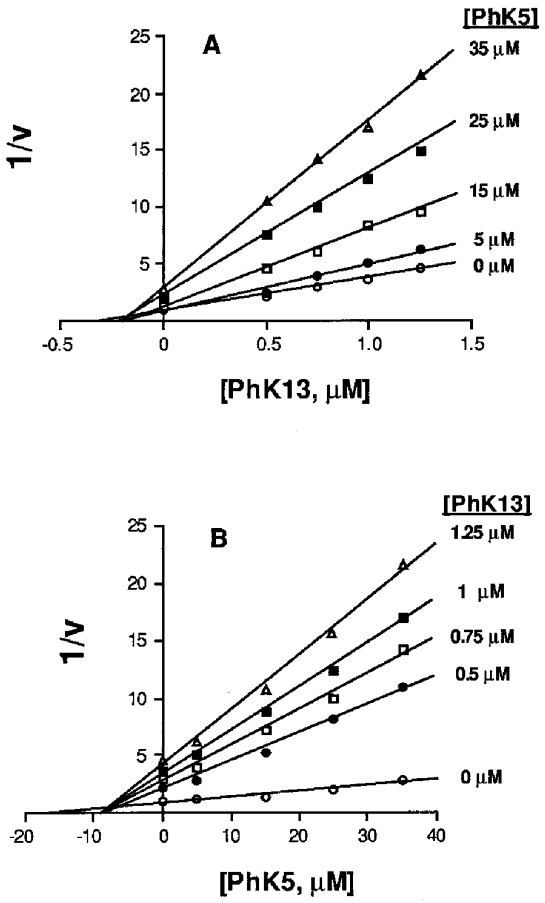

FIG. 3. **Inhibition of phosphorylase kinase activity by mixtures of PhK5 and PhK13.** Phosphorylase kinase activity was determined at the indicated concentrations of PhK5 and PhK13 and represented in Dixon plots. *Panel A* depicts the effects on phosphorylase kinase activity of fixed concentrations of PhK5 as a function of PhK13 concentration. The same data are replotted in *panel B*, but as a function of PhK5 concentration at fixed concentrations of PhK13. The concentrations of phosphorylase *b* and ATP used in these reactions were 30 μM and 1 mM, respectively. Other assay conditions are detailed under "Experimental Procedures."

DISCUSSION

Using a library of overlapping synthetic peptides based on the regulatory domain of the γ-subunit of phosphorylase kinase, two distinct noncontiguous regions, residues 302–326 (PhK13) and 342–356 (PhK5), have been identified as being putative pseudosubstrate autoinhibitory domains. Peptides from each of these two regions inhibited catalytic activity of phosphorylase kinase competitively with respect to the phos-

TABLE II

Effect of peptide length on calmodulin binding and inhibition of phosphorylase kinase activity by γ-subunit regulatory domain peptides

The peptide name, residue range, sequence, and K_i value for each synthetic peptide are shown. The K_i values and calmodulin dissociation constants were estimated by non-linear curve-fitting as described under "Experimental Procedures."

Name	Residues	Sequence	Calmodulin binding	Substrate antagonism
			K_D, nM	K_i, nM
PhK5	342–366	LRRLIDAYAFRIYGHWVKKGQQNR	20	20,000
PhK6A	352–361	RIYGHWVKKG	(>50,000)	(>100,000)
PhK6B	350–361	AFRIYGHWVKKG	20,000	(>100,000)
PhK6C	347–361	DAYAFRIYGHWVKKG	10,000	45,000
PhK6D	344–361	RLIDAYAFRIYGHWVKKG	1,600	40,000
PhK6E	342–361	LRRLIDAYAFRIYGHWVKKG	200	30,000
PhK6	337–361	YALRPLRRLIDAYAFRIYGHWVKKG	36	33,000
PhK13	302–326	GKFKVICLTVLASVRIYYQYRRVKP	6.5	300
PhK13A	312–326	LASVRIYYQYRRVKP	(>50,000)	(>100,000)
PhK13B	307–326	ICLTVLASVRIYYQYRRVKP	75	500
PhK13C	301–326	RGKFKVICLTVLASVRIYYQYRRVKP	1	200

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PhK(β)            420 KRNPGSQKRFPSNCGRD 436
                        .    ::  |  |
Phosphorylase-b     2 RPLSDQEKRKQISVRGLAGVENVTELKKNFNRHLHFTLVKDRNVATPRDYYFALAHTVRDHLVGRWIRTQQHYYEKDPKRIYYLSL  87
                      | .|   | | |::  || |  ..:.::      :  :::|    .   .  |   .  ::.|:|::  |         .  .:
PhK(γ)          296 RHFSPRGKFKVICLTVLASVRIYYQYRRVKPVTxREIVIRDPxxxYALRPLRRLIDAYAFRIYGHWVKKGQQQNRAALFENTPKAV 377
                            PhK13                                                PhK5
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FIG. 4. **Amino acid sequence alignment of phosphorylase *b*(2–87), and putative phosphorylase kinase pseudosubstrate autoinhibitory domains from the γ-subunit(296–377) and the β-subunit(296–377).** Amino acid identities are indicated by (|), close chemical similarities by (:), and distant similarities by (·). The sequences of PhK5 and PhK13 are *underlined* in *bold* and the interconvertible Ser^{14} of phosphorylase *b* is *underlined*. The alignment of β-subunit(296–377) is taken from Sanchez and Carlson (32).

phate acceptor substrate, phosphorylase *b*, and noncompetitively with respect to the phosphate donor substrate, Mg·ATP. Moreover, peptides from the two regions inhibited catalytic activity in a synergistic manner, indicating that both peptides can bind the γ-subunit at the same time. The inhibitory capacity of these peptides could be overcome by the addition of a molar excess of calmodulin, consistent with their previously being identified as calmodulin(δ-subunit)-binding domains (4). This appears to be the first example of a calmodulin-regulated protein kinase with multiple noncontiguous synergistic autoinhibitory/calmodulin-binding subdomains.

Reimann *et al.* (22) first suggested that the C-terminal 110 amino acids of the γ-subunit (residues 277–386) might constitute the regulatory domain of phosphorylase kinase. Subsequently, limited proteolysis (23) and genetically engineered truncation mutants (24, 25) of the γ-subunit were used to show that residues 298–386 contain an autoinhibitory domain. The two putative autoinhibitory domain sequences identified in the present study, 302–326 (PhK13) and 342–356 (PhK5), are both located in this region of the γ-subunit. Neither of these sequences corresponds to the putative pseudosubstrate domain proposed by Kemp and Pearson (3) who suggested that residues 332–353 might represent the pseudosubstrate domain based upon inspection of the γ-subunit sequence. Moreover, the peptide containing this latter sequence, PhK7 (residues 332–356), showed virtually no inhibitory activity in the present study (Table I).

Because PhK5 and PhK13 act as competitive inhibitors of phosphorylase *b*, some sequence similarity between these peptides and phosphorylase might be expected. Sequence alignment of phosphorylase with PhK5 and PhK13 reveals that both peptides contain short stretches of sequence that closely resemble the sequence of phosphorylase (Fig. 4). The two regions of highest sequence similarity correspond closely to the regions required for substrate inhibitory activity based on the data shown in Tables I and II. It is particularly noteworthy that a major portion of PhK13 shows sequence similarity with the sequence surrounding Ser^{14} in phosphorylase *b*, the phosphorylation site in phosphorylase *b*.

The most significant sequence similarity between PhK13 (Lys^{303} to Arg^{323}) and phosphorylase *b* (Lys^{9} to Lys^{29}) spans 21 amino acids. This sequence is only found in its entirety in PhK13, the peptide with the lowest K_i value (0.3 μM) of the inhibitory peptides in the region spanning PhK12 through PhK16 (Table I). The sequence Ile-Cys-Leu-Thr-Val appears to be especially important for inhibition because it is shared by all of the inhibitory peptides in this group and because truncation peptides based on PhK13 that lack this sequence are not inhibitory (Table II). Interestingly, the corresponding pentapeptide from phosphorylase *b* is a competitive inhibitor with a K_i value of about 6 mM (26), and Ile-Ser-Val-Arg-Gly-Leu (phosphorylase *b* (13–18)) is the shortest synthetic peptide substrate that can be phosphorylated by phosphorylase kinase (16). Other important determinants of substrate recognition (26) which are identical in the alignment of PhK13 and phosphorylase *b* include Lys^{9}(Lys^{303}), Lys^{11}(Lys^{305}), Ile^{13}(Ile^{307}), and Leu^{18}(Leu^{312}). Using γ-subunit(1–300), a truncated form of the γ-subunit that lacks a regulatory domain, Huang *et al.* (27) recently demonstrated that Glu^{111} and Glu^{154} are involved in binding peptide substrates at their P-3 and P-2 positions, respectively. Mutations of these residues increased the K_i value for PhK13 by 14- and 8-fold, respectively, indicating their involvement in binding PhK13, as well as peptide substrates. Thus, the sequence corresponding to PhK13 (residues 302–326) appears to represent a prototypical pseudosubstrate domain that regulates the catalytic activity of phosphorylase kinase by preventing the phosphorylation site of phosphorylase *b* access to the catalytic site.

The ability of PhK5 to act as a competitive inhibitor of phosphorylase kinase holoenzyme (Fig. 1) and its sequence similarity to a region of phosphorylase *b* approximately 50 residues C-terminal to Ser^{14} (Fig. 4) suggest that phosphorylase *b* may have a major binding site on the holoenzyme in addition to the binding site that recognizes the sequence around Ser^{14} and that this secondary binding site is also involved in autoinhibition. The sequence in PhK5 showing the

highest similarity to phosphorylase is Arg-Ile-Tyr-Gly-His-Trp-Val-Lys (residues 352–359) which aligns with His-Leu-Val-Gly-Arg-Trp-Ile-Arg (residues 62–69) in phosphorylase (Fig. 4). This sequence is shared by PhK4, PhK5, and PhK6, the only inhibitory peptides in this region of the γ-subunit (Table I). It is interesting to note that Graves and co-workers have shown that synthetic peptides corresponding to the sequence of phosphorylase *b* around its phosphorylation site are poor substrates compared to the intact protein (26) and that longer a CNBr fragment of phosphorylase *b* corresponding to residues 1–99 (16) exhibited a K_m value intermediate between that of the synthetic peptides and native phosphorylase *b*. The data obtained in the present study showing that PhK5 inhibits phosphorylase kinase activity synergistically with PhK13 (Fig. 3) provides further evidence that there are at least two distinct substrate binding sites for phosphorylase *b* and that both of these substrate binding sites might be used by autoinhibitory elements in the γ-subunit to regulate the catalytic activity of phosphorylase kinase. Inspection of the several x-ray crystal structures of phosphorylase (28, 29) show that the phosphorylation site and the PhK5-like region of phosphorylase are relatively close to one another on the surface of the protein, adding support to the idea that these two regions of phosphorylase might be simultaneously involved in interactions with phosphorylase kinase.

The kinetics of inhibition seen with PhK5 and PhK13 in the present study using phosphorylase kinase holoenzyme are in basic agreement with studies done using a γ-subunit truncation mutant, γ-subunit(1–300), which represents a minimal γ-subunit catalytic subunit that lacks calmodulin-binding and autoinhibitory pseudosubstrate domains (27). In the studies with γ-subunit(1–300), PhK5 and PhK13 were both found to be potent inhibitors, although both PhK5 and PhK13 were slightly less potent (3–6-fold) inhibitors of γ-subunit(1–300) than the holoenzyme. The patterns of inhibition of PhK5 and PhK13 toward γ-subunit(1–300) were identical to those seen in the present study using holoenzyme, except that PhK5 was a noncompetitive inhibitor with regard to phosphorylase *b* (as compared to being a competitive inhibitor in the case of the holoenzyme) and a simple noncompetitive inhibitor with regard to ATP (as compared to being a mixed noncompetitive inhibitor of the holoenzyme). Because the γ-subunit(1–300) represents a minimal γ-subunit catalytic subunit that lacks the many inter- and intrasubunit interactions present in the holoenzyme, it is not unexpected that some differences in potency and patterns of inhibition might be observed for PhK5 and PhK13 between the two forms of the enzyme.

In contrast to the findings of the present study, Newsholme *et al.* (30) have concluded that neither PhK5 nor PhK13 are likely to represent pseudosubstrate domains because both peptides appeared to be noncompetitive inhibitors of phosphorylase *b* in their experiments. However, the kinetic data obtained by these investigators for PhK13 could not conclusively discriminate between competitive and noncompetitive inhibition because of problems of solubility at high peptide concentrations. No such solubility problems were encountered in the experiments described here or in other studies involving relatively high concentrations of PhK5 and PhK13 (27, 31). The differences in results obtained cannot be readily explained but might be due to differences in assay conditions or the quality of peptides used in the two studies. PhK5 in particular has several amino acids that are especially sensitive to incomplete side chain deprotection and modification during synthesis, cleavage, and purification. All of the peptides used in the present study were subjected to peptide sequence analysis, quantitative amino acid analysis, and UV spectral analysis.

In addition to the two putative autoinhibitory domains in the γ-subunit, Sanchez and Carlson (32) have recently reported that residues 420–436 in the β-subunit of phosphorylase kinase may constitute a potential autoinhibitory domain. The proposed sequence alignment relative to the phosphorylation site in phosphorylase *b* is shown in Fig. 4. Kinetic analysis of a synthetic peptide based on the β-subunit 420–436 sequence indicated a pattern of inhibition that was competitive with respect to phosphorylase *b* (K_i = 921 μM) and uncompetitive with respect to ATP using the δ-γ enzyme complex. How these various autoinhibitory domains might interact in the holoenzyme remains to be answered. One possibility is that each domain acts independently to inhibit phosphorylase *b* binding. Another possibility is that the domain on the β-subunit acts by inducing conformational changes in the regions corresponding to PhK5 and PhK13, which in turn alter catalytic activity. Elucidation of the precise mechanisms by which these various autoinhibitory domains effect changes in the enzymatic activity of phosphorylase kinase will require detailed structural studies involving a variety of approaches including the use of synthetic peptide analogs, site-directed mutagenesis, and x-ray crystallography.

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