

Identification of the Substrate and Pseudosubstrate Binding Sites of Phosphorylase Kinase γ -Subunit*

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Using site-directed mutagenesis, we proposed that an autoinhibitory domain(s) is located at the C-terminal region (301–386) of the phosphorylase kinase γ -subunit (Huang, C.-Y. F., Yuan, C.-J., Livanova, N. B., and Graves, D. J. (1993) *Mol. Cell. Biochem.* 127/128, 7–18). Removal of the putative inhibitory domain(s) by truncation results in the generation of a constitutively active and calmodulin-independent form, γ_{1-300} . To probe the structural basis of autoinhibition of γ -subunit activity, two synthetic peptides, PhK13 ($\gamma_{303-327}$) and PhK5 ($\gamma_{343-367}$), corresponding to the two calmodulin-binding regions, were assayed for their ability to inhibit γ_{1-300} . Competitive inhibition of γ_{1-300} by PhK13 was found versus phosphorylase *b* ($K_i = 1.8 \mu\text{M}$) and noncompetitive inhibition versus ATP. PhK5 showed noncompetitive inhibition with respect to both phosphorylase *b* and ATP. Calmodulin released the inhibition caused by both peptides. These results indicate that there are two distinct autoinhibitory domains within the C terminus of the γ -subunit and that these two domains overlap with the calmodulin-binding regions. Two mutant forms of γ_{1-300} , E111K and E154R, were used to probe the enzyme-substrate-binding region using peptide substrate analogs corresponding to residues 9–18 of phosphorylase *b* (KRK¹¹Q¹²ISVRGL). The data suggest that Glu¹¹¹ interacts with the P-3 position of the substrate (Lys¹¹) and Glu¹⁵⁴ interacts with the P-2 site (Gln¹²). Both E111K and E154R were competitively inhibited with respect to phosphorylase *b* by PhK13, with 14- and 8-fold higher K_i values, respectively, than that observed with the wild-type enzyme. These data are consistent with a model for the regulation of the γ -subunit of phosphorylase kinase in which PhK13 acts as a competitive pseudosubstrate that directly binds the substrate binding site of the γ -subunit (Glu¹¹¹ and Glu¹⁵⁴).

Phosphorylase kinase catalyzes the phosphorylation and activation of glycogen phosphorylase *b* (for review see Refs. 1 and 2). Phosphorylase kinase has a subunit composition in white skeletal muscle of $(\alpha, \beta, \gamma, \delta)_4$. The α - and β -subunits are regu-

latory subunits. The δ -subunit is almost identical to bovine brain calmodulin and confers Ca^{2+} sensitivity upon the enzyme. The γ -subunit is catalytically active, and its N-terminal region (residues 1–276) shares sequence homology with the catalytic domains of other protein kinases (3). The C-terminal region of γ (residues 287–386) is thought to encompass two distinct, non-contiguous high affinity calmodulin-binding subdomains (4). This speculation is supported by the results that exogenous calmodulin can stimulate the γ -subunit isolated from rabbit skeletal muscle (5, 6) as well as the recombinant full-length γ forms (7–12), but not the truncated γ forms (11, 13, 14).

Several calmodulin-dependent protein kinases, *e.g.* myosin light chain kinase and Ca^{2+} /calmodulin-dependent protein kinase II, have been shown to contain an autoinhibitory domain or a pseudosubstrate sequence in their primary structures, which partly overlaps with their respective calmodulin-binding regions (reviewed in Refs. 15 and 16). In both instances, calmodulin plays an important role in releasing the inhibition imposed by the inhibitory domain. Because the autoinhibitory domain in calmodulin-dependent protein kinases usually overlaps with their calmodulin-binding domain, two calmodulin-binding peptides, PhK13 ($\gamma_{303-327}$) and PhK5 ($\gamma_{343-367}$), from the C terminus of the γ -subunit are potential models for studying autoinhibitory mechanisms in the γ -subunit. We have employed a simple system, the truncated constitutively active γ , γ_{1-300} , and the two synthetic peptides, PhK13 and PhK5, to investigate the possibility that those two regions of the γ -subunit regulatory domain might act as autoinhibitory domains. Two mutants of γ_{1-300} , E111K and E154R, which have been reported previously to have altered substrate binding affinity (18), were also used in this study to define substrate/pseudosubstrate binding sites in the catalytic domain of the γ -subunit.

EXPERIMENTAL PROCEDURES

Materials—[γ -³²P]ATP was purchased from ICN Biomedicals. Other reagents were purchased from Sigma. Phosphorylase *b* was prepared from rabbit skeletal muscle as described in Ref. 19. PhK13 (GKFKVCLTVLASVRIYYQYRRVKP), a peptide sequence derived from the calmodulin-binding domain $\gamma_{303-327}$; Phos(9–18)¹ peptide, corresponding to the phosphorylatable region (residues 9–18) of phosphorylase *b*; and analogs of Phos(9–18) peptides, E10, E11, and E12, were synthesized in the Protein Facility at Iowa State University and purified by reverse-phase high performance liquid chromatography with a C-18 column. PhK5 (LRRLIDAYAFRIYGHVVKKGGQQNR), corresponding to the calmodulin-binding domain $\gamma_{343-367}$, was synthesized at the University of Utah and purified as described previously (4).

Expression and Purification of Mutant γ -Subunits—Expression and purification of γ_{1-300} and its mutant forms, E111K and E154R, were done by using the T7 RNA polymerase-based expression system (20) as

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¹ The abbreviations used are: Phos(9–18) peptide, KRKQISVRGL; E10 peptide, KEKQISVRGL; E11 peptide, KREQISVRGL; E12 peptide, KRKEISVRGL; BSA, bovine serum albumin.

TABLE I
 K_i values and kinetic mechanisms for the inhibition of γ_{1-300} and its mutant forms

Data were obtained using non-linear curve-fitting procedures and linear secondary plots as described under "Experimental Procedures." Values are means \pm S.D. from at least three different experiments. Phos. *b*, phosphorylase *b*.

γ_{1-300} forms	Inhibitors	Substrates	Inhibition	K_i	K_{ii}
				μM	μM
Wild-type	PhK13	Phos. <i>b</i>	Competitive	1.8 \pm 0.1	
Wild-type	PhK13	ATP	Noncompetitive	30.9 \pm 1.5	29.8 \pm 1.3
Wild-type	PhK5	Phos. <i>b</i>	Noncompetitive	62.2 \pm 3.0	63.1 \pm 3.5
Wild-type	PhK5	ATP	Noncompetitive	119 \pm 4	116 \pm 3
E111K	PhK5	Phos. <i>b</i>	Noncompetitive	67.2 \pm 2.1	67.7 \pm 2.2
E154R	PhK5	Phos. <i>b</i>	Noncompetitive	101 \pm 6	106 \pm 4
E111K	PhK13	Phos. <i>b</i>	Competitive	25.8 \pm 1.3	
E111K	PhK13	ATP	Noncompetitive	91.9 \pm 4.6	90.0 \pm 4.5
E154R	PhK13	Phos. <i>b</i>	Competitive	15.0 \pm 1.2	
E154R	PhK13	ATP	Noncompetitive	69.7 \pm 5.1	71.8 \pm 4.8

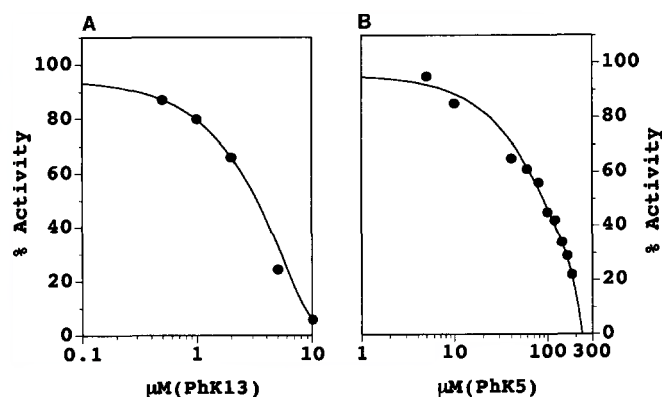


FIG. 1. Comparison of the inhibition of γ_{1-300} by PhK13 and PhK5. The final concentrations in the assay were 50 mM Tris, pH 8.2, 50 mM HEPES, 10 mM MgCl_2 , 4 μM phosphorylase *b*, 1 mM dithiothreitol, 1 mM [γ - ^{32}P]-ATP, 1 $\mu\text{g/ml}$ γ_{1-300} , and various concentrations of PhK13 (panel A) or PhK5 (panel B). The reaction was carried out at 30 $^\circ\text{C}$ for 5 min.

described previously (11). All of the recombinant proteins were purified to homogeneity as judged by SDS-gel electrophoresis (data not shown).

Protein and Peptide Concentration Determination—Protein concentrations were determined by the Bradford assay with commercially prepared reagents from Bio-Rad (21). Peptide solutions were prepared based on their dry weight.

Activity Assay—The protein kinase activity of the γ -subunit was determined by incorporation of ^{32}P into phosphorylase *b* as described previously with some modification (5, 22). The standard assay was performed at 30 $^\circ\text{C}$ for 5 min and contained final concentrations of 60 mM Tris, pH 8.2, 60 mM HEPES, 10 mM MgCl_2 , 100 μM phosphorylase *b*, 1 mM dithiothreitol, 1 mM [γ - ^{32}P]-ATP, and 0.1–2 $\mu\text{g/ml}$ γ_{1-300} (wild-type and mutant forms) in the reaction mixture. In the inhibition assays (Figs. 1 and 2), the standard assay was used except PhK13 or PhK5 were added and the final concentrations of phosphorylase *b* and γ_{1-300} were adjusted to 4 μM and 1 $\mu\text{g/ml}$, respectively. The calmodulin (or BSA) activation assay was done as follows. The standard assay mixture as described above containing PhK13 (10 μM) or PhK5 (250 μM) was first incubated at 30 $^\circ\text{C}$ for 5 min. Then, an equimolar concentration (10 μM or 250 μM) of calmodulin (or BSA) was added and incubated for another 10 min at 30 $^\circ\text{C}$. Reactions were stopped by spotting on ET-31 filter paper (Whatman) and by processing the filters as described by Reimann *et al.* (23). The radioactivity incorporated was determined by liquid scintillation counting.

In the kinetic analyses, the concentration ranges of substrate (ATP and phosphorylase *b*) were varied from 1/2 K_m to 5 times K_m . With wild-type γ_{1-300} , studies were done using (a) varying concentrations of phosphorylase *b* (10–90 μM) and inhibitor peptides (0–7.2 μM for PhK13 and 0–51 μM for PhK5) at a fixed concentration of ATP (400 μM), and (b) varying concentrations of ATP (40–400 μM) and inhibitor peptides (0–54 μM for PhK13 and 0–51 μM for PhK5) at a fixed concentration of phosphorylase *b* (90 μM). The K_m values for phosphorylase *b* using the E111K and E154R mutants of γ_{1-300} were both around 100 μM , but due

to insolubility it was impossible to obtain a final concentration of 500 μM phosphorylase *b* (5 times K_m). Therefore, a concentration of 225 μM phosphorylase *b* was used as the greatest concentration in the E111K and E154R mutant assay. In the case of the kinetic analyses using Phos(9–18) peptide and Phos(9–18) peptide analogs (Table II) as substrates, the concentration ranges of these peptides were varied from 0.5 to 15 mM.

Kinetic data were analyzed with the program, Enzfitter (Elsevier Science Publishers). The initial velocity data (Table II) were fitted to the equation for a two-substrate random Bi Bi mechanism. The inhibition data (Table I) with PhK13 and PhK5 were fitted to the equations for competitive or noncompetitive inhibition with the program Enzfitter. The inhibition constants, K_i for competitive inhibition and K_i and K_{ii} for noncompetitive inhibition, were calculated from the linear secondary plots as illustrated in Fig. 3 (A and B). All the kinetic analyses were repeated at least three times.

RESULTS

Effects of PhK13 and PhK5 on γ_{1-300} —To determine whether PhK13 and PhK5 could act as possible inhibitory domains, these peptides were tested for their ability to inhibit the constitutively active γ , γ_{1-300} . The results showed that both peptides could inhibit the kinase activity of γ_{1-300} . As shown in Fig. 1, PhK13 is a more potent inhibitor ($\text{IC}_{50} = 3 \mu\text{M}$) than PhK5 ($\text{IC}_{50} = 80 \mu\text{M}$). The inhibitory effects of both PhK13 and PhK5 were largely reversed by adding equimolar concentrations of calmodulin (Fig. 2). Another protein, BSA, had little effect on the inhibitory effects of PhK13 and PhK5. This result suggests that calmodulin is specifically required to prevent the inhibitory effect of PhK13 and PhK5.

Kinetic Analyses of the Inhibition of γ_{1-300} by PhK13 and PhK5—Kinetic analyses were undertaken to determine the mechanism of the inhibitory properties of PhK13 and PhK5 on γ_{1-300} . The results of these analyses showed that PhK13 is a competitive inhibitor of phosphorylase *b* phosphorylation and a noncompetitive inhibitor of ATP (Table I and Fig. 3). In contrast, PhK5 is a noncompetitive inhibitor of phosphorylase *b* and ATP. These results indicate the binding of PhK5 to the enzyme is not affected by the binding of substrate phosphorylase *b*. No further attempt was made to locate the possible binding site of PhK5 in γ_{1-300} because noncompetitive inhibition with respect to both substrates indicates PhK5 may be binding at a site other than the ATP and phosphorylase *b* binding sites.

Identification of the Specificity Determinants of Phosphorylase *b*—To gain further insight regarding the inhibitory mechanism, we first mapped substrate binding sites between γ_{1-300} and peptide substrate. This information was then used to evaluate where PhK13 binds and inhibits. Phos(9–18) peptide, $\text{K}^{\text{R}}\text{RKQISVRGL}^{18}$, containing the phosphorylation site, Ser 14 , of phosphorylase *b*, has been used as an alternative substrate of phosphorylase kinase holoenzyme (24) and the γ -subunit (5,

FIG. 2. Inhibition of γ_{1-300} activity by PhK13 or PhK5. The inhibition assay was performed as described under "Experimental Procedures." The final concentrations of PhK13 or PhK5 were 10 and 250 μM , respectively, and those of the other components were the same as described in Fig. 1. In the calmodulin (or BSA) activation assay, equimolar amounts (10 or 250 μM) of calmodulin (or BSA) relative to PhK13 or PhK5 were used in the reaction mixture. The assay conditions were described under "Experimental Procedures."

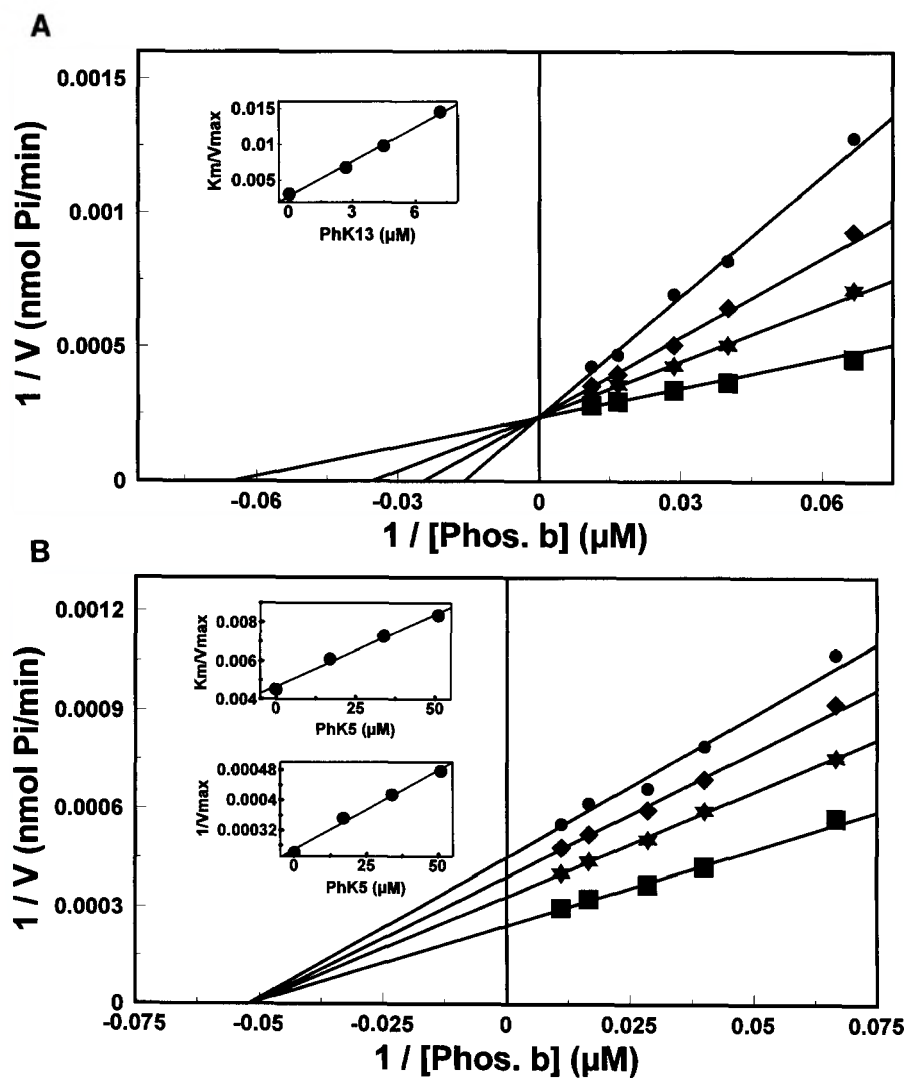
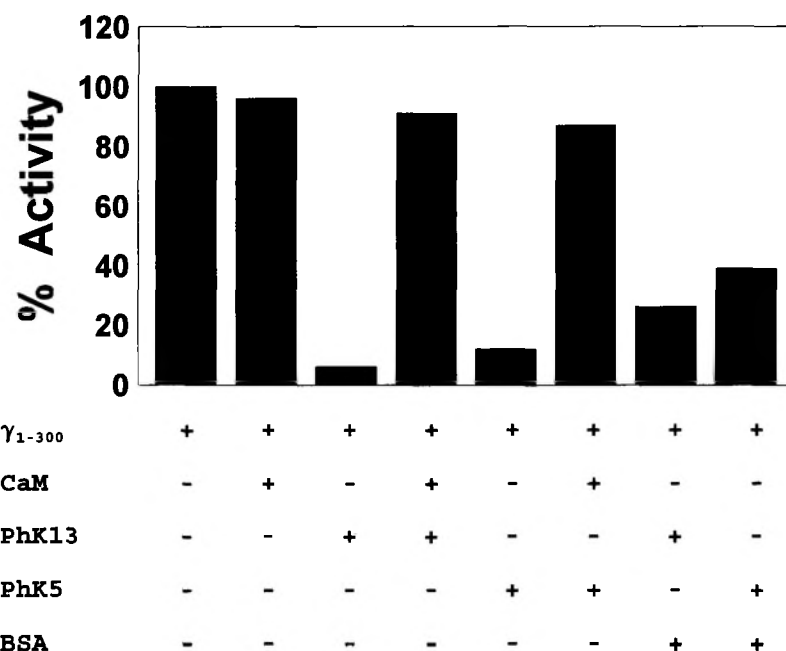


FIG. 3. Initial velocity patterns for inhibition of γ_{1-300} by PhK13 (A) and PhK5 (B). Activity was assayed as described under "Experimental Procedures." The final concentrations of peptides in the assay were PhK13 (0, 2.7, 4.5, and 7.2 μM) and PhK5 (0, 17, 34, and 51 μM). K_i and K_{ii} were estimated from the linear secondary plots (insets in A and B).

25). Previously, Graves and co-workers (26, 27) demonstrated that Lys¹¹ (P-3 site) and Gln¹² (P-2 site) of the Phos(9-18) peptide were important residues for phosphorylase kinase rec-

ognition N-terminal to Ser¹⁴ (P site). To test whether γ_{1-300} has the same substrate specificity determinants as phosphorylase kinase holoenzyme, three Phos(9-18) peptide analogs were

TABLE II

Efficiency of 9–18, E10, E11, and E12 peptides as substrates of wild-type γ_{1-300} and its charge-reversal mutant forms, E111K and E154R. Values are means \pm S.D. from three different experiments.

Enzyme	Substrate	K_m	V_{max}	Relative efficiency ^a
		mM	nmol P _i /min/nmol enzyme	
Wild-type	9–18	0.9 \pm 0.1	1400 \pm 300	1.0
	E10	0.9 \pm 0.1	1600 \pm 150	1.1
	E11	9.0 \pm 0.6	1500 \pm 200	0.11
	E12	2.9 \pm 0.4	1300 \pm 90	0.29
E154R	9–18	4.1 \pm 0.3	690 \pm 150	0.11
	E11	4.1 \pm 0.3	470 \pm 80	0.074
	E12	1.5 \pm 0.3	740 \pm 40	0.32
E111K	9–18	5.0 \pm 0.5	10 \pm 1	0.0013
	E11	1.5 \pm 0.2	110 \pm 3	0.047
	E12	4.6 \pm 0.5	12 \pm 1	0.0017

9–18: ⁹K-R-K-Q-I-S-V-R-G-L¹⁸

E10: K-E-K-Q-I-S-V-R-G-L

E11: K-R-E-Q-I-S-V-R-G-L

E12: K-R-K-E-I-S-V-R-G-L

^a Relative efficiency was calculated as: $(V_{max}/K_m)_{peptide}/(V_{max}/K_m)_{9-18}$, where $(V_{max}/K_m)_{peptide}$ represents the V_{max}/K_m ratio of the peptide being tested and $(V_{max}/K_m)_{9-18}$ represents the V_{max}/K_m ratio of the wild-type γ_{1-300} enzyme using phosphoryl (9–18) peptide as substrate.

prepared by replacing Arg¹⁰, Lys¹¹, and Gln¹² with glutamyl. These peptide analogs were termed E10, E11, and E12, respectively (Table II). Phos(9–18) and its glutamyl-substituted peptides were individually tested as substrates of γ_{1-300} to evaluate the effects on substrate recognition by γ_{1-300} .² Table II compares the results of these kinetic analyses and relative enzymatic efficiency $(V_{max}/K_m)_{peptide}/(V_{max}/K_m)_{Phos(9-18)}$. Peptide E10 showed little change in relative enzymatic efficiency (110%), indicating Arg¹⁰ in Phos(9–18) is not essential for γ_{1-300} recognition. Lower relative enzymatic efficiencies were found in E11 (11%) and E12 (29%), suggesting that E11 and E12 were poor substrates compared with Phos(9–18). Because the lower enzymatic efficiencies were mainly due to the greater K_m values, we suggest that Lys¹¹ (P-3 site) and Gln¹² (P-2 site) in the Phos(9–18) peptide are crucial for optimal substrate recognition.

Mapping the Substrate Binding Sites Using Peptide Analogs—Based on the crystal structure of cyclic AMP-dependent protein kinase-inhibitor complex (28, 29) and mutagenesis studies of yeast cyclic AMP-dependent protein kinase (30, 31), it has been concluded that Glu¹²⁷ and Glu¹⁷⁰ in cyclic AMP-dependent protein kinase (equivalent to Glu¹¹¹ and Glu¹⁵⁴ in γ) are involved in the recognition of Arg residues in the P-3 and P-2 positions of Kemptide, respectively. Interestingly, two mutant forms of γ_{1-300} , E111K and E154R, were shown to influence the binding of substrates (18). In the present study, we utilized peptide analogs (E11 and E12) in combination with charge-reversal mutant of γ_{1-300} (E111K and E154R) to identify possible interactions between Glu¹¹¹ and Glu¹⁵⁴ in γ_{1-300} and Lys¹¹ and Gln¹² in Phos(9–18) peptide.

To determine if either Glu¹¹¹ or Glu¹⁵⁴ in γ_{1-300} participates in the binding of Lys¹¹ at the P-3 site in Phos(9–18), wild-type γ_{1-300} and both E111K and E154R mutants were subjected to kinetic analyses by using Phos(9–18) and E11 peptides as substrates. If either residue Glu¹¹¹ or Glu¹⁵⁴ in γ_{1-300} interacts with the residue at the P-3 site of 9–18 peptide, it would be expected that charge-reversal mutations at these positions would negatively affect substrate kinetics and that charge reversal at the P-3 site in the peptide E11 (Lys¹¹ \rightarrow Glu) would reverse or attenuate these negative effects. The kinetic parameters and relative catalytic efficiencies calculated from these experiments are presented in Table II. Using wild-type γ_{1-300} , a 10-fold greater K_m value was observed with E11 as substrate

than Phos(9–18). There were no significant differences in the K_m or V_{max} values of the E154R mutant using either E11 or Phos(9–18) peptide. These data suggest that Glu¹⁵⁴ in γ_{1-300} does not interact with the P-3 residue in the substrate. Our results agree with the results of the co-crystal structure of cyclic AMP-dependent protein kinase and PKI, which shows that Glu¹⁷⁰ (or Glu¹⁵⁴ in γ) does not contact the P-3 site of PKI.

A dramatic increase in the relative efficiency of the E111K mutant toward E11 (33-fold) was found. The results indicate that the charge reversal in γ_{1-300} (Glu¹¹¹ \rightarrow Lys) was compensated by the peptide substrate charge reversal (Lys¹¹ \rightarrow Glu). We interpret these data to mean that Glu¹¹¹ in γ_{1-300} interacts with Lys¹¹ at the P-3 site of the Phos(9–18) peptide substrate.

To learn whether Glu¹⁵⁴ interacts with the P-2 site in the substrate as it does with cyclic AMP-dependent protein kinase, the kinetics of phosphorylation of Phos(9–18) and E12 peptides were analyzed. Charge reversal at residue 154 in γ_{1-300} (Glu¹⁵⁴ \rightarrow Arg) and substitution at the P-2 residue in the substrate peptide (Gln¹² \rightarrow Glu) might be predicted to improve substrate kinetics if there are interactions between residue 154 and the P-2 site in the substrate. The kinetic data confirmed this prediction (Table II). As shown previously, in wild-type γ_{1-300} , a 3-fold decrease in relative enzymatic efficiency was seen using E12 as a substrate compared with Phos(9–18). There was no significant difference in relative enzymatic efficiency with E111K toward Phos(9–18) (0.0013) or E12 (0.0017). A greater relative enzymatic efficiency was seen with E154R toward E12 peptide (0.32) than Phos(9–18) peptide (0.11). The results support the view that Glu¹⁵⁴ interacts with the P-2 site in the Phos(9–18) peptide.

Inhibitory Mechanism of PhK13—Previously we showed that PhK13 is a competitive inhibitor with respect to phosphorylase *b* and hence may bind directly to the substrate binding site of the γ -subunit. To provide additional evidence that PhK13 binds directly to γ_{1-300} and regulates activity via a pseudosubstrate mechanism, PhK13 inhibition was assayed with both the E111K and E154R mutants of γ_{1-300} . Both E111K and E154R were inhibited competitively by PhK13 with respect to phosphorylase *b*, and the K_i values of PhK13 toward E111K and E154R were increased 14- and 8-fold, respectively, compared with wild-type γ_{1-300} (Table I). Noncompetitive inhibition with respect to ATP with 3- and 2-fold increases in K_i values were found in E111K and E154R mutants, respectively (Table I). Because PhK5 is a noncompetitive inhibitor, we also analyzed the inhibition of both mutants by PhK5 to ensure that PhK13 specifically competed with phosphorylase *b* at its binding site.

² We have prepared several other Phos(9–18) peptide analogs and those analogs have been tested as substrates for γ_{1-300} . We will describe the effects of these analogs and the mapping results elsewhere.

TABLE III
Recognition sites

A, the substrate and proposed pseudosubstrate (or autoinhibitory) regions of phosphorylase kinase are aligned based on the phosphorylatable region of phosphorylase *b* residues. The boxes show the phosphorylation and the proposed pseudosubstrate (or autoinhibitory) sites. When compared to Phos(9–18), the conserved residues are underlined. B, comparison of peptide recognition sites in γ_{1-300} and in cyclic AMP-dependent protein kinase (cAPK). The residues corresponding to Glu¹²⁷ and Glu¹⁷⁰ in cyclic AMP-dependent protein kinase are conserved in the γ -subunit as Glu¹¹¹ and Glu¹⁵⁴. However, Glu²³⁰ in cyclic AMP-dependent protein kinase is replaced by Thr²²² in the γ -subunit.

A		References
Phos. <i>b</i> (9–18)	K-R-K-Q-I-S-V-R-G-L	
β (426–435)	Q-K-R-F-P-S-N-C-G-R	43
γ (328–336)	V-T-R-E-I-V-I-R-D	44
γ (334–343)	I-R-D-P-Y-A-L-R-P-L	15
PhK13/ γ (304–313)	K-F-K-V-I-C-L-T-V-L	this study and the first footnote

B	
γ Residues	E ¹¹¹ G ¹⁵⁴ /T ²²²
Phos(9–18)	K R K Q I S V R G L
Kemptide	L R R A S I G
cAMP Residues (mammalian cAPK)	G ¹⁷⁰ /E ²³⁰ E ¹²⁷

The analyses revealed that the K_i values of PhK5 toward phosphorylase *b* with either mutant were similar to that of wild-type γ_{1-300} (Table I). These results suggest that neither Glu¹¹¹ nor Glu¹⁵⁴ are involved in binding PhK5. Taking all of the data together, we propose that PhK13 acts as a pseudosubstrate and specifically binds to the phosphorylase *b* binding site of γ_{1-300} .

DISCUSSION

By using various combinations of peptide substrate analogs and mutants of the γ -subunit (γ_{1-300}), we have identified amino acids in the catalytic domain of the γ -subunit of phosphorylase kinase which recognize the substrate specificity determinants P-2 and P-3 of the phosphate acceptor substrate. The results indicate that the residues at P-3 and P-2 of the substrate (Lys¹¹ and Gln¹², respectively) may directly interact with residues Glu¹¹¹ and Glu¹⁵⁴ in the active site of γ . This result is consistent with the results of cyclic AMP-dependent protein kinase and myosin light chain kinase (30–33) that two conserved glutamyl residues in the active site region play an important role in substrate recognition. However, Glu²³⁰ in cyclic AMP-dependent protein kinase, which also interacts with the P-2 Arg of Kemptide, is replaced by Thr²²² in the γ -subunit (Table III, part B). If the same alignment exists in these two protein kinases at the P-2 site, H-bonding might occur between residues in the γ -subunit, *i.e.* Glu¹⁵⁴ and Thr²²², and Gln¹² at the P-2 site. Unlike Kemptide in the cyclic AMP-dependent protein kinase system, Arg¹⁶ at the P+2 site of Phos(9–18) was shown early on to be an important substrate specificity determinant (26, 27) for γ . The basic residues on both sides of Ser¹⁴ in phosphorylase seem to be required for substrate recognition for phosphorylase kinase, but the basic residue at P+2 is inhibitory for cyclic AMP-dependent protein kinase. This may partly explain why cyclic AMP-dependent protein kinase and phosphorylase kinase have different substrate specificity, although they share similar substrate recognition residues, Glu¹¹¹ and Glu¹⁵⁴.

The objective of the present study was to gain insight into the

structure-function relationships of phosphorylase kinase and its regulation. Previous studies suggested that two autoinhibitory domains may exist at the C terminus of γ , $\gamma_{301-386}$ (11). One is located before residue 331 and another after residue 331. Two γ -subunit calmodulin-binding peptides, PhK13 ($\gamma_{303-327}$) and PhK5 ($\gamma_{343-367}$), were tested and found to be inhibitory to γ_{1-300} . Based on the present findings, we found that the region $\gamma_{303-327}$ (PhK13) functions as a pseudosubstrate autoinhibitory inhibitor for γ_{1-300} and the region $\gamma_{343-367}$ (PhK5) acts as an allosteric autoinhibitory inhibitor. Both PhK13 and PhK5 can bind calmodulin simultaneously (4); however, these two peptides have different structures (4), suggesting they can interact with calmodulin by different ways and may have different functions in regulating activity. It is not surprising to see these two peptides have different inhibitory mechanisms observed here. Other studies³ (17) done using phosphorylase kinase holoenzyme instead of a truncated form of the γ -subunit, γ_{1-300} , gave different results suggesting that other subunits of the enzyme can influence PhK5 and PhK13 binding events.

Like other calmodulin-dependent protein kinases, the autoinhibitory effect of both PhK13 and PhK5 can be released specifically by calmodulin (Fig. 2). It is likely that the autoinhibitory action of this region is regulated by calmodulin or the δ -subunit. However, in our experiments no calcium was needed for good activation. This could be due to the fact that tight binding of calmodulin to the γ -subunit can occur in the absence of calcium (34). At this point, the physiological significance of this activation mechanism seen *in vitro* is not established.

Four protein kinase structures have been solved, including cyclic AMP-dependent protein kinase (28, 29, 35), cyclin-dependent kinase 2 (36), ERK2 (mitogen-activated protein kinase) (37), and twitchin kinase (38). Overall, the architecture of these kinases is similar. Co-crystallization of cyclic AMP-dependent protein kinase and the tight binding competitive inhibitory PKI (5–24) indicates that the amino acids of PKI participate in binding to the active site and maintain cyclic AMP-dependent protein kinase in an inactive state by steric inhibition. The pseudosubstrate inhibition mechanism has been proposed for myosin light chain kinase, Ca²⁺/calmodulin-dependent protein kinase II, and protein kinase C (reviewed in Ref. 15). Putative structural models for these protein kinases have been proposed based on homolog modeling using the cyclic AMP-dependent protein kinase and PKI as a template (33, 39–42). Recently, crystallization of the twitchin kinase, which contained the catalytic core and the autoinhibitory C-terminal tail, provides the direct evidence for the intrasteric mechanism of protein kinase regulation (38). Multicontact sites occur between the autoinhibitory tail and the active site of twitchin kinase. Based on the twitchin kinase crystal structure, Glu⁶⁰⁶⁷ and Glu⁶⁰²³ in the catalytic core of twitchin kinase (equivalent to Glu¹⁵⁴ and Glu¹¹¹ in γ) make an electrostatic contact with its autoinhibitory C-terminal tail. The data support our findings about the important role of these two glutamyl residues in γ regulation.

So far, there are at least three peptides ($\beta_{420-436}$ (43), $\gamma_{328-336}$ (44), and PhK13) derived from phosphorylase kinase that show competitive inhibition toward phosphorylase *b* using a catalytic subunit. However, there is no evidence suggesting there is more than one active site in the γ -subunit. This raises another question about which sequence is a real pseudosubstrate sequence regulating γ -subunit activity. Although PhK13 is a more potent inhibitor than $\beta_{420-436}$ and $\gamma_{328-336}$, the exact interpretation of the inhibitory mechanisms awaits future structural studies of the catalytic subunit and the holoenzyme.

³ M. Dasgupta and D. K. Blumenthal, submitted for publication.

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