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Activation and Inhibition of Phosphorylase Kinase by Monospecific Antibodies Raised against Peptides from the Regulatory Domain of the γ-Subunit*

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The C terminus of the catalytic γ-subunit of phosphorylase kinase comprises a regulatory domain that contains regions important for subunit interactions and autoinhibitory functions. Monospecific antibodies raised against four synthetic peptides from this region, PhK1 (362–386), PhK5 (342–366), PhK9 (322–346), and PhK13 (302–326), were found to have significant effects on the catalytic activities of phosphorylase kinase holoenzyme and the γ·δ complex. Antibodies raised against the very C terminus of the γ-subunit, anti-PhK1 and anti-PhK5, markedly activated both holoenzyme and the γ·δ complex, in the presence and absence of Ca^{2+}. In the presence of Ca^{2+} at pH 8.2, anti-PhK1 activated the holoenzyme more than 11-fold and activated the γ·δ complex 2.5-fold. Activation of the holoenzyme and the γ·δ complex by anti-PhK5 was 50–70% of that observed with anti-PhK1. Prior phosphorylation of the holoenzyme by the cAMP-dependent protein kinase blocked activation by both anti-PhK1 and anti-PhK5. Antibodies raised against the peptides from the N terminus of the regulatory domain, anti-PhK9 and anti-PhK13, were inhibitory, with their greatest effects on the γ·δ complex. These data demonstrate that the binding of antibodies to specific regions within the regulatory domain of the γ-subunit can augment or inhibit structural changes and subunit interactions important in regulating phosphorylase kinase activity.

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) catalyzes the conversion of phosphorylase *b* to *a* and plays a key role in regulating glycogen breakdown in response to adrenergic and neuronal stimuli. The skeletal muscle isozyme is a multimeric enzyme with the subunit composition $(\alpha\beta\gamma\delta)_4$ and a molecular weight of 1.3×10^6 (reviewed in Ref. 1). The α-, β-, and δ-subunits are regulatory subunits, whereas the γ-subunit is the catalytic subunit (2). Interactions of the regulatory subunits with the γ-subunit serve to modulate the activity of the enzyme in response to the second messengers, cAMP and Ca^{2+}. Phosphorylation of the α- and β-subunits by the cAMP-dependent protein kinase and by autophosphorylation activates the enzyme at pH 6.8. The unphosphorylated α- and β-subunits are thought to inhibit the catalytic site from exhibiting maximum catalytic potential, since inhibition can be relieved by increasing pH to 8.2, by phosphorylation, by proteolysis, or by dissociation of these subunits from the holoenzyme complex. Ca^{2+} dependence is conferred upon the enzyme by the δ-subunit, which is identical to calmodulin (3).

Residues 19–276 of the γ-subunit represent the catalytic domain of the enzyme based on sequence homology with other protein kinases (2). Two crystal structures of a constitutively active catalytic core of the γ-subunit (residues 1–298) have recently been solved to a resolution of 3.0 Å or better (4). Overall, these two γ-subunit structures are very similar to the catalytic cores of other protein kinases. The C-terminal 110 amino acids of the γ-subunit (277–386) are thought to contain pseudosubstrate/autoinhibitory domains and subunit interaction domains based on this region's lack of sequence similarity to other protein kinases (2) and studies involving limited proteolysis (5) and site-directed mutagenesis (6–8). By using a library of overlapping synthetic γ-subunit peptides, the regions corresponding to γ302–326 and γ342–366 have been identified as being regulatory subdomains that act in concert to mediate interactions between the γ-subunit and the catalytic domain of the γ-subunit (6, 9, 10). Peptides corresponding to these two regions (termed PhK13 and PhK5, respectively) bind calmodulin with high affinity (9) and competitively inhibit phosphorylase kinase catalytic activity (6, 10). Site-directed mutagenesis experiments (6) and small-angle scattering studies (11) have provided limited structural information regarding the interactions of PhK5 and PhK13 with the γ-subunit catalytic domain (6) and the δ-subunit (11). However, detailed information regarding the structure of the γ-subunit regulatory domain is not presently available. It has also been proposed that the regulatory domain of the γ-subunit might interact directly with the α- and β-subunits (2), but the sites of interaction on the γ-subunit for the α- and β-subunits have yet to be firmly established.

The present investigation was undertaken to better define the potential regulatory functions of specific regions within the regulatory domain of the γ-subunit of phosphorylase kinase. Antibodies were raised against each of four peptides, PhK1 (γ362–386), PhK5 (γ342–366), PhK9 (γ322–346), and PhK13 (γ302–326), that together span the C-terminal 85 amino acids of the γ-subunit (Fig. 1). These monospecific antibodies were then affinity-purified and assayed for their ability to activate or inhibit the catalytic activity of several different forms of phosphorylase kinase. The approach of using antipeptide antibodies as probes to assess the functional properties of putative regulatory domains within protein kinases has previously been

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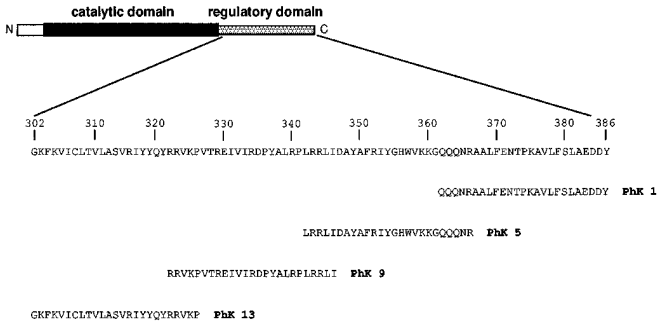


FIG. 1. **Nomenclature of synthetic γ-subunit (PhK) peptides used for raising monospecific antisera.** Each peptide also carries a C-terminal Gly-amide residue, which is not shown.

used to characterize the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase (12), the pseudosubstrate domain of protein kinase C (13), and potential regulatory domains in casein kinase II (14), the insulin-like growth factor-I receptor kinase (15, 16), and rhodopsin kinase (17). The results of the studies presented here indicate that binding of monospecific antibodies to the regulatory domain of the γ-subunit can have profound effects on catalytic activity and provide important insights into structural features involved in regulatory interactions in the phosphorylase kinase holoenzyme complex.

EXPERIMENTAL PROCEDURES

Preparation of Phosphorylase b, Phosphorylase Kinase Holoenzyme, and δ·γ Complex—Phosphorylase kinase holoenzyme was prepared by the procedure of Cohen (18), and phosphorylase *b* was prepared using the procedure of Fischer and Krebs (19). Protein concentrations were determined spectrophotometrically using values of $E^{1\%}_{280\,nm}$ of 12.4 (18) and 13.2 (20) for phosphorylase kinase and phosphorylase *b*, respectively. The γ-subunit of phosphorylase kinase was purified using reversed-phase HPLC[1] as described by Crabb and Heilmeyer (21), except that a Vydac C-4 analytical column (5 mm, 0.46 × 25 cm) was used instead of a Vydac C-18 column. The subunit elution pattern obtained using the C-4 column was similar to that reported for the C-18 column. The active δ·γ complex was prepared from HPLC-purified γ-subunit using the reactivation procedure described by Kee and Graves (22). The reactivation buffer contained 50 mM Tris, 50 mM β-glycerophosphate, pH 8.0, 2 mM dithiothreitol, 0.1 mM $CaCl_2$, 3 mM calmodulin, 1 mg/ml bovine serum albumin, and HPLC-purified γ-subunit (diluted 10-fold into the reactivation buffer). Reactivation was carried out at 0 °C for 18 h.

Peptide Synthesis—Synthesis of peptides was done by standard solid-phase techniques using *t*-butoxycarbonyl chemistry as described previously (9). Peptide purification was performed using reversed-phase HPLC, and each sequence was confirmed by amino acid analysis and protein sequence analysis.

Antibody Production and Purification—Monospecific polyclonal antibodies were raised in rabbits against the synthetic peptides PhK1 (γ362–386), PhK5 (γ342–366), PhK9 (γ322–346), and PhK13 (γ302–326), which span the C-terminal 85 residues of the γ-subunit (see Fig. 1). In the case of PhK1, PhK5, and PhK9, the peptides were coupled to keyhole limpet hemocyanin using glutaraldehyde, and then each peptide was used to immunize two rabbits using the Ribi Adjuvant System (Hamilton, MT). Booster injections were given on days 14 and 28 and every month thereafter. Rabbits were bled each week (3 times a month) between immunizations. In the case of PhK13, the peptide was coupled to Imject maleimide-activated keyhole limpet hemocyanin (Pierce) and used to immunize two rabbits. These rabbits were immunized and bled by Bethyl Laboratories (Montgomery, TX) according to their recommended schedule. Initial screening using an enzyme-linked immunosorbent assay (ELISA) was performed to select the rabbits giving the best responses and the bleeds with the highest titer against phosphorylase kinase holoenzyme. These antisera were used for the subsequent workup described below.

[1] The abbreviations used are: HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; ELISA, enzyme-linked immunosorbent assay.

Each antiserum was subjected to the following prepurification procedure to remove contaminating lipids and lipoproteins. To each milliliter of antiserum, 0.05 ml 5% (w:v) sodium dextran sulfate (Pharmacia Biotech Inc.) was added, stirred, then allowed to sit on ice for 1 h. To this solution was added 0.09 ml of 11.1% (w/v) $CaCl_2$/ml of antiserum. The solution was allowed to settle on ice for 1–2 h, and the precipitate was removed by centrifugation at 5000 × *g* for 20 min. To further purify the antipeptide antibodies, the supernatant was subjected to ammonium sulfate fractionation by slowly adding, while stirring, 0.667 ml of saturated ammonium sulfate (pH 7.3)/ml of antibody solution. The solution was allowed to stir for 1 h on ice and then was centrifuged at 5000 × *g* for 25 min. The precipitated pellet was resuspended in a minimal volume of Milli-Q water and then dialyzed overnight against Milli-Q water at 4 °C. The dialysate was then subjected to affinity purification using phosphorylase kinase immobilized to Sepharose 4B.

Phosphorylase kinase holoenzyme was immobilized on CNBr-activated Sepharose 4B at a density of 5 mg of protein/ml of gel to produce affinity columns for the isolation of each of the antipeptide antibodies. Dry CNBr-activated Sepharose (1.14 g; Sigma) was suspended in 1 mM HCl to dissolve stabilizers and hydrate the gel. After about 1 min, the suspension was filtered, and to the moist gel was added 20 mg of phosphorylase kinase dissolved in 0.1 M borate, 0.5 M NaCl, pH 8.3. The gel and phosphorylase kinase were gently shaken for 24 h at 4 °C, after which the suspension was mixed with 1 M ethanolamine and shaken at room temperature for another 2 h to block unreacted sites on the gel. The gel was washed with 0.1 M borate, 0.5 M NaCl, pH 8.3, followed by 0.1 M acetic acid, 0.5 M NaCl, pH 4.0, and was then aliquoted into four 1-ml columns. The columns were stored in 50 mM MOPS, 2 mM EDTA, pH 7.0, at 4 °C. Before use, the columns used for purification of anti-PhK1 and anti-PhK5 were equilibrated with buffer containing 50 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 1 μM leupeptin. The columns used for anti-PhK9 and anti-PhK13 were preequilibrated with the above buffer containing 200 μM $CaCl_2$. Antipeptide antibodies were applied to the columns in the presence (anti-PhK9 and anti-PhK13) or absence (anti-PhK1 and anti-PhK5) of 200 μM Ca^{2+}. The columns were then washed with equilibration buffer until the A_{280} of the column effluent was less than 0.05. The anti-PhK1 and anti-PhK5 antibodies were eluted with 500 mM $MgCl_2$, and the anti-PhK9 and anti-PhK13 antibodies were eluted with 50 mM MOPS, pH 7.0, 1 mM dithiothreitol, 1 μM leupeptin, and 2 mM EDTA. Eluates were desalted using Bio-Gel P6 DG (Bio-Rad) desalting columns and stored in phosphate-buffered saline at 4 °C. Antibody concentrations were determined spectrophotometrically using a $E^{1\%}_{280\,nm}$ value of 15 for IgG (23).

Fab fragments were prepared from affinity-purified anti-PhK1 antibody according to the procedure of Gibson *et al.* (24) as briefly described here. A sample of affinity-purified antibody (0.5 mg) was dialyzed overnight at pH 7.5 against 50 mM Tris-HCl, 0.15 M NaCl, and 2 mM EDTA. After dialysis, dithioerythritol was added to a final concentration of 1 mM. A suspension of mercuripapain (Sigma) was prepared in the above buffer in the presence of 10 mM dithioerythritol. The solutions of protease and antibody were mixed in a ratio of 1:33 (w:w) and incubated at 37 °C for 30 min. The reaction was stopped by the addition of dehydroascorbic acid to a final concentration of 1 mM, and the solution was allowed to sit for 15 min. The solution was filtered (0.22-μm filter, Pharmacia), and the Fab fragments were separated by fast protein liquid chromatography using a Superdex 200 prep grade (HiLoad 16/60) column.

A sandwich ELISA method was used to determine the ability of anti-PhK1 Fab fragments to bind phosphorylase kinase and the γ·δ complex. The procedure used the reagents and protocol provided in the ELISAmate kit (Kirkegaard and Perry Laboratories). Polystyrene microtiter plates (96-well flat-bottom, Corning) were first coated with the indicated concentrations of anti-PhK1 or anti-PhK1 Fab fragment for 1 h at room temperature. Either phosphorylase kinase holoenzyme (0.9 μg/ml) or γ·δ complex in 1% (w/w) bovine serum albumin was then added and allowed to bind for 1 h before the plate was emptied and rinsed with 1% bovine serum albumin. Antibody raised against a synthetic multiple antigen peptide corresponding to the N terminus of the γ-subunit, GKSHSGPLAADRT, was then added (0.7 μg/ml in 1% bovine serum albumin) and allowed to bind for 1 h. The plate was then emptied and washed three times with 0.02% Tween 20 (v/v). Peroxidase-labeled goat anti-rabbit IgG (0.3 μg/ml in 1% bovine serum albumin) was added and incubated for 1 h. The plate was then washed three times (0.02% Tween 20) before adding the color development reagents, 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide. The plate was read in kinetic mode on a UVmax microplate reader (Molecular Devices) at 650 nm using SOFTmax (version 2.01) for data acquisition and analysis.

Phosphorylase Kinase and γ·δ Complex Activity Assay—A radioactive

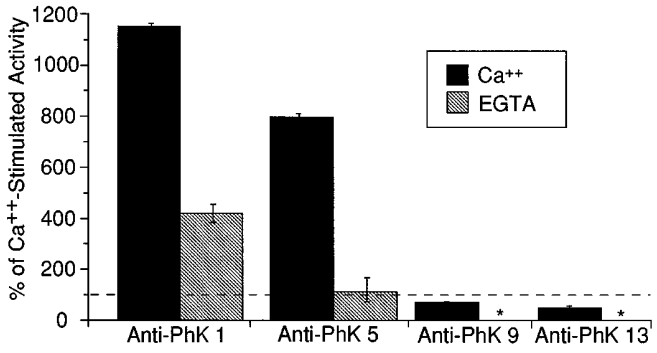


FIG. 2. **Effect of overnight incubation with affinity-purified antipeptide antibodies on phosphorylase kinase holoenzyme activity.** Phosphorylase kinase holoenzyme was assayed at pH 8.2 in the presence of antipeptide antibodies as described under "Experimental Procedures." The *horizontal dashed line* at 100% represents the Ca^{2+}-stimulated activity in the absence of antibody. *Error bars* represent the standard error of the mean. *, not determined.

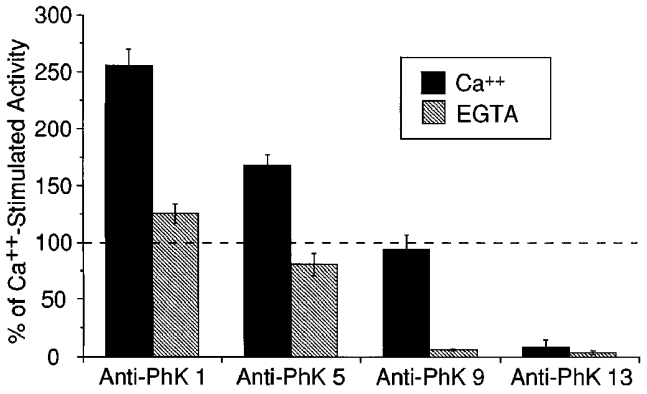


FIG. 3. **Effect of affinity-purified antipeptide antibodies on γ·δ complex activity in the presence of Ca^{2+} or EGTA.** The γ·δ complex was incubated in the presence of antipeptide antibodies and assayed at pH 8.2 as described under "Experimental Procedures." The *horizontal dashed line* represents the Ca^{2+}-stimulated activity of the γ·δ complex in the absence of antibody. *Error bars* represent the standard error of the mean.

protein kinase assay was used to monitor antipeptide antibody interactions with phosphorylase kinase holoenzyme and the γ·δ complex. Enzymatic activities of the holoenzyme and the γ·δ complex were determined by measuring the rate of ^{32}P incorporation from [γ-^{32}P]ATP into phosphorylase *b* or a synthetic peptide substrate (SDQEKRKQISVRGLG). The reaction mixture (50 μl) contained 50 mM HEPES, 42 mM Tris, 1 mM dithiothreitol, pH 8.2 or 6.8 (as indicated), 10 mM magnesium acetate, 200 μM $CaCl_2$, 25 μM phosphorylase *b* or 250 μM peptide substrate (as indicated), 100 ng of phosphorylase kinase, and 1 mM [γ-^{32}P]ATP (300 cpm/pmol; DuPont NEN). The reaction mixtures were preincubated for 5 min at 30 °C before the reactions were started by the addition of ATP. Aliquots of 10 or 20 μl of each reaction were removed after 5 and 15 min and spotted on Whatman 3MM or P81 filter paper squares. Whatman 3MM paper squares were used for the protein substrate, and P81 filter paper squares were used for the synthetic peptide substrate. The paper squares were immediately placed in 10% trichloroacetic acid, 4% sodium pyrophosphate (3MM paper) or 75 mM H_3PO_4 (P81 paper). The papers were washed at least three times, rinsed in 95% ethanol, dried, and counted in a scintillation counter following the addition of Opti-fluor (Packard Instrument Co.) scintillation mixture. Assays containing antibodies or Fab fragments were performed as described above except that the enzyme was preincubated on ice (in a volume of 10 μl) with the indicated concentrations of antibody or Fab fragment for the indicated times before addition to the protein kinase reaction mixture.

cAMP-dependent Protein Kinase Activation—Phosphorylase kinase (2 mg/ml) was activated by incubation with 0.02 milliunits of cAMP-dependent protein kinase catalytic subunit (Promega; units of activity are as defined by Promega) and 1 mM ATP for 30 min in a volume of 100 μl in the presence of the pH 6.8 kinase assay buffer described above. The protein kinase-activated phosphorylase kinase was then preincubated with antibody and assayed as described above.

Statistical Analysis—Error bars associated with each data set represent standard errors of the mean calculated from duplicate or triplicate assay tubes, each of which was sampled at two time points. In some cases, the data represent means and standard errors from more than one experiment. Nonlinear least squares curve-fitting was performed using MacCurveFit (version 1.03) to estimate K_m and V_{max} values and to determine partial inhibition constants.

RESULTS

Activation of Phosphorylase Kinase by Antipeptide Antibodies—The ability of each affinity-purified antipeptide antibody to alter the enzymatic activity of phosphorylase kinase holoenzyme (Fig. 2) and γ·δ complex (Fig. 3) at pH 8.2 was examined. Two of the antibodies, anti-PhK1 and anti-PhK5, induced activation of both phosphorylase kinase and the γ·δ complex in the presence and absence of Ca^{2+}. In the presence of Ca^{2+}, anti-PhK1 antibody increased holoenzyme activity by approximately 1150% and the γ·δ complex activity by 250%. The degree of holoenzyme activation induced by anti-PhK5 antibody was approximately 70% of that seen with anti-PhK1 antibody and somewhat less (50%) for the γ·δ complex. The extent of holoenzyme and γ·δ complex activation induced by anti-PhK1 in the presence of 3 mM EGTA was greater than that seen in the presence of Ca^{2+} with no antibody present. The extent of stimulation of holoenzyme and γ·δ complex activity by anti-PhK5 in the presence of 3 mM EGTA was nearly equal to the Ca^{2+}-stimulated activity in the absence of antibody. The concentration of anti-PhK1 required for activation was comparable for the holoenzyme and the γ·δ complex, in the presence or absence of Ca^{2+}, with maximal activation occurring at an antibody concentration of 0.3 mg/ml (data not shown).

To determine whether the activation of phosphorylase kinase by anti-PhK1 required antibody with intact Fc regions, Fab fragments of anti-PhK1 were tested for their ability to activate the holoenzyme and the γ·δ complex. Other antipeptide antibodies were not investigated due to difficulties in purifying these other antibodies in sufficient quantities to conduct such studies. The anti-PhK1 Fab fragments were unable to activate either the holoenzyme or the γ·δ complex (Fig. 4). A sandwich ELISA was used to determine whether the inability of the Fab fragments to activate was due to the loss of binding capability. It was found that the anti-PhK1 Fab fragments were still able to bind holoenzyme and the γ·δ complex as tightly as the intact antibody (Fig. 5).

The time dependence of antibody activation was assessed by measuring holoenzyme and γ·δ complex activity after overnight and 1-h incubations. Antibody-induced activation by anti-PhK5 occurred in a time-dependent manner with holoenzyme but not with the γ·δ complex (data not shown). Anti-PhK1 exhibited no difference between the overnight and 1-h incubations in the extent of activation of either holoenzyme or the γ·δ complex.

To examine the possible mechanisms for antibody-induced activation, kinetic analyses were performed with anti-PhK1 and anti-PhK5 antibody using both the holoenzyme and the γ·δ complex in the presence and absence of Ca^{2+} at pH 6.8. The K_m and V_{max} values determined from these analyses are shown in Table I. In the presence of Ca^{2+}, the effects of both anti-PhK1 and anti-PhK5 on holoenzyme activity were primarily attributable to changes in V_{max}, with only modest or minimal effects on K_m values for ATP or phosphorylase *b*. Because of the intrinsic difficulty in accurately determining K_m values, it is difficult to know whether the 2-fold decrease in K_m value for ATP seen with anti-PhK1 and the comparable effect of anti-PhK5 on the K_m value for phosphorylase *b* are due to normal assay variability or whether these lower K_m values reflect real but modest effects of these antibodies on enzyme-substrate interactions. The effects of anti-PhK1 and anti-PhK5 on hol-

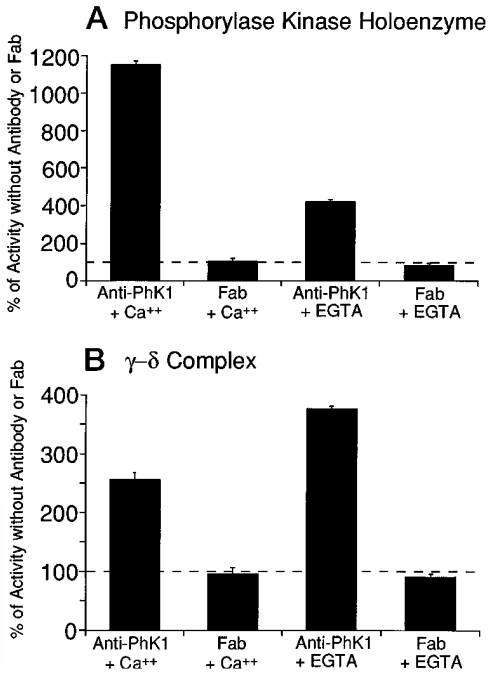


FIG. 4. **Activity of phosphorylase kinase holoenzyme and the γδ complex in the presence of Fab fragments of anti-PhK1.** Phosphorylase kinase holoenzyme (*A*) or the γδ complex (*B*) were preincubated with affinity-purified anti-PhK1 antibody or anti-PhK1 Fab fragments in the presence of Ca^{2+} (1 mM) or EGTA (3 mM) and then assayed at pH 8.2 as described under "Experimental Procedures." *Error bars* represent the standard error of the mean from three independent assays (each performed in duplicate). The *horizontal dashed line* represents Ca^{2+}-stimulated enzyme activity in the absence of antibody.

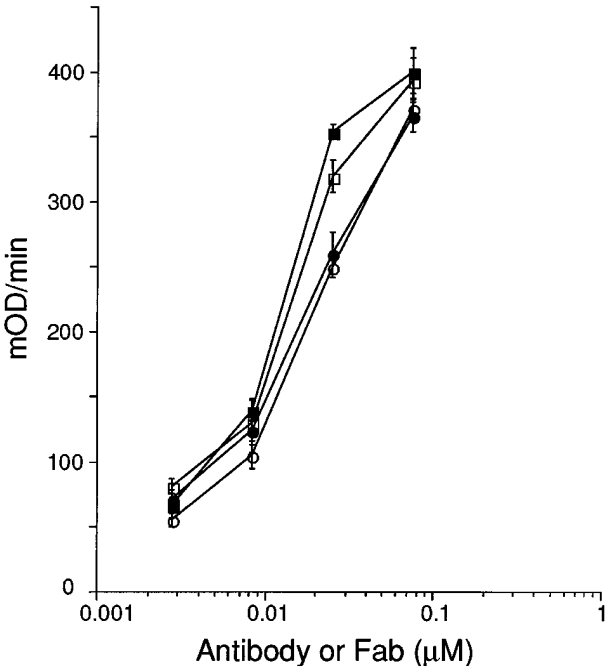


FIG. 5. **Binding of anti-PhK1 antibody (*filled symbols*) and anti-PhK1 Fab fragments (*open symbols*) to phosphorylase kinase holoenzyme and the γδ complex as determined by sandwich ELISA.** Data obtained using anti-PhK1 antibody are represented by *filled symbols*, and anti-PhK1 Fab data are represented by *open symbols*. Data obtained using holoenzyme are depicted by *square symbols*, while those obtained using the γδ complex are represented by *circles*. Details of the sandwich ELISA are described under "Experimental Procedures." Each point represents the mean of three independent assays (each with quadruplicate wells) ± S.D.

oenzyme V_{max} values were much more robust, with anti-PhK1 increasing V_{max} values 10–13-fold and anti-PhK5 increasing V_{max} values 7–9-fold.

Slightly different kinetic patterns were observed for holoenzyme activation when the Ca^{2+} concentration was lowered using EGTA, although the effect of antibody was still predominantly a V_{max} effect. Assays of the holoenzyme with varying concentrations of phosphorylase *b* in the presence of 3 mM EGTA resulted in a 9.3-fold increase in V_{max} with anti-PhK1 antibody but an increase of only 3.2-fold with anti-PhK5 antibody (Table I). There were also 4-fold and 2.4-fold decreases in the K_m value for phosphorylase *b* with anti-PhK1 antibody and anti-PhK5 antibody, respectively. Kinetic analyses using ATP as the varied substrate indicated there were no significant differences in K_m values for ATP, but there were 22.5- and 7-fold increases in V_{max} values with anti-PhK1 and anti-PhK5 antibodies, respectively. These observations indicate that in the absence of Ca^{2+}, anti-PhK1 and anti-PhK5 both activate the holoenzyme through a mixed type mechanism, which is mostly due to an effect on V_{max}, but with some rate-enhancing effects on the K_m for phosphorylase *b* and no effect on the K_m for ATP.

The kinetic patterns observed for activation of the γδ complex by anti-PhK1 and anti-PhK5 were similar overall to those seen with the holoenzyme, with activation being predominantly a V_{max} effect (Table I). Differences between holoenzyme activation and γδ complex activation were primarily seen in the extent of activation and the somewhat more complex pattern of γδ complex activation observed with anti-PhK1 in the presence of Ca^{2+}. A 4-fold decrease in the K_m value for phosphorylase *b* and a 2-fold increase in the V_{max} was seen when the γδ complex was assayed with anti-PhK1 antibody in the presence of Ca^{2+}, indicating a mixed type activation, which differs from the pure V_{max} effect seen with the holoenzyme. A 1.7-fold increase in V_{max} was seen with the anti-PhK5 antibody, with no significant change in K_m for phosphorylase *b*. With regard to ATP, there was no significant change in K_m value observed with anti-PhK1 and a 2-fold increase with anti-PhK5, whereas the V_{max} value increased 3- and 1.7-fold with anti-PhK1 and anti-PhK5, respectively. The effects of anti-PhK1 and anti-PhK5 on the K_m and V_{max} values of the γδ complex in the presence of EGTA were qualitatively the same as those seen in the presence of Ca^{2+}, with V_{max} effects always being observed and K_m effects being seen to a variable degree.

Phosphorylase kinase is activated by cAMP-dependent protein kinase-catalyzed phosphorylation of its α- and β-subunits. In order to determine if activation by phosphorylation affects the activation induced by the anti-PhK1 and anti-PhK5, antibody activation assays were performed using holoenzyme activated by cAMP-dependent protein kinase (Fig. 6). Activation by cAMP-dependent protein kinase resulted in a 4-fold stimulation of activity, which is comparable with the 3.8-fold stimulation in V_{max} induced by raising the pH from 6.8 to 8.2 with this preparation of phosphorylase kinase (data not shown). The activation induced either by anti-PhK1 (13-fold) or by anti-PhK5 (8-fold) was significantly greater than the activation seen with cAMP-dependent protein kinase. Interestingly, cAMP-dependent protein kinase activation of phosphorylase kinase completely blocked subsequent activation of the holoenzyme by either antibody (Fig. 6).

Inhibition of Phosphorylase Kinase Activity by Antipeptide Antibodies—In contrast to the activation seen with anti-PhK1 and anti-PhK5, both anti-PhK9 and anti-PhK13 caused inhibition of holoenzyme and γδ complex activity (Figs. 2 and 3; Table I). Inhibition of holoenzyme activity by anti-PhK9 was modest (20%) and maximal after 1 h of incubation (data not shown). Holoenzyme inhibition by anti-PhK13 was more pro-

TABLE I

Summary of kinetic data for antibody activation and inhibition of holoenzyme and γδ complex activity

V_{max} and K_m values for ATP and phosphorylase *b* at pH 6.8 were determined by nonlinear curve fitting of initial rate data using MacCurveFit (version 1.03). The errors associated with each value are 95% confidence intervals. Values in parentheses were determined from linear regression of double reciprocal plots. Initial rates were determined as described under "Experimental Procedures" using phosphorylase *b* concentrations varying from 10 to 100 μM at a fixed concentration of ATP (1 mM) and ATP concentrations ranging from 0.25 to 1 mM at a fixed concentration of phosphorylase *b* (25 μM). All experiments were done in duplicate at two time points and repeated at least three times. Control reactions were handled in the same manner as those with antibody, except that no antibody was added.

	Phosphorylase b		ATP	
	K_m	V_{max}	K_m	V_{max}
	μM	μmol/min/mg	μM	μmol/min/mg
Phosphorylase kinase holoenzyme in the presence of Ca^{2+} (1 mM)				
Control	64.0 ± 3.16 (53.2)	2.23 ± 0.05 (2.01)	0.25 ± 0.03 (0.33)	1.37 ± 0.06 (1.55)
Anti-PhK1	55.8 ± 8.11 (55.9)	28.8 ± 1.96 (28.9)	0.13 ± 0.01 (0.21)	13.9 ± 0.04 (15.7)
Anti-PhK5	32.7 ± 7.51 (38.6)	14.8 ± 1.26 (15.9)	0.26 ± 0.01 (0.25)	12.7 ± 0.24 (12.6)
Anti-PhK13	65.9 ± 13.8 (53.3)	1.34 ± 0.14 (1.20)	0.31 ± 0.05 (0.48)	0.79 ± 0.05 (0.96)
Phosphorylase kinase holoenzyme in the absence of Ca^{2+} (3 mM EGTA)				
Control	69.0 ± 11.2 (49.7)	0.49 ± 0.04 (0.61)	0.19 ± 0.06 (0.17)	0.39 ± 0.04 (0.38)
Anti-PhK1	17.5 ± 10.1 (23.1)	4.56 ± 0.77 (5.04)	0.45 ± 0.05 (0.73)	8.79 ± 0.46 (11.4)
Anti-PhK5	28.9 ± 10.2 (24.2)	1.57 ± 0.20 (1.50)	0.42 ± 0.06 (0.50)	2.70 ± 0.15 (2.94)
γδ complex in the presence of Ca^{2+} (1 mM)				
Control	47.9 ± 9.57 (31.5)	1.76 ± 0.15 (1.46)	0.13 ± 0.02 (0.15)	1.02 ± 0.03 (1.06)
Anti-PhK1	12.5 ± 3.00 (17.0)	3.81 ± 0.22 (4.13)	0.10 ± 0.01 (0.09)	3.00 ± 0.06 (2.94)
Anti-PhK5	31.2 ± 11.2 (38.5)	2.94 ± 0.38 (3.17)	0.27 ± 0.05 (0.27)	1.71 ± 0.11 (1.72)
γδ complex in the absence of Ca^{2+} (3 mM EGTA)				
Control	59.5 ± 9.37 (72.4)	0.41 ± 0.03 (0.45)	0.21 ± 0.04 (0.27)	0.37 ± 0.02 (0.41)
Anti-PhK1	16.7 ± 5.25 (26.2)	1.43 ± 0.12 (1.65)	0.14 ± 0.01 (0.15)	1.49 ± 0.03 (1.52)
Anti-PhK5	85.2 ± 26.9 (96.9)	2.06 ± 0.36 (2.16)	0.11 ± 0.02 (0.12)	0.75 ± 0.03 (0.78)

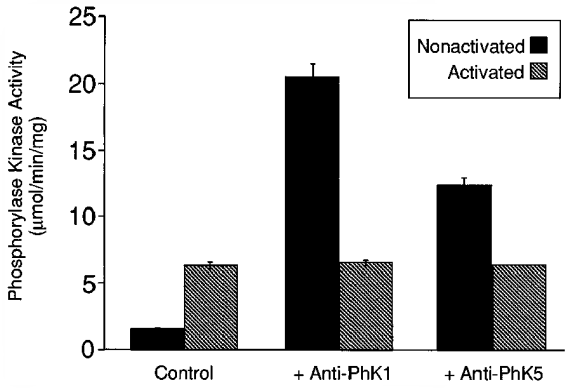


FIG. 6. **Activity of cAMP-dependent protein kinase-activated phosphorylase kinase holoenzyme in the presence of anti-PhK1 and anti-PhK5 antibody.** Phosphorylase kinase was activated by cAMP-dependent protein kinase and then incubated with or without anti-PhK1 or anti-PhK5 antibody, as indicated, before assaying for activity at pH 6.8. Conditions used for activation and for the phosphorylase kinase assay are described under "Experimental Procedures." *Error bars* represent the standard error of the mean of three independent assays (each performed in duplicate).

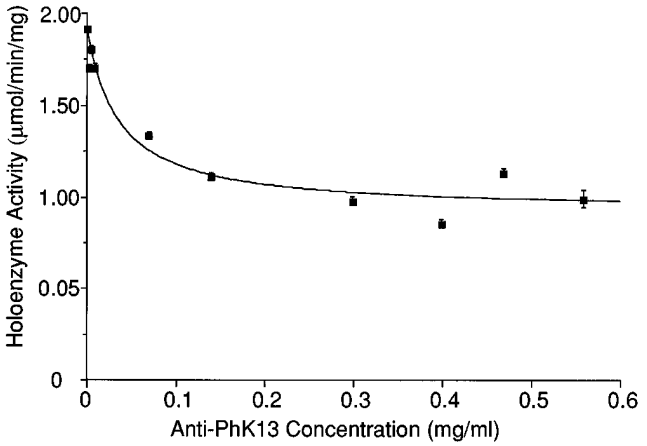
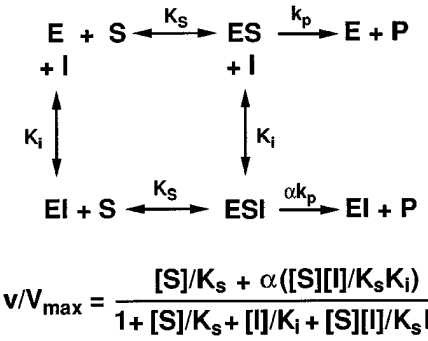


FIG. 7. **Concentration dependence of phosphorylase kinase holoenzyme activity by affinity-purified anti-PhK13 antibody.** Phosphorylase kinase holoenzyme was incubated overnight with the indicated concentrations of anti-PhK13 antibody and then assayed at pH 8.2 as described under "Experimental Procedures." The data were fit to the equation for partial noncompetitive inhibition (Fig. 8). The curve shown was calculated using the equation shown in Fig. 8 and the best fit values determined by MacCurveFit (version 1.03). Each point represents the mean of three independent assays (each performed in duplicate) ± the standard error of the mean.

nounced but slower, with less than 20% inhibition occurring after 1 h and ~50% inhibition being reached after 24 h (data not shown). When either anti-PhK9 or anti-PhK13 was incubated with the γδ complex, a relatively slow onset of inhibition was observed (data not shown). After a 1-h incubation of the γδ complex with anti-PhK9 antibody there was only slight inhibition (~10%), whereas anti-PhK13 elicited nearly complete inhibition (90%) after 1-h. Both anti-PhK9 and anti-PhK13 caused nearly complete inhibition (98%) of γδ complex activity with overnight incubation.

Kinetic studies of anti-PhK13 inhibition of holoenzyme activity indicate a noncompetitive mode of inhibition with a 1.7-fold decrease in V_{max} value and little or no effect on the K_m value for either ATP or phosphorylase *b* (Table I). Inhibition of holoenzyme by anti-PhK13 was concentration-dependent and saturable (Fig. 7). With saturating concentrations of anti-PhK13, only a partial (~50%) inhibition of the holoenzyme was obtained (Fig. 7), whereas essentially complete inhibition (99%) was seen with the γδ complex (Fig. 3). Thus, anti-PhK13 acts as a partial inhibitor of the holoenzyme (25) but a full inhibitor of the γδ complex. A kinetic scheme illustrating partial noncompetitive inhibition of holoenzyme activity as seen with anti-PhK13 is depicted in Fig. 8. For the sake of simplicity, only one substrate is shown. In this scheme, anti-PhK13 antibody is represented as *I*, which is able to bind free enzyme (*E*) as well as the enzyme-substrate complex (*ES*). Inhibitor binding to the enzyme has no effect on substrate binding (K_s), and conversely, substrate binding has no effect on inhibitor binding (K_i). The catalytic efficiency of the enzyme-substrate-inhibitor complex (*ESI*), is reduced relative to the enzyme-substrate complex, and this is reflected in the term α, the partial inhibition coefficient.



$$v/V_{max} = \frac{[S]/K_s + \alpha([S][I]/K_sK_i)}{1 + [S]/K_s + [I]/K_i + [S][I]/K_sK_i}$$

FIG. 8. **Kinetic scheme and equation for partial noncompetitive inhibition of phosphorylase kinase holoenzyme activity by anti-PhK13 antibody.** [*S*] is the concentration of phosphorylase *b*; K_s is the Michaelis constant for phosphorylase *b*; v/V_{max} is the fractional activity; [*I*] is the concentration of anti-PhK13 antibody; K_i is the inhibition constant (in mg/ml); and α is the partial inhibition coefficient. For the sake of simplicity and because anti-PhK13 had no effect on the K_m for ATP, the kinetic scheme and equation do not include the substrate, ATP. The values for α and K_i were determined by nonlinear curve fitting of the data in Fig. 7 using MacCurveFit (version 1.03).

In the case of pure noncompetitive inhibition, $\alpha = 0$. Estimates of α (0.589 ± 0.028) and K_i (0.035 ± 0.014 mg/ml) were determined for anti-PhK13 from nonlinear least squares curve fitting of the data in Fig. 7 using the equation shown in Fig. 8. The curve calculated using these best fit values is plotted in Fig. 7.

Because it has recently been shown that the pseudosubstrate/autoinhibitory domain of the γ-subunit is rather extensive and involves the regions corresponding to both PhK5 and PhK13 (6, 10), it was important to determine whether using a minimal length phosphate-acceptor substrate might have any effect on antibody-induced activation or inhibition. Fig. 9 shows the results of assays performed in the presence of anti-PhK1, anti-PhK5, and anti-PhK13 using a tetradecapeptide peptide substrate corresponding to the phosphorylation site of phosphorylase *b* in comparison with phosphorylase *b* as substrate. There was no difference in the activation or inhibition observed between the two substrates, using either phosphorylase kinase holoenzyme or the γ·δ complex, with regard to any of the three antibodies examined.

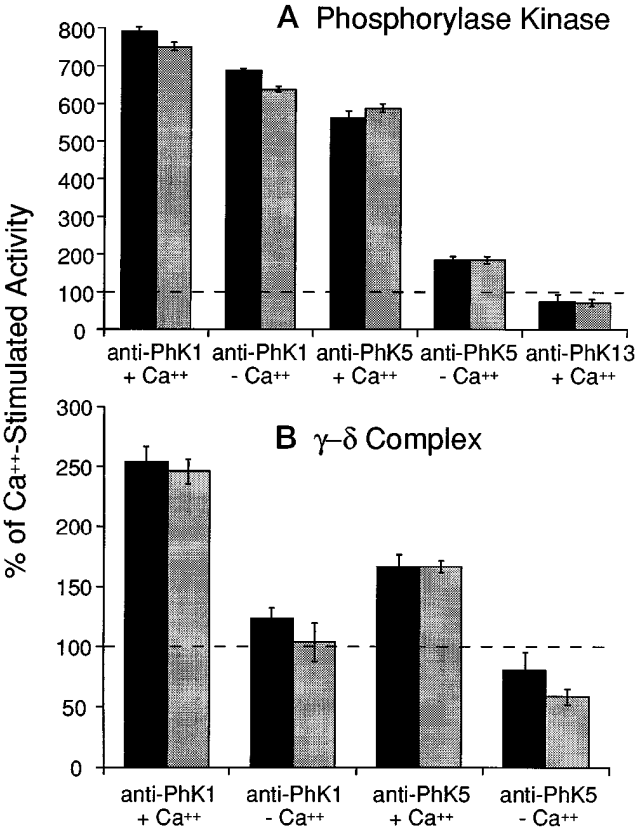


FIG. 9. **Antibody activation and inhibition of phosphorylase kinase holoenzyme and γ·δ complex with phosphorylase *b* or synthetic peptide substrate.** Phosphorylase kinase (*A*) or the γ·δ complex (*B*) was assayed at pH 8.2 following incubation with anti-PhK1, anti-PhK5, or anti-PhK13 antibody and using either phosphorylase *b* (*filled bars*) or peptide substrate (*open bars*) in the presence or absence of Ca^{2+} as indicated. Conditions of the assay are described under "Experimental Procedures." *Error bars* represent the standard error of the mean of three independent assays (each performed in duplicate).

DISCUSSION

The purpose of this study was to characterize monospecific antibodies raised against peptides spanning the regulatory domain of the γ-subunit of phosphorylase kinase to determine whether any of these antibodies might have an effect on the biochemical properties of the enzyme. Antipeptide antibodies have previously been used to identify important functional domains in other protein kinases by characterizing the effects of antibody binding on enzyme activation and inhibition (12–17). Antibodies capable of activating or inhibiting phosphorylase kinase have also been raised using native phosphorylase kinase holoenzyme and denatured subunits of phosphorylase kinase as antigens (26–28). However, most of the epitopes of these antiphosphorylase kinase antibodies have not been precisely localized, so that it is difficult to interpret the effects of these antibodies in terms of their binding to specific structural elements within the holoenzyme. In the present study, the effects of each antibody on enzymatic activity can be interpreted in terms of that antibody's binding to a specific region on the regulatory domain of the γ-subunit, since the antibody's epitope is defined by the peptide antigen used in raising it.

Because of the complex subunit structure and enzymology of phosphorylase kinase, the effects of each regulatory domain antibody were determined using two different catalytic forms of the enzyme, the holoenzyme and the γ·δ complex. The former catalytic form contains a full complement of subunits, whereas the latter catalytic form lacks the regulatory α- and β-subunits. All of the γ-subunit regulatory domain antipeptide antibodies were found to have some sort of effect on the enzymatic activity of phosphorylase kinase, with the C-terminal regulatory domain antibodies (anti-PhK1 and anti-PhK5) stimulating activity and the antibodies raised against the N-terminal region of the regulatory domain (anti-PhK9 and anti-PhK13) being inhibitory.

The activating effects of the two antibodies specific for the C terminus of the regulatory domain of the γ-subunit, anti-PhK1 and anti-PhK5, were qualitatively the same in nearly every respect. Both were able to stimulate the activity of holoenzyme and γ·δ complex in the presence and absence of Ca^{2+} (Figs. 2 and 3), with anti-PhK1 consistently having the greater effect. Calcium ion was required for maximal antibody activation with both antibodies, regardless of which enzymatic form of phosphorylase kinase was used. In the absence of Ca^{2+}, both antibodies were able to stimulate enzymatic activity to levels comparable with that seen with Ca^{2+} in the absence of antibody. These data suggest that anti-PhK1 and anti-PhK5 affect the activity of phosphorylase kinase through a similar mechanism that involves a conformational change within the regulatory domain of the γ-subunit. Since the region corresponding to PhK5 is thought to serve as a calmodulin-binding subdomain (9) and as a pseudosubstrate/autoinhibitory subdomain (6, 10),

it seems likely that antibody-induced structural changes that overcome the inhibitory capacity of the PhK5 region are responsible for activating phosphorylase kinase. Interestingly, the region corresponding to PhK1 appears to have no measurable calmodulin-binding activity or pseudosubstrate-like activity (9, 10). However, it is not difficult to imagine that anti-PhK1 binding to the γ-subunit might be capable of inducing structural changes in the proximal PhK5 region (cf. Fig. 1). The greater effects of anti-PhK1 compared with anti-PhK5 are difficult to explain except that perhaps anti-PhK1 binding induces larger structural changes in the regulatory domain than the binding of anti-PhK5. The effects of anti-PhK1 and anti-PhK5 on catalytic activity were predominantly or entirely through an effect on V_{max}, although some rate-enhancing effects on the K_m for phosphorylase *b* were also seen with anti-PhK1, particularly with the γ·δ complex (Table I). These results are consistent with the data of Newsholme and Walsh (29), which showed that activation of phosphorylase kinase at pH 6.8 by Ca^{2+} and by phosphorylation of the α- and β-subunits was predominantly due to changes in V_{max}. It is interesting to note that Jennissen *et al.* (28) also observed activation of the holoenzyme through a V_{max} mechanism with antibodies raised against purified γ- and β-subunits of phosphorylase kinase. The epitopes of these polyclonal antibodies were not determined.

The degree to which the C-terminal region of the γ-subunit regulatory domain (γ342–386) interacts with the α- and β-subunits can be inferred from differences in activation seen between the holoenzyme and the γ·δ complex and the effects of α- and β-subunit phosphorylation on antibody-induced activation. The most obvious difference between antibody-induced activation of holoenzyme *versus* γ·δ complex is that the magnitude of activation observed with holoenzyme is generally severalfold greater than that seen with the γ·δ complex (Table I). One possibility for this difference is that in the nonactivated holoenzyme, the α- and β-subunits are acting to suppress activity and that anti-PhK1 and anti-PhK5 relieve this supression. Indeed, it is generally thought that the α- and β-subunits are inhibitory, since their dissociation, phosphorylation, or proteolysis results in enzyme activation (reviewed in Ref. 1). Phosphorylation of the α- and β-subunits by incubation with cAMP-dependent protein kinase and MgATP effectively blocked antibody-induced activation (Fig. 6), providing additional evidence that the regions corresponding to PhK1 and PhK5 are involved in α- and/or β-subunit regulation of holoenzyme activity. The mechanism by which phosphorylation inhibits antibody-induced activation is presently unknown, but there are two likely possibilities. The first is that phosphorylation prevents the antibodies from binding to the γ-subunit regulatory domain. The second is that prior phosphorylation of the α- and/or β-subunit locks the γ-subunit regulatory domain into a conformation that cannot be altered by antibody binding. Studies are presently underway to distinguish these two possibilities, but regardless of the mechanism involved, these data suggest a tight coupling of structural changes in the α- and/or β-subunits to structural changes in the C-terminal 45 residues of the γ-subunit.

The activation of phosphorylase kinase by anti-PhK1 appears to require antibody with intact Fc domains based on studies using Fab fragments of this antibody (Fig. 4). Neither the holoenzyme nor the γ·δ complex were activated by anti-PhK1 Fab fragments, even though the Fab fragments were able to bind both phosphorylase kinase and the γ·δ complex as shown by a sandwich ELISA (Fig. 5). These data suggest that binding of the second arm of an antibody molecule to an adjacent γ-subunit regulatory domain is required for activation. Thus, antibody-induced activation may be a consequence of both arms of an intact antibody molecule simultaneously binding to the enzyme, which then imparts a leveraging action to the γ-subunit regulatory domain. It is interesting to consider whether some sort of mechanical activation such as this might be an important but heretofore unrecognized mechanism for activating muscle phosphorylase kinase in response to contractile activity. Based on the effects of anti-PhK1 and anti-PhK5 reported here, mechanical activation could be superimposed on Ca^{2+}-induced activation, would be relatively independent of cellular pH, and would be blocked by phosphorylation of the enzyme. We are currently designing experiments to test whether mechanical activation might represent an important physiological means of regulating phosphorylase kinase activity in muscle. It is not clear why intact antibody should also be required for activation of the γ·δ complex (Fig. 4), since this complex is thought to exist as a simple heterodimer (30). One possibility is that antibody binding might facilitate the formation of a $(\gamma\cdot\delta)_2$ or $(\gamma\cdot\delta)_4$ complex that is then activated by antibody as in the holoenzyme complex.

In contrast to the activating effects of anti-PhK1 and anti-PhK5, the antibodies raised against the N terminus of the γ-subunit regulatory domain exhibited inhibitory effects on phosphorylase kinase activity. The effects of anti-PhK9 were very weak with regard to inhibition of the holoenzyme and the γ·δ complex (Figs. 2 and 3), although overnight incubation of anti-PhK9 with the γ·δ complex elicited essentially complete inhibition (data not shown). The antibody to PhK13 could only partially inhibit the activity of the holoenzyme (Figs. 1, 3, and 5) but was able to completely inhibit the activity of the γ·δ complex (Figs. 2 and 5). The differences in inhibition with respect to phosphorylase kinase holoenzyme and the γ·δ complex are most likely due to the presence of the α- and β-subunits in the holoenzyme, which may interfere with the ability of these antibodies to elicit complete inhibition.

There are at least two mechanisms by which anti-PhK9 and anti-PhK13 might inhibit the activity of phosphorylase kinase. The first possibility is that these antibodies might trap the regulatory domain in a conformational state that prevents activation. This seems a likely possibility, since PhK13 is thought to function as both a calmodulin-binding subdomain (9) and as an autoinhibitory/pseudosubstrate subdomain (10). Moreover, this mechanism of antibody-induced inhibition has previously been shown to operate in the case of tryptophan synthase, where an antibody to the β_2-subunit prevented or restricted molecular movements associated with substrate binding during the catalytic cycle (31). An alternative explanation for inhibition by anti-PhK9 and anti-PhK13 is that antibody binding to the N-terminal region of the regulatory domain causes steric hindrance of the catalytic site. Kinetic analysis of anti-PhK13 inhibition should make it possible to distinguish between these two alternative mechanisms. Steric hindrance would be expected to result in a change in K_m for phosphorylase *b*, whereas a mechanism involving restriction of conformational changes would be predicted to cause a reduction in V_{max}. The kinetic data obtained for anti-PhK13 (Table I) are consistent with this antibody acting through a mechanism that restricts conformational changes in the regulatory domain, since anti-PhK13 acted as a noncompetitive inhibitor of phosphorylase kinase activity. If the mechanism of inhibition by anti-PhK13 were to involve steric hindrance of the protein substrate, then the use of a smaller phosphate-acceptor substrate might at least partially alleviate the inhibitory effects of the antibody. However, the use of a tetradecapeptide phosphate-acceptor substrate instead of phosphorylase *b* had no effect on the efficacy of anti-PhK13 with regard to either holoenzyme or γ·δ inhibition (Fig. 6). This adds further support to the idea that anti-PhK13

acts by preventing conformational changes in the γ-subunit regulatory domain important for full catalytic efficiency. It is worth noting that a γ-subunit monoclonal antibody whose epitope has been identified as being just N-terminal to PhK13 (residues 277–290) activates the holoenzyme approximately 2-fold at pH 6.8 (32).[2] This region of the γ-subunit is thought to be a linker region between the catalytic and regulatory domains. The kinetic mechanism of the activation by this antibody is not yet known, but it is interesting that it binds closely to where anti-PhK13 binds but elicits an opposite effect on activity.

Experiments comparing the effect of incubation time on antibody-induced activation and inhibition (data not shown) suggest that certain conformational states in phosphorylase kinase are relatively slow to form. In the case of anti-PhK5, significantly greater activation of the holoenzyme was observed after 24 h of incubation with the antibody than after a 1-h incubation. This effect appeared to be dependent upon the presence of the α- and/or β-subunits, since no such time-dependent effect was observed for anti-PhK5 with the γδ complex. Proteolysis of the α- and/or β-subunits is known to result in activation of the holoenzyme (reviewed in Ref. 1), but this is unlikely to be the mechanism for slow activation with anti-PhK5, since no significant time-dependent activation was seen with the control incubation or with the other antibodies. Moreover, inhibition of holoenzyme by anti-PhK13 was also markedly time-dependent, as was inhibition of the γδ complex by anti-PhK9. The slow rates of activation and inhibition noted above are unlikely to be due to diffusional effects, since an equilibrium between bound and free antibody should be reached in well under an hour with the concentrations of antibody and enzyme used in the incubations. The fact that different time-dependent effects were observed with different forms of phosphorylase kinase also argues against slow diffusional effects being the reason for slow activation or inhibition. One possible mechanism to explain the delayed effects of antibody on catalytic activity is that the enzyme may need to undergo a slow conformational change before antibody can bind. Another possibility is that the enzyme undergoes a slow conformational change after antibody is bound. A third possibility is that slow-acting antibodies are only able to bind to a very infrequent or low probability conformational state in a highly mobile region of the enzyme. At present, we are unable to distinguish among these several mechanisms. However, because gradual changes in phosphorylase kinase catalytic activity could have important physiological consequences, it will be important to characterize the underlying mechanisms in future studies and to determine whether they are operative *in situ*.

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Note Added in Proof—It has come to our attention that the idea for the possibility of the mechanical activation of phosphorylase kinase linked to muscle contraction has been independently proposed by Dr. Gerald M. Carlson and co-workers of the University of Tennessee, Memphis based on structural studies that localized a region of the enzyme's inhibitory β-subunits to near the contact points between dimers in the tetrahedral dimer of dimers, which describes the holoenzyme. It was hypothesized that slight mechanical rotation of the lobes could activate the enzyme by mimicking the structural changes that typically occur when tetrahedral dimers of dimers undergo T to R transitions in response to ligands.

[2] G. M. Carlson, personal communication.

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