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The Regulatory β Subunit of Phosphorylase Kinase Interacts with Glyceraldehyde-3-phosphate Dehydrogenase†

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Abstract

Skeletal muscle phosphorylase kinase (PhK) is an $(\alpha\beta\gamma\delta)_4$ hetero-oligomeric enzyme complex that phosphorylates and activates glycogen phosphorylase *b* (GP*b*) in a Ca^{2+}-dependent reaction that couples muscle contraction with glycogen breakdown. GP*b* is PhK's only known in vivo substrate; however, given the great size and multiple subunits of the PhK complex, we screened muscle extracts for other potential targets. Extracts of P/J (control) and I/lnJ (PhK deficient) mice were incubated with [γ-^{32}P]ATP with or without Ca^{2+} and compared to identify potential substrates. Candidate targets were resolved by two-dimensional polyacrylamide gel electrophoresis, and phosphorylated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified by matrix-assisted laser desorption ionization mass spectroscopy. In vitro studies showed GAPDH to be a Ca^{2+}-dependent substrate of PhK, although the rate of phosphorylation is very slow. GAPDH does, however, bind tightly to PhK, inhibiting at low concentrations (IC_{50} ~ 0.45 μM) PhK's conversion of GP*b*. When a short synthetic peptide substrate was substituted for GP*b*, the inhibition was negligible, suggesting that GAPDH may inhibit predominantly by binding to the PhK complex at a locus distinct from its active site on the γ subunit. To test this notion, the PhK–GAPDH complex was incubated with a chemical cross-linker, and a dimer between the regulatory β subunit of PhK and GAPDH was formed. This interaction was confirmed by the fact that a subcomplex of PhK missing the β subunit, specifically an $\alpha\gamma\delta$ subcomplex, was unable to phosphorylate GAPDH, even though it is catalytically active toward GP*b*. Moreover, GAPDH had no effect on the conversion of GP*b* by the $\alpha\gamma\delta$ subcomplex. The interactions described herein between the β subunit of PhK and GAPDH provide a possible mechanism for the direct linkage of glycogenolysis and glycolysis in skeletal muscle.

Phosphorylase kinase (ATP:phosphorylase *b* phosphotransferase, EC 2.7.1.38), hereafter termed PhK,[1] is in fast-twitch skeletal muscle a hexadecameric complex with a subunit composition of $(\alpha\beta\gamma\delta)_4$ and a mass of 1.3×10^6 Da (reviewed in ref 1). The activity of its catalytic γ subunit (44.7 kDa) is controlled by its regulatory α (138.4 kDa), β (125.2 kDa),

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SUPPORTING INFORMATION AVAILABLE

Construction of transfer vectors and preparation of recombinant α-, γ-, and δ-baculoviruses, expression of PhK subunits, and purification of the recombinant rabbit muscle $\alpha\gamma\delta$ subcomplex. This material is available free of charge via the Internet at http://pubs.acs.org.

[1]Abbreviations: 1D, one-dimensional; 2D, two-dimensional; PhK, phosphorylase kinase; GP*b*, glycogen phosphorylase *b*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-3-P, DL-glyceraldehyde 3-phosphate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone; GMBS, *N*-(γ-male-imidobutyryloxy) succinimide ester; BV, baculovirus; rBV, recombinant baculovirus.

and δ (16.7 kDa) subunits, the last being calmodulin, which is tightly bound to the complex even in the absence of Ca^{2+}. PhK phosphorylates and activates glycogen phosphorylase *b* (GP*b*), and the absolute dependence of this reaction on Ca^{2+} couples muscle contraction with energy production via the glycolytic breakdown of glucose 1-phosphate released from glycogen by glycogen phosphorylase upon its activation by PhK (2). For more than 50 years, GP*b* has remained PhK's only known in vivo substrate, although several proteins have been reported to be phosphorylated in vitro by PhK with varying levels of effectiveness: glycogen synthase (3), troponin I (4), troponin T (4), and PhK itself (5). On the other hand, PhK has been reported to bind to, but not necessarily phosphorylate, several other proteins under less artificial conditions. When the C-terminus of PhK's α subunit (residues 1060–1237) was used as bait in a two-hybrid system to screen a rabbit skeletal muscle cDNA library, Cdc42-interacting protein 4 (CIP4) was identified as associating with PhK, and this was corroborated in vivo via immunocytochemistry (6). Via immunoprecipitation from cell extracts, PhK has also been reported to interact with the glycogen targeting protein PTG (7), which promotes glycogen storage by activation of protein phosphatase 1, which activates glycogen synthase and inactivates PhK (8). Using various techniques, several laboratories have also reported the interaction of PhK with actin (9, 10). Despite its very large mass, variety of subunits, and high concentration in muscle (11), few potential targets have been identified for PhK. The goal of this study was to find and identify additional PhK targets in mouse skeletal muscle. The findings reported herein show a strong interaction between PhK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), potentially directly linking glycogenolysis to glycolysis, which is discussed. Further, evidence is presented that this interaction occurs through the regulatory β subunit of PhK, which aside from its phosphorylation by cAMP-dependent protein kinase is the least characterized of PhK's four subunits.

EXPERIMENTAL PROCEDURES

Materials and Reagents

[γ-^{32}P]ATP was from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Rabbit muscle GAPDH (lyophilized powder and ammonium sulfate suspension), Triton X-100, Sephacryl S-300 HR, and DEAE-Sepharose were from Sigma Chemical Co. (St. Louis, MO). TSK gel Toyopearl HW-55F was from Toyo Soda Manufacturing Co., Ltd. (Tokyo, Japan). All other chromatography resins were from Amersham Biosciences (Piscataway, NJ). DL-Glyceraldehyde 3-phosphate (G-3-P) was from MP Biomedicals, Inc. (Solon, OH), and β-NAD was from Research Organics, Inc. (Cleveland, OH). Reagents, materials, and molecular weight standards for 1D SDS–PAGE were from Bio-Rad Laboratories (Hercules, CA). Materials and equipment for 2D SDS–PAGE and all other reagents used in this work were from Fisher Scientific (Pittsburgh, PA).

Mouse Inbred Strains

P/J (wild-type) and PhK deficient I/lnJ mice (12–14) were from Jackson Laboratory (Bar Harbor, ME).

Preparation of Mouse Skeletal Muscle Extracts and in Situ Phosphorylation Reaction

Back and leg skeletal muscles were excised from 6–8-week-old mice and placed in an ice-cold 2 mM EDTA solution. Muscles were then weighed, minced on ice, and homogenized on ice using a tissue tearor (500 mg of tissue/mL of buffer) in 50 mM Hepes-NaOH (pH 7.5), 30 mM KCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 2 mM benzamidine, 1 mM Pefabloc, 0.1 mM TPCK, 0.05 mM TLCK, 1 μg/mL (2.3 μM) leupeptin, and 1 μg/mL (1.45 μM) pepstatin A. The homogenate was filtered through cheese cloth to remove fat and connective tissue and centrifuged at 2000*g* for 10 min to collect a postnuclear supernatant

containing solubilized proteins (clarified extract). Alternatively, extraction of proteins from mouse skeletal muscle was performed in the presence of Triton X-100 (0.5%) to improve the solubilization of cytoskeletal proteins. Clarified extract was then dialyzed overnight in a Slide-A-Lyzer dialysis cassette (3500 MWCO; Pierce Biotechnology, Inc., Rockford, IL) against 50 mM Hepes-NaOH (pH 7.5), 5% sucrose, 1 mM EDTA, 1 mM DTT, and 2 mM benzamidine. The dialysis buffer also contained 0.1% Triton X-100 if used in the extraction. After dialysis, the extracts (typically ~10 mg/mL total protein) were supplemented with protease inhibitors (as in homogenization buffer) and used immediately or stored at –80 °C.

In situ phosphorylation in I/lnJ and P/J mouse extracts was performed in the presence of 50 mM Tris, 50 mM β-glycerophosphate, 5 mM Hepes (pH 8.0), 12 mM $Mg(CH_3CO_2)_2$, 0.5% sucrose, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM DTT, 0.2 mM benzamidine, 14 mM β-mercaptoethanol, and 1.8–2.4 mM [γ-^{32}P]ATP (1800–2345 Ci/mol). Each reaction mixture was supplemented with either 0.5 mM $CaCl_2$ (with Ca^{2+} conditions) or 0.5 mM EGTA (without Ca^{2+} conditions). Endogenous phosphorylation was initiated by addition of muscle extract (100–150 μg of total protein) supplemented with a phosphatase inhibitor cocktail (2 μL/mg of total protein; P-2850, Sigma). Purified exogenous rabbit muscle PhK (1 μg/100 μg of total protein) was added to appropriate reaction mixtures to enhance the phosphorylation effect of the endogenous PhK in P/J extracts and to visualize possible PhK targets in I/lnJ extracts.

Protein Purification

Glycogen phosphorylase *b* (GP*b*) was isolated from rabbit skeletal muscles as described previously (15) and passed through charcoal three times to remove residual AMP. PhK was purified from psoas muscle of New Zealand white rabbits as previously reported (2, 16). The tetradecapeptide alternative substrate (SDQEKRKQISVRGL) was synthesized and purified by the Alberta Peptide Institute (Department of Biochemistry, University of Alberta, Edmonton, AB).

Enzymatic Assays and Inhibition Experiments

Phosphorylase kinase activity was measured using a phosphocellulose P81 (Whatman) paper assay (17) as described previously (18). For all experiments, GAPDH was dissolved in 50 mM β-glycerophosphate (pH 6.8) and 14 mM β-mercaptoethanol buffer and prior to use was dialyzed overnight against the same buffer at 4 °C. When an ammonium sulfate suspension of GAPDH was used, the protein was first pelleted at 4 °C for 15 min at 10000*g* and processed as described above. Incorporation of phosphate into GP*b* or into GAPDH by PhK or its αγδ subcomplex was assessed at 30 °C in reaction mixtures containing 50 mM Tris, 50 mM β-glycerophosphate (pH 8.2), 12 mM $Mg(CH_3CO_2)_2$, 1.5–1.8 mM [γ-^{32}P]ATP (400–480 Ci/mol), 0.09 mM EGTA, 0.2 mM $CaCl_2$, 2.36 mg/mL GP*b* (12 μM) or 1.73 mg/mL (12 μM) GAPDH, and either 3.25 μg/mL PhK (2.5 nM) or 2.0 μg/mL αγδ trimer (10 nM). The concentration of the catalytic γ subunit in the reaction mixtures was 0.47 μg/mL for both forms of the enzyme. The PhK and αγδ 10× working solutions were prepared in 40 mM β-glycerophosphate (pH 6.8), 0.1 mM EGTA, 28 mM β-mercaptoethanol buffer and used immediately to initiate the reactions. The counts in reactions where PhK and αγδ were tested alone (autophosphorylation controls) and counts in controls to detect the potential presence of endogenous PhK activity in GAPDH or GP*b* fractions were subtracted from the corresponding total counts in appropriate experimental assays.

The in vitro phosphorylation of GAPDH for analyses by 2D SDS–PAGE (Figure 2C) was carried out for 30 min as described above, except for the following concentration differences: 1.0–1.2 mM [γ-^{32}P]ATP (300–320 Ci/mol), 84 μg/mL GAPDH (~0.6 μM), and 3.0 μg/mL PhK (2.3 nM). Reactions were terminated by addition of UPPA-I buffer from a

Perfect-Focus kit (G-Biosciences, St. Louis, MO). Samples were processed according to the manufacturer's instructions and resolved by 2D PAGE.

GAPDH activity was determined spectrophotometrically at room temperature essentially as described previously (19) using G-3-P as the substrate. Other slight modifications of the assay, such as decreasing the concentration of both β-NAD and G-3-P, were adopted from the protocol published in the manual from Worthington Biochemical Corp. (Lakewood, NJ).

PhK and αγδ inhibition assays were conducted in 50 mM Tris, 50 mM β-glycerophosphate (pH 8.2), 12 mM $Mg(CH_3CO_2)_2$, 1.7–1.8 mM [γ-^{32}P]ATP (400–430 Ci/mol), 0.09 mM EGTA, 0.2 mM $CaCl_2$, and either 12 μM GP*b* or 100 μM tetradecapeptide. For these experiments, separate GAPDH solutions were prepared in 50 mM β-glycerophosphate (pH 6.8), 14 mM β-mercaptoethanol buffer. Either PhK (25 nM) or αγδ (100 nM) was mixed 1:1 with the appropriate GAPDH solutions (concentration varied from 0.025 to 250 μM) and incubated for 5 min at room temperature prior to the assay. Reaction mixtures containing all components except enzymes were preincubated at 30 °C for 3 min. Reactions were initiated by addition of the 10× PhK–GAPDH or αγδ–GAPDH solution described above to the master mix and carried out at 30 °C for 2 min. Aliquots (20 μL) were spotted onto P81 filters, washed with 0.5% H_3PO_4, and counted in a scintillation counter.

For GAPDH inhibition experiments, test solutions of PhK (from 17.5 nM to 2.69 μM) were prepared in 50 mM Hepes (pH 6.8), 0.2 mM EDTA, and 10% sucrose by dilution of a concentrated 3.5 mg/mL stock. GAPDH for these experiments was prepared as a 1 mg/mL solution in 25 mM Tris (pH 8.2) and 0.2 mM DTT, dialyzed overnight, diluted to 10 μg/mL (~70 nM) with 0.015 M sodium pyrophosphate and 0.03 M sodium arsenate buffer (pH 8.5), and then mixed 1:4 with the appropriate PhK solutions. These enzyme mixtures were incubated at room temperature for 5 min before being added to cuvettes containing reaction buffer, β-NAD, and DTT. Reactions at pH 8.5 were initiated by addition of G-3-P, and mixtures contained 12 mM sodium pyrophosphate, 24 μM sodium arsenate, 225 μM β-NAD, 3 mM DTT, 0.15 mM G-3-P, 1.75 nM GAPDH, and 1.75–269 nM PhK. The 100% GAPDH control activity was obtained with buffer replacing the PhK. All activity measurements were repeated a minimum of three times using three different PhK preparations and two αγδ purified fractions. In all experiments, both lyophilized powder and an ammonium sulfate suspension of GAPDH were tested and gave comparable results.

Electrophoresis and Immunoblotting

1D SDS–PAGE was carried out as described by Laemmli (20). 2D SDS–PAGE was conducted according to the manual of Amersham Biosciences for 2D PAGE, using the Broad Range Molecular Weight Standards from Promega Corp. (Madison, WI). All gels were prepared using a 30% Duracryl solution purchased from Genomic Solutions, Inc. (Ann Arbor, MI), instead of the commonly used acrylamide/bis-*N,N*′-methylene-bis-acrylamide mixture.

Anti-PhK α, β, and γ subunit-specific monoclonal antibodies (mAbs) were those previously described (21, 22). Anti-calmodulin serum (clone CaM85) was from Zymed Laboratories, Inc. (South San Francisco, CA). The polyclonal antibody to the PhK complex that detects α, β, and γ subunits was raised in guinea pigs (23). The goat polyclonal antibody against GAPDH was from Imgenex Corp. (San Diego, CA). All other detection alkaline phosphatase conjugates were from Southern Biotechnology (Birmingham, AL). For Western blotting, proteins were resolved by either 1D or 2D SDS–PAGE, transferred onto a 0.45 μm PVDF membrane, stained with antibodies, and visualized with Bio-Rad nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (24).

Pull-Downs from Muscle Extract by Immobilized GAPDH

Psoas muscle from New Zealand white rabbits was excised, briefly chilled in ice-cold 1 mM EDTA (pH 7.5), passed once through a meat grinder at 4 °C, blended at high speed for 1 min with 2.5 volumes (w/v) of 4 mM EDTA (pH 7.5), and centrifuged at 8000*g* for 40 min at 4 °C. The supernatant was filtered through glass wool, dialyzed overnight against 800 volumes of 40 mM β-glycerophosphate (pH 6.8), 0.1 mM EGTA, 28 mM β-mercaptoethanol buffer, and used fresh in pull-down experiments.

The covalent immobilization of GP*b*, GAPDH (Sigma, G 2267), and control BSA (Fisher Scientific, BP 1605) on cyanogen bromide-activated Sepharose-4B (GE Healthcare) was performed according to the manufacturer's instructions in 0.1 M potassium phosphate buffer (pH 8.3) and 5 mM EDTA (immobilization buffer) for 3 h at room temperature with mixing. Any remaining reactive groups on the Sepharose were then blocked with 50 mM glycine for an additional 1 h at room temperature. Any unbound protein was then removed with immobilization buffer containing 2 mM DTT until no protein could be detected in the eluate. Control empty beads were prepared under identical conditions with 200 mM glycine as the ligand. To ensure successful conjugation, the concentration of proteins covalently bound to the beads was determined by the modified procedure of Bradford (25).

Pull-down assays were carried out at 4 °C for 10 min on a rotating wheel in 1.5 mL centrifuge tubes. Sepharose beads (100 μL settled gel) were incubated with 1 mL of the muscle extract or with a purified PhK control solution (0.25 mg/ mL) and washed three times with 1 mL of 40 mM β-glycerophosphate (pH 6.8), 0.1 mM EGTA, and 28 mM β-mercaptoethanol. The beads were than divided into two equal parts. One half was stripped with 2× SDS sample buffer, and proteins present were analyzed by 1D SDS–PAGE and immunoblotting. The other half was directly tested for PhK activity using the filter paper assay (17, 18). Incorporation of phosphate into GP*b* by proteins pulled from the extract was also visualized by autoradiography.

Chemical Cross-Linking

All cross-linkers were from Pierce Biotechnology, Inc. PhK was cross-linked with *N*-(γ-maleimidobutyryloxy)succinimide ester (GMBS) in the presence and absence of GAPDH for 5 min at 30 °C at pH 8.2. Final concentrations in the reaction were as follows: PhK, 0.47 μM holoenzyme; GAPDH, 0.47 μM holoenzyme; GMBS, 9.4 μM; Hepes, 50 mM (pH 8.2); and EDTA, 0.2 mM. GAPDH was cross-linked in the absence of PhK under identical conditions. Reactions were initiated by adding GMBS and terminated by adding an equal volume of SDS buffer [0.125 M Tris (pH 6.8), 20% glycerol, 5% β-mercaptoethanol, and 4% SDS], followed by brief vortexing. The PhK subunits were separated on 6 to 18% linear gradient polyacrylamide gels and stained with Coomassie Blue or subjected to Western blotting. Cross-linking of the complexes with formaldehyde (26), 1,5-difluoro-2,4-dinitrobenzene (27), *p*-phenylenedimaleimide (28), and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (29) and *N*-5-azido-2-nitrobenzoylsuccinimide (30) was carried out as described previously. Densitometry of gels was performed using a Fluorochem 8900 analyzer (Alpha Innotech, San Leandro, CA).

Determination of Protein Concentrations and Other Techniques

The concentrations of GP*b* and PhK were determined spectrophotometrically using their absorbance indexes of 13.0 (31) and 12.4 (32), respectively, for 1% solutions at 280 nm. The concentrations of the purified αγδ subcomplex and total protein during its purification were determined by the Bradford method (33) with Bio-Rad concentrated dye reagent and bovine serum albumin (A-9647, Sigma) as the standard. Protein samples containing Triton X-100

and/or DTT were analyzed using the modified BCA assay (Pierce) in the presence of iodoacetamide according to Pierce's technical handbook.

Protein staining on 1D and 2D gels was carried out either with a Coomassie Brilliant Blue R-250 and Bismarck Brown R mixture to increase the sensitivity of the dye (34) or with silver, using a Silver Quest staining kit from Invitrogen Corp. (Carlsbad, CA).

RESULTS

Identification of PhK Targets in Mouse Skeletal Muscle

Our initial approach was to search for previously unidentified protein substrates for PhK in skeletal muscle using a phosphoproteomics approach, specifically 2D SDS–-PAGE followed by mass spectrometry. The availability of an inbred strain of mice (I/lnJ) with a naturally occurring PhK deficiency, resulting from a mutation causing premature truncation of the α subunit (14), greatly facilitated this approach by allowing identification (and elimination) of those targets that were phosphorylated by other Ca^{2+}-dependent protein kinases besides PhK. Skeletal muscle extracts from these mice contain only 0.2% of the PhK activity found in their wild-type counterparts (13). The in situ radiolabeling of proteins with Mg[γ-^{32}P]ATP with or without Ca^{2+} was carried out in I/lnJ and wild-type (wt) muscle extracts, with the additional control of purified rabbit muscle PhK being spiked into both. A set of four related criteria were then used in evaluating the phosphoproteins formed as potential targets of PhK. (i) To be considered as a PhK substrate, the phosphorylated protein should not be visible on phosphor images in I/lnJ experimental extracts, regardless of the presence of Ca^{2+}. (ii) Adding exogenous rabbit muscle PhK to such extracts should give a visible signal in the presence of Ca^{2+} ions. (iii) The phosphoprotein candidate must be missing in P/J (control) mice extract when the reaction is carried out without Ca^{2+} but visible when Ca^{2+} ions are included. (iv) The phosphoprotein signal in P/J homogenates should be enhanced when the reaction mixture is spiked with exogenous PhK.

Applying these criteria, we observed that a phosphoprotein with a molecular mass of ~42 kDa and a pI of ~6.6 occurred in the wild-type P/J extract with Ca^{2+} but was absent in the I/lnJ extract with Ca^{2+} (Figure 1A,B, row b, phosphor images). When exogenous rabbit muscle PhK was added to I/lnJ extract prior to 2D PAGE analysis, a 42 kDa phosphorylated protein with a pI of ~6.6 was present (Figure 1B, row c), and it was therefore identified as a potential substrate of PhK. Furthermore, this phosphoprotein was missing when both extracts were phosphorylated in the absence of Ca^{2+}, thus indicating the Ca^{2+} dependency of its phosphate incorporation (Figure 1A,B, row a). This candidate and the other phosphoproteins with a mass of ~42 kDa, but with pI values of ~8.0, 7.6, and 7.0, were identified by MALDIMS (described in detail in the Supporting Information) as GAPDH. A protein with a similar mass and a pI of ~8.5, which is visible on Coomassie-stained gels but missing on phosphor images (Figure 1), was also identified as GAPDH and was presumed to represent a nonphosphorylated form of the enzyme. When P/J and I/lnJ extracts were tested by 2D PAGE directly (i.e., no prior in situ phosphorylation reaction was performed), both modified (pI ~ 8.0, 7.6, 7.0, and 6.6) and unmodified (pI ~ 8.5) species of GAPDH were present on the gels, with smaller immunoreactive fragments, presumably proteolytic fragments, running beneath each spot (Figure 1C). Our results are in agreement with earlier studies reporting that phosphorylated GAPDH exists in skeletal muscle, via autophosphorylation (35) or phosphorylated by protein kinases (19, 36) on tyrosine (37–39) or serine and threonine residues (40).

pH and Ca^{2+} Dependence of GAPDH Phosphorylation

Besides requiring Ca^{2+} ions, the activity of nonphosphorylated PhK toward GP*b* is significantly greater at pH 8.2 than at pH 6.8 (1, 32); we therefore tested whether PhK's in vitro phosphorylation of GAPDH was similarly affected by these variables and found that it was (Figure 2A,B). The rate of GAPDH phosphorylation, however, was very slow, only 0.17% of that of GP*b* at pH 8.2 and the same molar concentrations of substrates. When the in vitro phosphorylated GAPDH was resolved by 2D SDS–PAGE, several modified forms of it were observed by autoradiography and had pI values of 8.0, 7.6, 7.0, and 6.6 (Figure 2C).

Although we did not attempt to identify the sites phosphorylated on GAPDH, its Ser-189, Thr-234, Ser-238, Ser-263, Ser-290, and Ser-309 have high phosphorylation probabilities according to the post-translational modification prediction of the NetPhos 2.0 server (www.cbs.dtu.dk/services/NetPhos). Furthermore, all these residues are exposed and therefore accessible to PhK (44). Ser-263 is within a sequence (K_{260}QASEGPL$_{267}$) that is similar to PhK's known phosphorylation site on GP*b* [K_{11}QISVRGL$_{18}$ (41)], and it is surrounded by basic and aliphatic residues that are favored by PhK (42, 43); nevertheless, even after incubation for 60 min with PhK, only 4.4 ± 0.2 mmol of phosphate was incorporated per mole of GAPDH monomer in a linear reaction. Considering the existence of at least four phosphorylation sites for PhK on GAPDH in vitro (Figure 2C), the stoichiometry of phosphorylation of each site is much lower than the total; that fact coupled with the very slow rate of phosphorylation makes it highly unlikely that this process could be physiologically important.

Interaction of GAPDH and PhK in Pull-Downs from Muscle Extract

Although the phosphorylation of GAPDH by PhK was poor, that implies nothing about the strength of their interaction. For example, could a complex between them be sufficiently stable to be isolated from muscle extract? Both GAPDH and GP*b* immobilized to Sepharose beads were found to pull down PhK from muscle extracts (Figure 3A, lanes 3 and 4, respectively), whereas control empty beads and immobilized BSA did not (Figure 3A, lanes 1 and 2, respectively). Subjecting the pulled-down beads to assay conditions with GP*b* as a substrate showed strong PhK activity for the GAPDH and GP*b* beads, but none for the two controls (Figure 3C), confirming the Western blots (Figure 3A). To rule out the possibility that the immobilized GAPDH might be interacting only indirectly with PhK via binding of GP*b*, which in turn would interact with and pull down PhK, we also performed pull-downs from a solution of purified PhK. As with the muscle extract, the immobilized GAPDH and GP*b* did pull down PhK, whereas the two controls did not (Figure 3B). The survival of the complex through extensive washing and the similar amounts of PhK pulled down by GP*b* and GAPDH suggested a surprisingly strong interaction between PhK and GAPDH, perhaps being equivalent to that between PhK and its natural substrate, GP*b*.

PhK and GAPDH Inhibition Experiments

The very low rate of GAPDH phosphorylation by PhK prohibited determination of a K_m value as an estimate of binding affinity; therefore, to estimate their binding avidity, we asked whether the two enzymes could influence each other's activity in a concentration-dependent manner. Thus, we tested rabbit muscle PhK and GAPDH activities in vitro toward GP*b* and G-3-P, respectively, and all at fixed concentrations, while varying the concentration of the second enzyme as described in Experimental Procedures. We found that PhK activity toward GP*b* was inhibited by inclusion of GAPDH in the assays (Figure 4A). In fact, strong inhibition of PhK activity occurred even at nanomolar concentrations of GAPDH, and the corresponding monophasic semilogarithmic plot (45) yielded an apparent IC_{50} of 0.45 μM (Figure 4A, inset). These results suggested that PhK binds GAPDH even

more tightly than it does its natural substrate, GP*b*, although at that point we could not rule out the possibility that GAPDH inhibited GP*b* phosphorylation by binding directly to it and by so doing making it a poorer substrate for PhK. This theoretical possibility was eliminated when we observed that PhK also exhibited a similar potent inhibitory effect on GAPDH activity (Figure 4B), with an apparent IC_{50} of 0.1 μM (Figure 4B, inset). Nevertheless, we observed that when a tetradecapeptide analogue (termed S peptide and containing the residues that correspond to and encompass the phosphorylation site of GP*b*, specifically residues 5–18) was used as the substrate for PhK, the inhibitory effect of GAPDH was nearly abolished [IC_{50} ~ 3.5 mM (data not shown)]. The sum of these data suggested that GAPDH inhibits PhK's activity primarily by binding at a site distinct from the active site cleft of its catalytic γ subunit, perhaps at a locus on one of its regulatory subunits (α, β, or δ).

Chemical Cross-Linking

To determine which subunits of the PhK complex other than γ may interact with GAPDH, we attempted to form cross-linked GAPDH–PhK adducts in a 1:1 molar mixture of the two proteins utilizing chemical cross-linkers whose actions on PhK have been characterized. Under the reaction conditions previously described for those cross-linkers, incubation with bifunctional reagents that selectively target the α subunit [*N*-5-azido-2-nitrobenzoyloxysuccinimide (30) and formaldehyde (26)] failed to yield detectable conjugates between GAPDH and any subunit of PhK (data not shown). Likewise, no cross-linked adducts were observed when using reagents known to react with other PhK subunits, namely, 1,5-difluoro-2,4-dinitrobenzene (27), *p*-phenylenedimaleimide (28), and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (29). In contrast, GMBS, an affinity-based cross-linker of PhK that selectively targets its β and γ subunits (46), rapidly formed a conjugate between the β subunit of PhK and GAPDH at minimal stoichiometries (5 mol of GMBS, 1 mol of GAPDH monomer, and 1 mol of PhK αβγδ protomer) (Figure 5, lane 3, solid arrow). Its estimated mass (~171 kDa) and antibody cross-reactivity indicated that the β-GAPDH conjugate formed was dimeric and did not contain the catalytic γ subunit of PhK. These results suggest that the regulatory β subunit of PhK is involved in the binding of GAPDH.

Activity of the αγδ Subcomplex of PhK

To confirm that GAPDH interacts with the β subunit of Phk, we studied the behavior of a PhK complex lacking the β subunit, namely, an αγδ trimer (23, 47). A baculovirus (BV)-mediated expressed recombinant rabbit αγδ subcomplex of PhK was purified (described in the Supporting Information), and its potential phosphorylation of GAPDH was assessed in extended reactions. In such experiments, a concentration of the αγδ subcomplex that exhibited considerable catalytic activity toward GP*b* (9.5 mmol of ^{32}P incorporated per mole of GP*b* monomer per minute) was unable to phosphorylate GAPDH (data not shown), again suggesting that the major binding site for GAPDH is indeed located on the β subunit. To further confirm this conclusion, we tested the ability of GAPDH to inhibit the GP*b* conversion activity of the αγδ subcomplex, as in Figure 4 with the PhK complex, and found that the inhibition by GAPDH was completely eliminated (data not shown). Considered together, our results indicate that GAPDH binds to PhK via its regulatory β subunits.

DISCUSSION

Our observation that GAPDH is an in situ substrate for PhK was not completely surprising, given that once before it had been reported that under unspecified in vitro conditions a small amount of phosphate was incorporated into purified GAPDH by PhK (48). Prior to that, the EGF receptor tyrosine kinase had been shown to phosphorylate GAPDH in vitro to

relatively high yields (49). Moreover, other Ser/Thr protein kinases have been reported to relatively efficiently phosphorylate GAPDH, namely, protein kinase C ιλ (36) and musclespecific calmodulin-dependent protein kinase (19). Thus, the action of a number of different protein kinases could explain the multiple GAPDH spots in Figure 1 that have the same mass but different pI values. In fact, the migration pattern of in situ phosphorylated mouse muscle GAPDH reported herein is very similar to one observed in 2D PAGE for rabbit muscle GAPDH following its in vitro phosphorylation by protein kinase C, resulting in multiple phosphorylated species of GAPDH (40).

Even though the efficiency of phosphorylation of GAPDH by PhK is low, it nevertheless does raise questions about the contribution of PhK's regulatory β and catalytic γ subunits toward that phosphorylation. The cross-linking of GAPDH to β and the inability of the αγδ subcomplex to phosphorylate GAPDH or to be inhibited by it suggest the possibility that the β subunit may anchor one region of GAPDH to the PhK complex while another region is simultaneously phosphorylated by the γ subunit. It might be noted in this regard that the β and γ subunits are structurally and functionally coupled to each other and are adjacent (22, 46). Such a joint interaction with two subunits would not be unexpected in that there is evidence that the regulatory α subunit may interact with PhK's substrate, GP*b* (50).

Due to the low rate of incorporation of phosphate into GAPDH by PhK under optimal in vitro conditions, it seems highly unlikely that this phosphorylation catalyzed by PhK is significant to the regulation of GAPDH in vivo. It seems more likely that the strong interaction between these enzymes may be important in linking glycogenolysis and glycolysis in muscle. Glycogen and the enzymes of glycogenolysis, including PhK, are known to be associated with the sarcoplasmic reticulum (SR) in skeletal muscle (51–54). The activation of PhK by Ca^{2+} released from the SR is thought to couple muscle contraction with energy production via the glycolytic breakdown of glucose 1-phosphate released from glycogen by phosphorylase upon its activation through phosphorylation by PhK (2, 53). Consistent with this idea, all enzymes of both glycogenolysis and glycolysis, including of course PhK and GAPDH, are found in glycogen particles isolated from skeletal muscle (51, 55). The mechanism through which glycogen particles interact with the SR is complex, dynamic, and not fully characterized (56); however, given that PhK interacts with both the SR (57, 58) and glycogen (59–62), it could potentially participate in bridging the glycogen particle to the SR. The interaction of PhK with glycogen could be mediated by GP*b*, which binds PhK and glycogen (61, 63), or by the protein phosphatase I subunit PTG (protein targeting to glycogen), which binds PhK in addition to glycogen (7, 64, 65). Alternatively, since GAPDH also associates with the SR, particularly with the foot protein of triad junctions (66–69), it could indirectly bridge the glycogen particle to the SR by interacting with PhK. Another potential player relevant to these associations is the skeletal muscle-specific calmodulin-dependent protein kinase, which is anchored to the SR and binds both PhK and GAPDH (19), potentially adding further complexity to the downstream effects of the Ca^{2+} released from the SR. Regardless, however, of the mechanism through which glycogen particles associate with the SR, the strong interaction of PhK and GAPDH reported herein provides a possible mechanism for the direct linkage of the glycogenolytic and glycolytic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

I.G.B. dedicates this work to Y. V. Rodionov (1948–1997), supervisor and mentor, Senior Research Scientist in the Microbiology Department of Moscow State University, Moscow, Russia.

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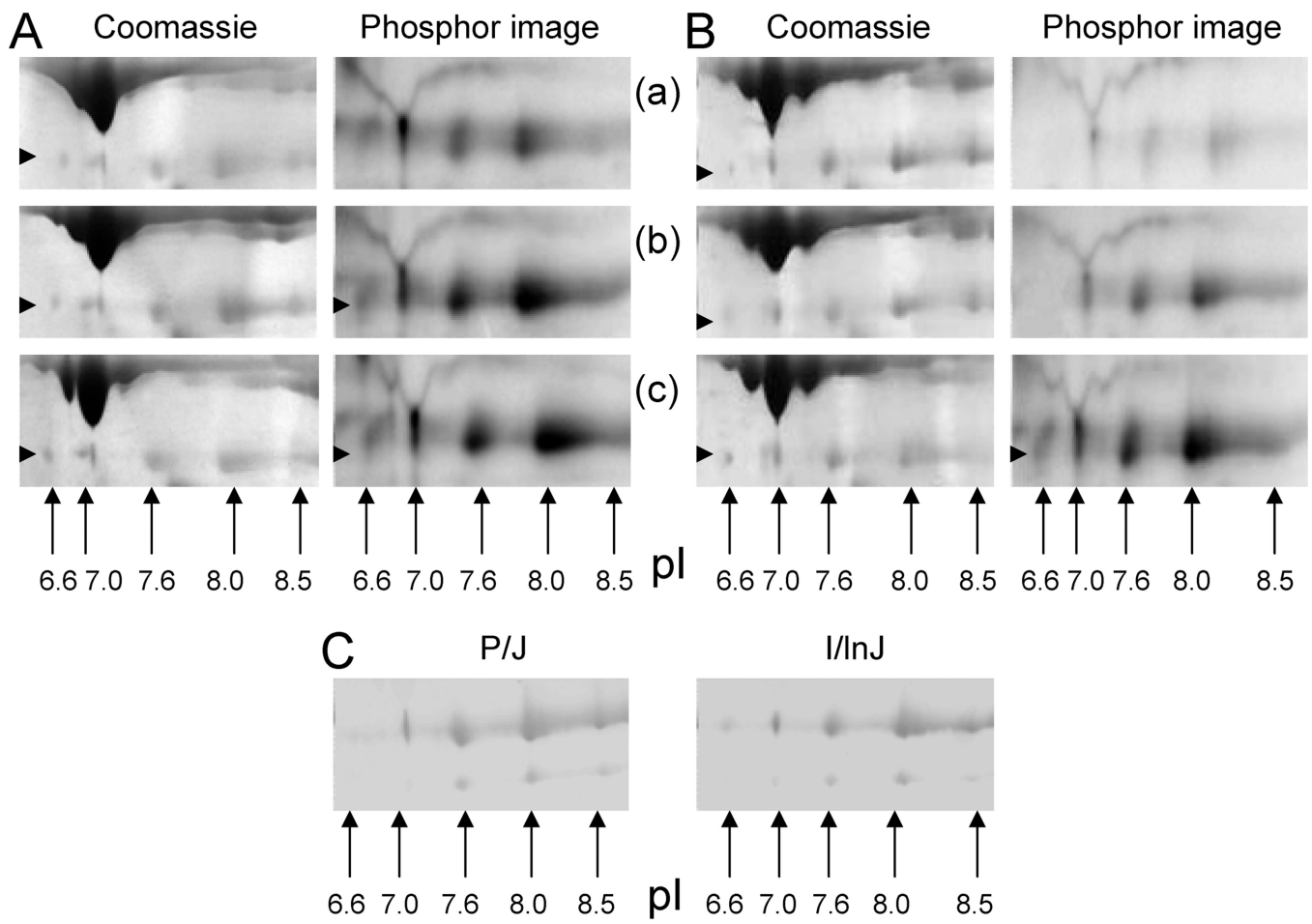

Figure 1.

In situ phosphorylation in mouse skeletal muscle extracts. P/J (A) and I/lnJ (B) extracts were incubated either with Mg[γ- ^{32}P]ATP under different conditions [(a) without Ca^{2+} and without exogenous PhK, (b) with Ca^{2+} without exogenous PhK, and (c) with Ca^{2+} and exogenous PhK] or without exogenous ATP (C). Reactions were carried out at 30 °C for 30 min and terminated by addition of UPPA-I buffer, the first component of the Perfect FOCUS kit purchased from Genotech Corp. (St. Louis, MO). Low-conductivity protein samples for isoelectric focusing were then prepared according to the manufacturer's protocol. Proteins were resolved by 2D PAGE on linear gradient pH 3 to 10 strips (13 cm) from GE Healthcare (Uppsala, Sweden), followed by a second-dimension separation on a 5 to 17.5% T gradient gel. Gels were then stained, destained, dried, photographed, and exposed to a phosphor screen for 7–9 days. Phosphorylation patterns were visualized by autoradiography using an Amersham Biosciences Typhoon 9410 Imager and Image Quant verion 5.2 (Molecular Dynamics Corp., Sunnyvale, CA). Only the central part of the 16 cm × 18 cm gels is shown. A potential PhK target with a mass of ~42 kDa and a pI of ~6.6 (marked with an arrowhead within the gel image) was subjected to MALDI-MS analysis as described in Experimental Procedures. Proteins with similar masses and pI values of ~7.0, 7.6, 8.0, and 8.5 were also digested and analyzed by mass spectrometry. When no exogenous, radiolabeled ATP was used, proteins resolved by 2D PAGE were transferred to PVDF and stained with an anti-GAPDH polyclonal antibody (C).

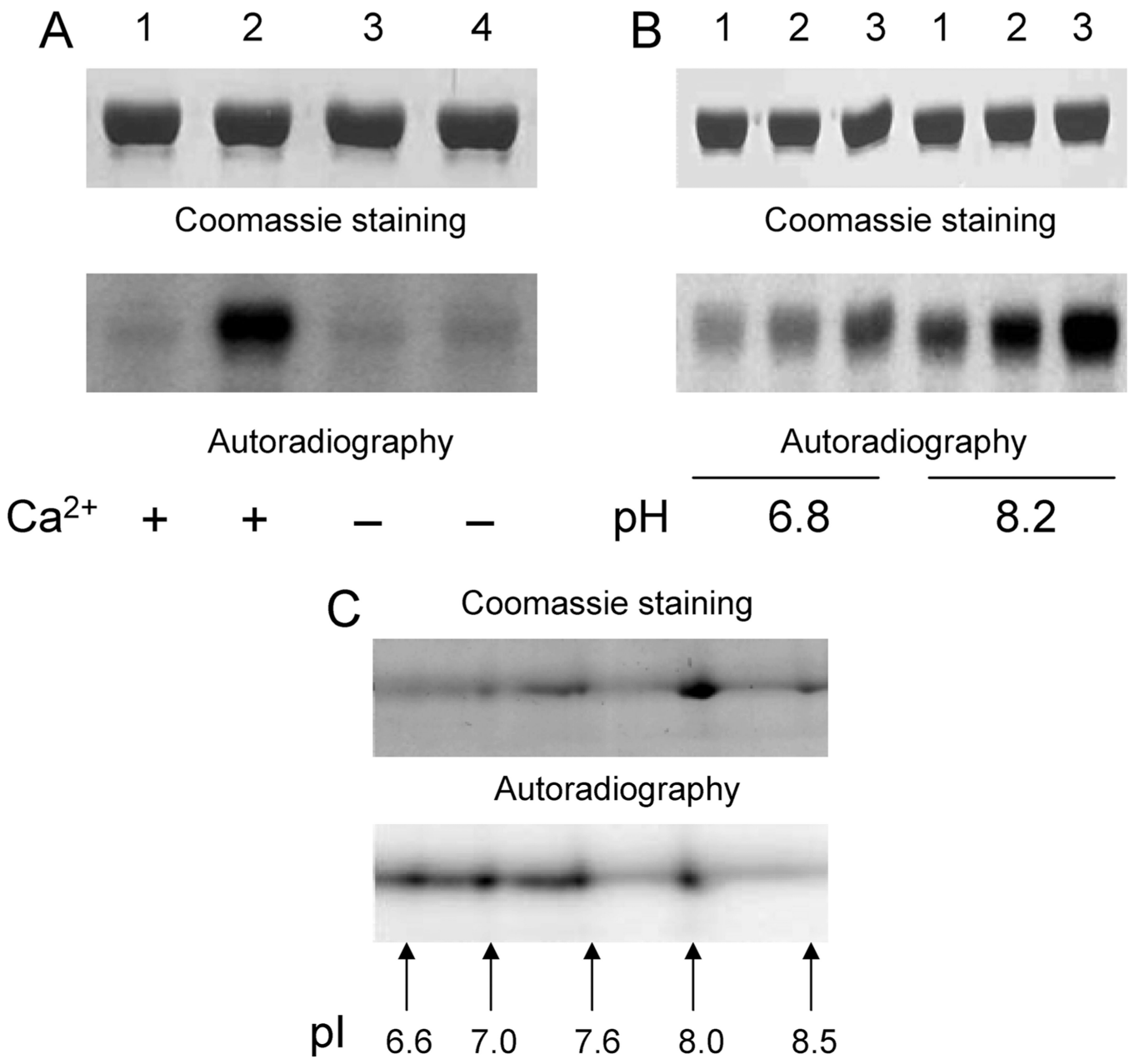

Figure 2.

GAPDH phosphorylation by PhK in vitro. Phosphorylation reactions were carried out for 20 min as described in Experimental Procedures in the presence of Ca^{2+} (0.2 mM $CaCl_2$) or absence of Ca^{2+} (0.2 mM EGTA) at pH 8.2 (A) and at pH 6.8 and pH 8.2 (B) in the presence of Ca^{2+} (0.2 mM $CaCl_2$) for 12 min (lane 1), 24 min (lane 2), and 36 min (lane 3). GAPDH was tested either alone (A, lanes 1 and 3) or in the mixture with PhK (A, lanes 2 and 4). At appropriate times, aliquots of each reaction mixture were either spotted onto P81 filters and counted on a scintillation counter or mixed with an equal volume of 2× SDS–PAGE sample buffer. Radiolabeled proteins were then resolved by 1D (A and B) or 2D SDS–PAGE for the phosphorylation at pH 8.2 with Ca^{2+} (C) and visualized by autoradiography.

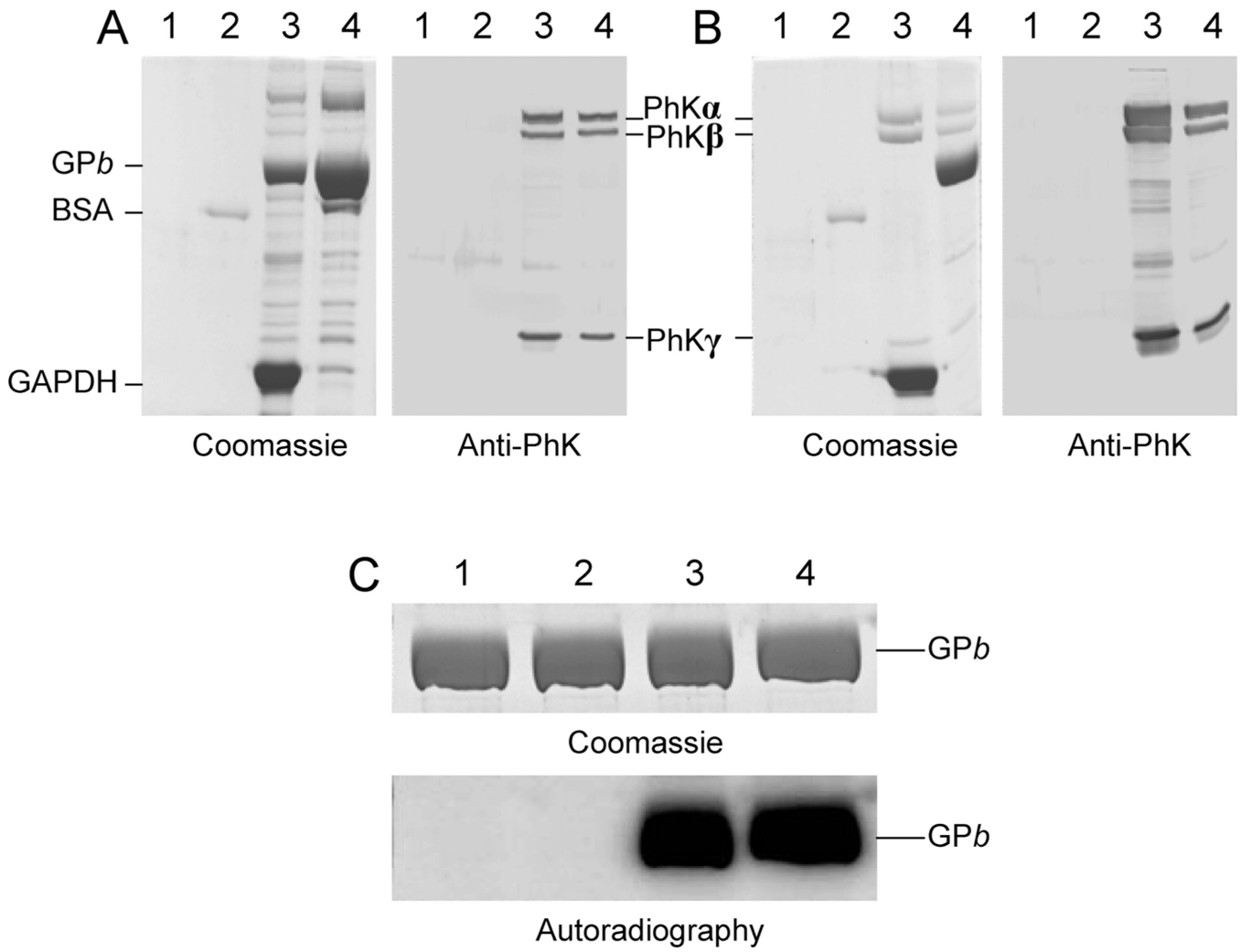

Figure 3.

Pull-down of PhK by immobilized GAPDH and GP*b*. Empty beads (lane 1), BSA beads (lane 2), GAPDH beads (lane 3), and GP*b* beads (lane 4) were incubated with rabbit muscle extract (A) or with a purified solution of PhK (B) as described in Experimental Procedures. After being extensively washed, the proteins associated with the beads were analyzed on a 7.5% T separating gel and Coomassie stained and immunoblotted with anti- PhK guinea pig polyclonal antibodies. Beads incubated with the muscle extract were also tested for PhK activity toward GP*b* in a 10 min assay at 30 °C in reactions initiated by a 1:1 suspension of each bead. Aliquots of the product supernatant were mixed with an equal volume of 2× SDS–PAGE sample buffer, and the ^{32}P-radiolabeled GP*b* was visualized via 1D SDS–PAGE by autoradiography (C).

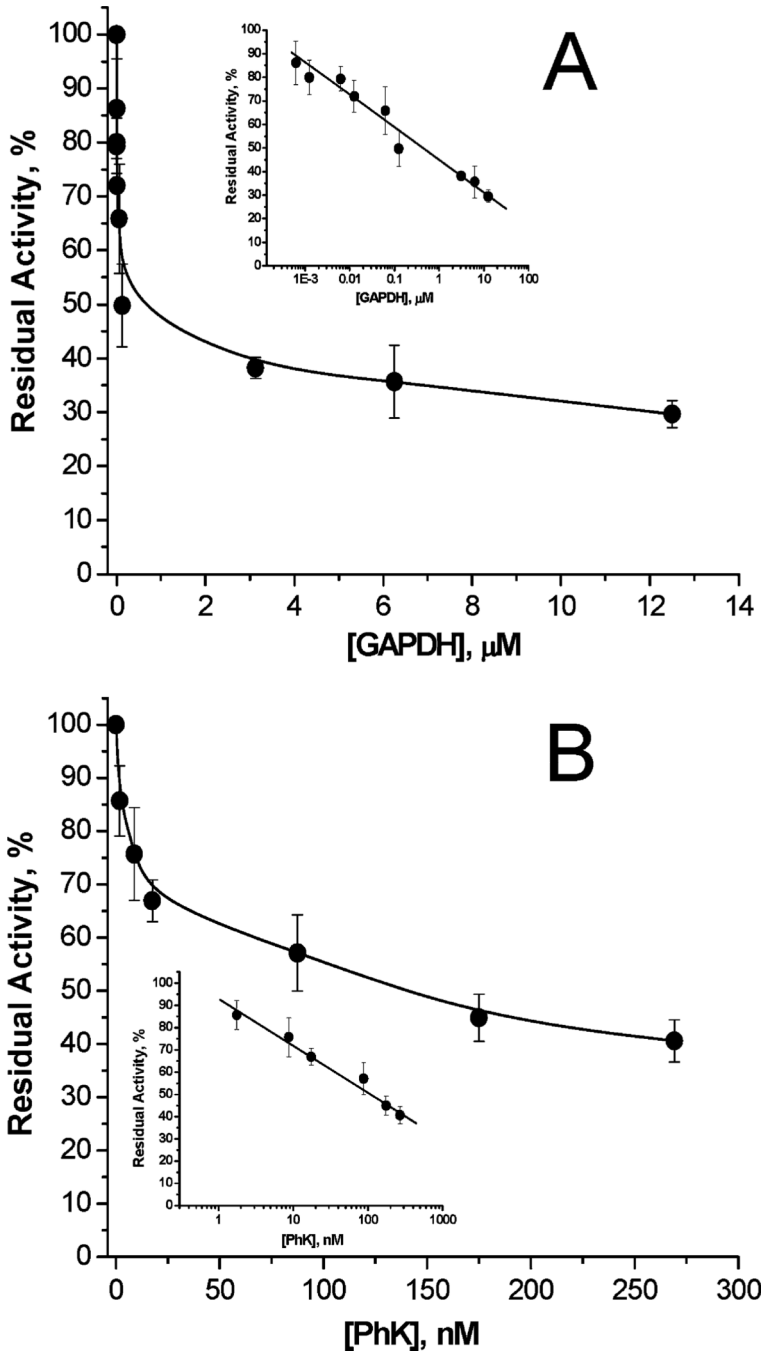

Figure 4.

Mutual inhibition of PhK and GAPDH. The activity of PhK in the presence of GAPDH and vice versa was determined as described in Experimental Procedures. (A) Effect of GAPDH on PhK activity and (B) effect of PhK on GAPDH activity. The enzymatic activity in control assays in which the inhibitory protein was replaced by the same amount of appropriate buffer was regarded as 100% activity, and the activities of each enzyme premixed with the inhibitor were compared against this initial control activity. The error bars represent the standard deviation of at least three separate activity measurements.

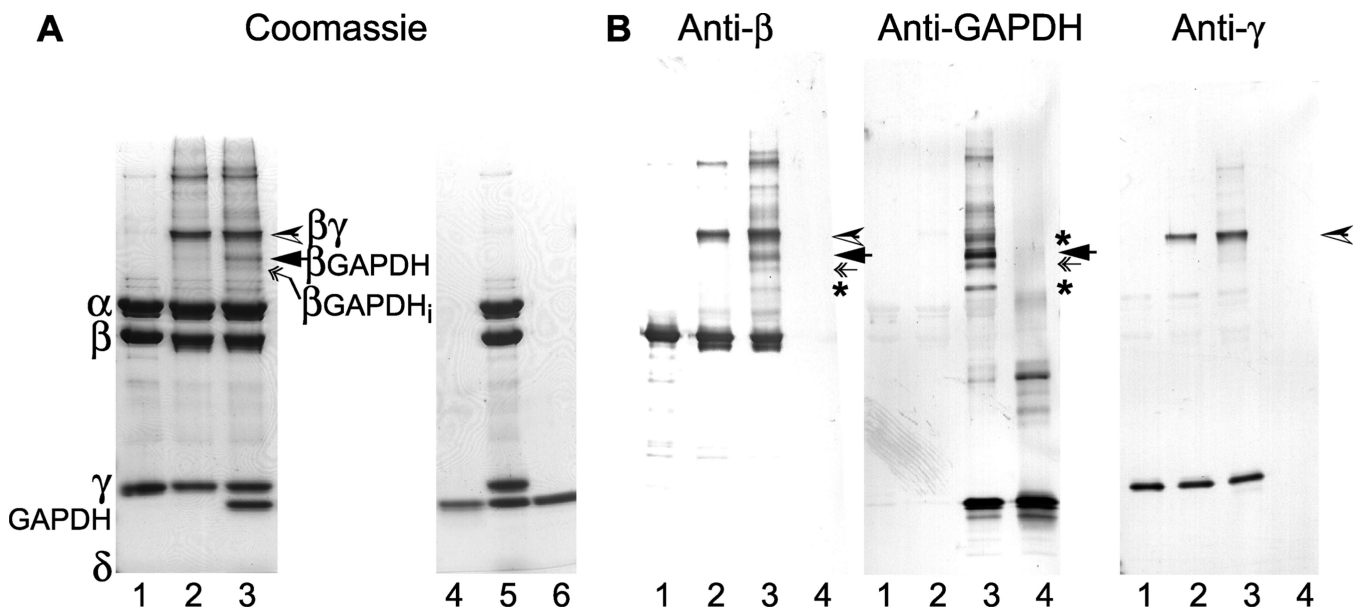

Figure 5.

Chemical cross-linking of PhK and GAPDH by GMBS. (A) Native PhK (lane 1) and GAPDH (lane 6) were incubated together in the absence (lane 5) and presence (lane 3) of GMBS and then resolved by SDS–PAGE using a slab gel with a 5 to 18% linear gradient of acrylamide. Under identical conditions, GAPDH (lane 4) and PhK (lane 2) were each treated separately with GMBS, with the latter reaction forming large amounts of the previously reported βγ heterodimer [black and white arrow (45)]. In addition to the conjugates observed in these control cross-linking reactions (lanes 2 and 4), a relatively large amount of a new band with a mass corresponding to a β-GAPDH dimer (154.7 kDa; 4% error) was observed when PhK and GAPDH were incubated with GMBS (lane 3, solid arrow). A second, but faint, new band (lane 3, double-headed arrow) is also observed migrating slightly faster than the apparent β-GAPDH dimer (solid arrow). Because the β subunit is known to readily undergo intrasubunit cross-linking, which results in its faster migration (26), we tentatively assigned this second faint band also as a β-GAPDH dimer (141.8 kDa, 12% error). (B) Parallel samples were transferred to PVDF membranes and probed with mAbs against all the PhK subunits and an anti-GAPDH polyclonal Ab as described in Experimental Procedures. All conjugates with apparent masses greater than that previously reported for the PhK βγ dimer [170 kDa (45)] cross-reacted with combinations of the PhK anti-β and -γ mAbs, as well as the GAPDH polyclonal Ab (lane 3); however, no significant cross-reactivity was observed in blots probed with either PhK anti-α or -δ mAbs (data not shown). Although not readily apparent in Coomassie-stained gels, cross-linked conjugates of GAPDH itself are observed in the immunoblot (lane 4). The identity of the two bands assumed to be β-GAPDH dimers (solid and double-headed arrows) on the basis of their apparent mass in panel A was confirmed by their cross-reactivity against only the anti-β and anti-GAPDH Abs (lane 3). Additional conjugates in lane 3 that cross-react with both of these antibodies (designated with an asterisk) could potentially result from additional intrasubunit cross-linking of β in a β-GAPDH dimer or more than one GAPDH monomer in a β-GAPDH complex, resulting from intersubunit cross-linking of GAPDH, as observed in lane 4.