Baculovirus-Mediated Overexpression of the Phosphorylase *b* Kinase Holoenzyme and $\alpha\gamma\delta$ and $\gamma\delta$ Subcomplexes

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ABSTRACT: Recombinant baculoviruses were created and used to coexpress rat phosphorylase kinase (Phk) α, γ, and δ subunits and rabbit β subunit in insect cells. Coexpression allowed creation of the $(\alpha\beta\gamma\delta)_4$ hexadecamer, the $\alpha\gamma\delta$ heterotrimer, and the $\gamma\delta$ heterodimeric subcomplexes. Neither the individual α, β, or γ subunit nor any complex containing the β subunit other than the hexadecameric holoenzyme was obtained in soluble form. The expressed complexes exhibited pH- and $[Ca^{2+}]$-dependent specific activities that were similar to those of the Phk holoenzyme purified from rabbit skeletal muscle (SkM Phk). SkM Phk, expressed Phk, and the $\alpha\gamma\delta$ subcomplex were activated by exogenous calmodulin and underwent Ca^{2+}-dependent autophosphorylation. In some of these features there were subtle differences that could likely be attributed to differences in the covalent modification state of the baculovirus-driven expressed protein. Our results provide an important avenue to probe the detailed characterization of the structure of Phk and the function of the individual domains of the subunits using baculovirus-mediated expression of Phk and Phk subcomplexes.

Phosphorylase *b* kinase (Phk;[1] EC 2.7.1.38) controls the rate-limiting step in glycogenolysis (*1*). It contains one catalytic (γ) and three regulatory (α, β, and δ) subunits and is one of the most structurally complex protein kinases known (*1*). It is generally presumed that the four subunits form a hexadecameric holoenzyme with $(\alpha\beta\gamma\delta)_4$ stoichiometry. The γ catalytic subunit contains structural elements common in protein kinases, as well as unique structural features likely to be involved in intersubunit interactions and regulation. The α and β regulatory subunits do not share significant sequence homology with any other known proteins. The δ subunit shares identity with calmodulin (CaM) (*1*−*3*).

cAMP-dependent protein kinase is a physiological regulator of Phk, and there is at least one site for PKA phosphorylation on each α and β subunit (*1*). The phosphorylation of these sites significantly increases Phk phosphotransferase activity leading to the activation of phosphorylase. There is evidence for additional PKA phosphorylation sites, but they have not yet been characterized (*1*). Phk activity is also Ca^{2+}-dependent. It is well established that the δ subunit is largely responsible for mediating Ca^{2+} effects. The binding of CaM to its target enzymes is most typically Ca^{2+}-dependent, and CaM dissociates when the $[Ca^{2+}]$ is lowered to submicromolar levels. The δ subunit is unusual in that it remains an integral part of the Phk holoenzyme even when the $[Ca^{2+}]$ is below submicromolar levels (*1*), and binding of Ca^{2+} leads to a conformational change in the structure of the enzyme (*4*). There is evidence that δ interacts with a putative autoinhibitory sequence in the C-terminal domain of the γ subunit (*5*−*8*). Specific high-affinity calmodulin binding sites also exist on the α and β subunits (*1*, *9*). These are possible sites for the binding of exogenous CaM (so-called δ'), in what appears to involve classic Ca^{2+}-dependent interactions, but they may also be involved in binding of the endogeneous δ subunit as part of an activation cascade of the holoenzyme. Phosphorylation and Ca^{2+} act synergistically, and consequently, Phk is a potential point for cross talk between two prominent eucaryotic signal transduction pathways (*1*, *10*). In vitro, Phk is activated by increased pH, autophosphorylation, limited proteolysis, and ADP, but the physiological significance of these activation mechanisms is unknown (*1*). Phk can also be extensively covalently modified, with up to seven phosphorylation sites in the α subunit and four in the β subunit (*11*) and farnesylation sites on the carboxy termini of both the α and β subunits. These modifications clearly must have physiological implications in Phk's control and function, but what they control remains to be elucidated.

An overall structure of Phk has been deduced by electron microscopy (*12*) with subunit localization by immunodetection (*13*). Understanding of the interactions between the subunits will likely only be obtained by reconstructing the holoenzyme from the individual subunits. Earlier studies have obtained partial complexes or free γ subunit, but the amounts have not been sufficient enough for biophysical characterization of the enzyme complex (*14*−*16*). Here we describe a baculovirus (BV) overexpression system for producing recombinant Phk and the $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes. The Phk and subunit subcomplexes obtained with this system are

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; Phk, phosphorylase *b* kinase; SkM Phk, phosphorylase *b* kinase purified from rabbit muscle tissue; BV, baculovirus.

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Table 1

Name	Sequence (5→3')
pα5'	CCTTCTAGAATATAAATATGAGGAGCGGAG *Xba I*
pα3'	CCCACGGCCGTCACTTGCATGGCACAGAGGCTGTG *Eag I*
pβ3040$_f$	CCTGAGCTAGAATTTCAAGACAAAGTAG
pβ3'	GGCTTTCCTTTTGCGGCCGCTACTACTAGCTAACCAGACACGAGTCCTC *Eag I*
pβ5'	CGCGGATCC**TATAAA**TATGGCGGGGGGCGACGGGGCTCATGGC *Bam HI*
pβ450$_r$	GTGAAGACACGTTGTTGGGCGTGGATCCTG
γ E-5'	CCTGGGATCC**ATATAAATATG**ACCCGGGATGAGGCCCTCCCTGACTCT *Bam HI*
γ E-3'	GGCGAGATCTCTGTTTGCTTTCTCCCCTGTGCCCCGCCAC
pδ 5'	CGGGATCCCTATAAATATGGCTGACCAACT *Bam HI*
pδ 3'	GATCTAGATCACTTCGCTGTCATCATCTG *Xba I*
γ His-5'	CCGGCGGTACCCTACTCAGAAGTCCTCCTCCTCAGCCA *Kpn I*
γ His-3'	GGCGAATTCATGACCCGGGATGAGGCCCTCCCTGAC *Eco RI*

Restriction sites are labeled. Bold sequence denote the baculovirus "Kozak-type" sequence. Where appropriate, the ATG translation start site is underlined.

quite similar in properties to those of the enzyme purified from muscle tissue (SkM Phk), but they also have some distinctive features. The BV expression system that we have created can be used to obtain sufficient quantities of enzyme for biophysical structural characterization and will therefore be extremely useful for further elucidating the nature of the molecular interactions involved in Phk holoenzyme assembly and regulation.

MATERIALS AND METHODS

Materials and Reagents. Chromatography resins and all DNA restriction enzymes, Taq polymerase, and T4 DNA ligase were purchased from Amersham Biosciences (Piscataway, NJ). Rabbit skeletal muscle glycogen phosphorylase *b* and rabbit skeletal muscle Phk (SkM Phk) were purified as described (*17*, *18*). Calmodulin (CaM) was obtained as a lyophilized powder from Sigma (St. Louis, MO), resuspended in dialysis buffer (50 mM Hepes, pH 7.5, 15 mM β-mercaptoethanol, 10 mM NaCl, and 5% sucrose), dialyzed at 4 °C overnight against 1 L of dialysis buffer, and stored at −20 °C. Oligonucleotide primers were synthesized by the Molecular Structure Facility at the University of California (Davis, CA), and their sequences are provided in Table 1. All other reagents and materials were obtained as indicated elsewhere in this paper were from standard sources and were of the highest purity available.

Polymerase Chain Reaction (PCR). PCR reactions contained 200 μM each of dATP, dTTP, dGTP, and dCTP, 2.5 units of Taq polymerase, 1 nmol of template DNA, and 50 pmol of each oligonucleotide primer in reaction buffer (supplied with the polymerase). DNA was amplified for 25 cycles (94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min), and PCR products were purified prior to cloning using a Wizardprep PCR purification kit (Promega, Madison, WI).

Preparation of Phk Subunit cDNAs. The complete rat SkM Phk α subunit cDNA (Accession Number AF197561) was amplified by PCR from a λgt11 skeletal muscle cDNA library (Stratagene, La Jolla, CA) by PCR with oligonucleotide primers pα5′ and pα3′ (Table 1). The PCR product was digested with restriction enzymes *Xba*I and *Eag*I and ligated into the pVL1393 BV transfer vector (Invitrogen Corp., Carlsbad, CA) using T4 DNA ligase to create the pVL-α recombinant plasmid construct.

The rabbit SkM Phk β subunit cDNA was the generous gift of Dr. Manfred Kilimann (Institut für Physiologische Chemie, Ruhr-Universität Bochum, Bochum, Germany). The cDNA was digested with *Eco*RI and *Xba*I, and the resulting β fragment (base pairs 372−3070) was isolated from a Seaplaque agarose (FMC Bioproducts, Rockland, ME) gel following electrophoresis using the Wizardprep PCR purification kit. The β subunit cDNA was also amplified by PCR with oligonucleotide primers pβ3040$_f$ and pβ3′ (Table 1). The PCR product was digested with *Eag*I and *Xba*I. This PCR product and the $\beta_{372-3070}$ fragment were simultaneously ligated into the *Eco*RI and *Eag*I sites in vector pVL1393, and the pVL$\beta_{372-3282}$ recombinant plasmid was isolated. The β cDNA was used as template for a second round of PCR amplification with oligonucleotides pβ5′ and pβ450$_r$ (Table 1). The PCR product was digested with *Bam*HI and ligated into the *Bam*HI site in pVL$\beta_{372-3282}$ to create the pVL-β recombinant plasmid construct.

The rat SkM Phk γ subunit cDNA was previously cloned and sequenced (*19*). Oligonucleotide primers γE-5′ and γE-3′ were used to amplify the γ subunit cDNA by PCR. The PCR product was digested with *Bam*HI and *Bgl*II and ligated into *Bam*HI and *Bgl*II sites in the pBlueBac III vector (Invitrogen Corp., Carlsbad, CA) to create the γ-BlueBac recombinant plasmid.

To generate the His-tagged γ subunit expression vector, the coding region of the γ subunit of phosphorylase kinase was amplified by PCR using oligonucleotide primers γHis-5′ and γHis-3′ (Table 1) with *Eco*RI and *Kpn*I sites at the 5′ and 3′ ends, respectively. The amplified product was then subcloned into the pAcHLT-A vector (Pharmingen, San Diego, CA).

The CaM (δ subunit) cDNA was the generous gift of Dr. Anthony Means (Duke University Medical Center, Durham, NC). The δ subunit cDNA was amplified by PCR using the pδ5′ and pδ3′ oligonucleotide primers (Table 1). The PCR product was digested with *Bam*HI and *Xba*I and ligated into the *Bam*HI and *Xba*I sites in vector pVL1393 to create the pVL-δ plasmid.

Escherichia coli strain HB101 was used as a host to select and amplify all recombinant plasmid constructs. Recombinant plasmids were purified from *E. coli* using a midiprep kit (Qiagen, Valencia, CA). All clones were verified by dideoxy chain terminator sequencing performed by the Protein and Nucleic Acid Core Facility at Stanford University (Palo Alto, CA).

Cell Culture. Sf9 insect cells (Invitrogen Corp., Carlsbad, CA) were maintained in monolayer cultures at 28 °C in complete Hink's TNM-FH medium (Pharmingen, San Diego, CA). Hi-5 insect cells (Invitrogen Corp., Carlsbad, CA) were maintained in a monolayer culture at 28 °C in Excel 405 medium (JRH Biosciences, Lenexa, KS). All media were supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

Preparation of Recombinant Baculoviruses (BVs). The γ-BlueBac plasmid was cotransfected into Sf9 insect cells along with linearized Ac*M*NPV viral DNA (Invitrogen Corp., Carlsbad, CA) according to instructions provided by Invitrogen. The pVL-α, pVL-β, and pVL-δ recombinant BV transfer vectors were cotransfected into Sf9 insect cells along with linearized Baculogold DNA (Pharmingen, San Diego, CA) according to instructions supplied by Pharmingen. Five days posttransfection the recombinant viruses were plaque-purified from the culture medium, amplified, and concentrated into high-titer stocks as described (*20*).

Protein Expression in Insect Cells. Hi-5 cells were seeded into 225 cm^2 flasks and placed at 27 °C until ~60% confluent. The medium was then removed and replaced with 10 mL of inoculum per flask. The inoculum consisted of Excel 405 medium containing a sufficient quantity of each recombinant, α, β, γ, His-γ, and/or δ, virus stock to provide a multiplicity of infection of 5 for each virus, as shown to be optimum for Phk holoenzyme and subcomplex formation by combinatorial transfection at various levels of multiplicity of infection with the individual subunit vectors. The flasks were rocked on a platform rocker for 1 h, and then ~20 mL of fresh Excel 405 medium was added to bring the total volume of medium to ~30 mL per flask. The cells were placed at 28 °C to allow protein expression. Typically 15 flasks were used for a single expression experiment.

Protein Purification. At ~48−55 h postinfection infected cells were scraped from the flasks and harvested by centrifugation at 800*g*. Cell pellets were rinsed once in ice-cold PBS and resuspended in 10 mL of ice-cold buffer A [50 mM Hepes, 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM NaCl, and 45 mM β-mercaptoethanol, plus protease inhibitors (1 mM benzamidine, 1 μg/mL aprotinin, 10 μM leupeptin, 1 μM pepstatin A, 1 μg/mL E64, and 1 mM AEBSF), pH 7.0 for the $\alpha\gamma\delta$ complex and pH 7.5 for the $\gamma\delta$ complex] for purification of the $\alpha\gamma\delta$ and $\gamma\delta$ complexes, or 10 mL of ice-cold buffer B (30 mM β-glycerophosphate, pH 6.8, 50 mM NaCl, 10% sucrose, 2 mM EDTA, and 15 mM β-mercaptoethanol, plus protease inhibitors as in buffer A), for purification of Phk. Cell suspensions were placed in a Parr N_2 cavitation bomb and equilibrated on ice for 15 min at 300 psi N_2. Cells were lysed by extrusion from the bomb, the lysate was centrifuged at 10000*g*, 4 °C, for 40 min, and the supernatants (cytosolic fractions) were recovered.

For purification of the expressed Phk holoenzyme complex, the cytosolic fraction (~10 mL, ~30 mg of protein/mL) was centrifuged first at 17000*g* for 30 min and then at 100000*g*, 4 °C, for 90 min. An ice-cold solution of 47% poly(ethylene glycol) (PEG, MW 8000) was added to the supernate to achieve a final PEG concentration of 3%. After being stirred on ice for 10 min, the resulting precipitate was collected by centrifugation at 12000*g*, 4 °C, for 10 min. The 3% (w/v) PEG supernate was adjusted to ~10% PEG by further addition of 47% PEG (w/v) stock solution. This was stirred at 4 °C for 10 min, and again the precipitate was collected by centrifugation. The pellet was dissolved in ~2 mL of fresh buffer B and chromatographed on a 2.6 cm × 60 cm S300 gel filtration column equilibrated in buffer C (30 mM Hepes, pH 6.8, 50 mM NaCl, 10% sucrose, 2 mM EDTA, 4.5 mM $CaCl_2$, and 15 mM β-mercaptoethanol, plus protease inhibitors as in buffer A). S300 column fractions were assayed for Phk activity, and two peaks of activity were recovered and pooled (the second peak containing some $\alpha\gamma\delta$ subcomplex). The first peak that contained holoenzyme was chromatographed on a Bio-Rad Q2 anion-exchange column equilibrated in buffer C. Phk was eluted from the Q2 column with a 45 mL linear 50−500 mM NaCl gradient. Fractions with activity were pooled (typical pool ~12 mL, 0.2 mg of protein/mL) and concentrated in an ultrafiltration device (Amicon) to ~1.5 mg/mL protein (total volume of ~1.6 mL). The concentrated pool was dialyzed overnight at 4 °C in buffer C and stored at −20 °C.

For purification of the $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes (from their separate expression systems), a 2 cm × 16 cm DEAE-Sepharose fast-flow column was equilibrated in buffer D (50 mM Hepes, 0.2 mM $CaCl_2$, 10 mM NaCl, 15 mM β-mercaptoethanol, and 1 mM benzamidine) at pH 7.0 for the $\alpha\gamma\delta$ subcomplex and pH 7.5 for the $\gamma\delta$ subcomplex. Equilibration buffer was also supplemented with 5% sucrose for purification of the $\alpha\gamma\delta$ subcomplex. The cytosolic fraction was loaded onto the column, and the column was washed with ~5 bed volumes of equilibration buffer. The column was eluted with a 600 mL linear 10−750 mM NaCl gradient. Fractions were assayed for Phk activity and pooled (typical pool volume ~135 mL, ~0.6 mg of protein/mL).

To further purify the $\alpha\gamma\delta$ subcomplex, the $\alpha\gamma\delta$ DEAE pool was concentrated in a Jumbosep 10K MWCO ultrafiltration device and combined with an equal volume of buffer C containing 2 mM EDTA instead of 1 mM $CaCl_2$. This mixture was centrifuged at 10000*g*, 4 °C, for 10 min, and the supernatant was chromatographed on a Sephacryl 2.6 cm × 60 cm S300 HR gel filtration column. Fractions were assayed for Phk activity, pooled (typical pool ~50 mL, ~1 mg of protein/mL), and concentrated to ~5 mL in a Jumbosep 10K MWCO ultrafiltration device. The pool was adjusted to 2 mM $CaCl_2$ and applied to a CaM−Sepharose 4B column (10 mL bed volume, ~1.2 mg/mL CaM) equilibrated in buffer E (50 mM Hepes, pH 6.8, 2 mM $CaCl_2$, 5% sucrose, 150 mM NaCl, and 15 mM β-mercaptoethanol) at 4 °C. The CaM column was washed with 10 bed volumes of buffer E and eluted with buffer F (50 mM Hepes, pH 6.8, 5 mM EGTA, 5% sucrose, 150 mM NaCl, and 15 mM β-mercaptoethanol). The eluted fractions that contained most protein and high Phk activity were pooled. The pool was dialyzed at 4 °C overnight in 1 L of buffer G (100 mM Hepes, pH 6.8, 100 mM NaCl, 10% sucrose, and 30 mM β-mercaptoethanol). The dialysate was concentrated to ~2 mg/mL protein (final volume ~5 mL) in a Jumbosep 10K MWCO ultrafiltration device and combined with an equal volume of 100% glycerol. The final preparation (typically ~1 mg/mL protein in ~10 mL total volume) was stored at −20 °C.

For further purification of the $\gamma\delta$ subcomplex, the DEAE pool was concentrated in a Jumbosep 10K MWCO ultrafiltration device and chromatographed on an S300 gel filtration column. The column fractions were assayed for Phk activity and pooled (typical volume of pool was ~25 mL, ~0.1 mg of protein/mL). The pool was dialyzed at 4 °C overnight in 1 L of buffer G. The dialysate was concentrated to ~1 mL (~2 mg/mL protein) in a Jumbosep 10K MWCO ultrafiltration device and combined with an equal volume of 100% glycerol. The final mixture (typically ~1 mg/mL protein in 2 mL total volume) was stored at −20 °C.

For purification of His-tagged $\alpha\gamma\delta$ and His-tagged $\gamma\delta$ subcomplexes the cytosolic fractions were added to a 50% Ni-NTA−agarose slurry (Qiagen Inc., Valencia, CA) equilibrated in buffer H (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 15 mM βME, 10% glycerol, 5% sucrose, and 1 mM benzamidine hydrochloride) plus 10 mM imidazole and incubated on rotation for 1 h at 4 °C. The slurry was then centrifuged at 500*g* for 5 min at 4 °C and washed twice in buffer H plus 20 mM imidazole, and the protein was eluted twice in 4 mL of buffer I (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 15 mM βME, 5% sucrose, 1 mM benzamidine hydrochloride, and 250 mM imidazole) by incubating in buffer for 5 min on rotation and centrifuged at 500*g* for 5 min at 4 °C. The eluted protein was loaded onto a HiPrep FPLC 26/60 Sephacryl S-300 column equilibrated in buffer E at a flow rate of 1 mL/min, and 4 mL fractions were collected. Twelve microliters of each fraction was run on a 4−16% gel (Novex, Invitrogen), and the fractions that contained high protein were pooled. The protein pool from the His-tagged $\alpha\gamma\delta$ subcomplex expression was dialyzed for 16 h against 2× buffer E and concentrated to 2 mg/mL using the Centriprep 30 concentrators (Amicon). An equal volume of glycerol was added, and the pool was stored at −20 °C. The protein pool from the His-tagged $\gamma\delta$ complex expression was dialyzed against buffer A, batched onto the Ni-NTA−agarose slurry, and eluted twice in 500 μL of buffer I as described earlier. The eluted protein was dialyzed against 2× buffer E; an equal volume of glycerol was added, and the pool was stored at −20 °C.

Phk Activity Assay. Phk activity was assayed as described (*14*, *21*), and the components of reaction mixes are provided in the legend to Figure 4. The reaction rate was confirmed to be linear for the duration of the assay. Specific activity is always expressed as moles of [γ-^{32}P]ATP transferred to phosphorylase *b* per minute per mole of γ subunit. Activity was expressed in these units so as to allow direct comparison of all enzyme species. To express specific activity in these terms, the concentration of SkM Phk was first determined experimentally, and the concentration of the γ subunit was then calculated on the basis of the known mass and stoichiometry of the holoenzyme and the individual γ subunit that comprise the holoenzyme. Each expressed enzyme species and SkM Phk were then resolved by SDS−PAGE, and stained gels were subjected to scanning densitometry (as described in the section on SDS−PAGE). The relative amount of γ subunit present in each sample was then calculated on the basis of comparison to the known amount of γ subunit present in SkM Phk.

Autophosphorylation Reactions. The expressed holoenzyme and the $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes and rabbit SkM Phk were diluted to ∼1 nM in 5% glycerol, 25 mM Tris, 25 mM β-glycerophosphate, 10 mM NaCl, 15 mM β-mercaptoethanol, and 100 mM NaF adjusted to either pH 6.8 or pH 8.2. To initiate the autophosphorylation reaction, five parts of diluted enzyme was combined with one part of the [γ-^{32}P]ATP mix (6 mM [γ-^{32}P]ATP, ∼100 dpm/pmol, 10 mM magnesium acetate, 1 mM $CaCl_2$, or 2 mM EGTA), and the reaction tube was immediately placed at 30 °C. At regular intervals following initiation of the reaction an aliquot of the reaction mix was either combined with an equal volume of 2× SDS−PAGE buffer (150 mM Tris, pH 6.8, 2% SDS, 15 mM β-mercaptoethanol, and a trace of bromphenol blue) and stored at −20 °C for SDS−PAGE or spotted onto Whatman (Rockland, MA) ET31 filter papers and processed as described (*14*, *21*).

SDS−PAGE and Immunoblotting. Proteins were routinely resolved by electrophoresis in precast Novex 8−16% denaturing polyacrylamide gels (Invitrogen, San Diego, CA) in the presence of β-mercaptoethanol. Proteins in the gels were visualized with Coomassie brilliant blue R-250 (*22*). A Molecular Dynamics densitometer SI and Imagequant image analysis software (Molecular Dynamics Corp., Sunnyvale, CA) were used for densitometric analysis of Coomassie-stained proteins. Immunoblotting was used to verify the identity of expressed proteins. Briefly, following SDS−PAGE proteins were transferred to a nitrocellulose membrane and detected with a guinea pig anti-rabbit SkM Phk antibody as described (*14*). Radiolabeled proteins from phosphorylation reactions were also resolved by SDS−PAGE. The gels were stained with Coomassie brilliant blue R-250, dried, photographed, and autoradiographed on Kodak X-Omat film. Radiolabeled proteins in dried gels were analyzed with a Molecular Dynamics Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA) and Imagequant image analysis software.

Protein Concentration Determination. Protein concentrations were typically estimated with the Bio-Rad (Hercules, CA) protein assay reagent according to the manufacturer's instructions.

RESULTS

Purification of Expressed Complexes. The expressed Phk and the $\alpha\gamma\delta$ subcomplex were prepared and isolated as described in Materials and Methods and obtained with a high degree of purification (∼90% pure as judged by SDS−PAGE and scanning densitometry; Figure 1) and yield. The expression was also confirmed by immunoblotting (data not shown). Densitometry of SDS−PAGE gels indicated that the expressed holoenzyme and $\alpha\gamma\delta$ subcomplex were 1:1:1:1 and 1:1:1, respectively (within ±10%). The molecular weights (MW) of the expressed enzyme species and Phk holoenzyme purified from rabbit skeletal muscle (SkM Phk) were measured by gel filtration chromatography (Figure 2). The expressed holoenzyme exhibited an apparent MW of $\sim 1.3 \times 10^6$ similar to that of SkM Phk (indicated by arrow). The $\alpha\gamma\delta$ subcomplex exhibited an apparent MW of ∼200000, which is the predicted MW of an $\alpha\gamma\delta$ trimer based on the molecular weight of its constituent subunits.

The $\gamma\delta$ subcomplex has so far proven somewhat refractory to purification and, although expressed in significant quantities, was only partially purified (∼50% pure) by the methods described in Materials and Methods. By incorporating a hexahistidine tag into γ and using nickel chelate affinity chromatography, we were able to achieve a high degree of purification of the $\gamma\delta$ subcomplex, >90% (Figure 3). The hexahistidine-tagged $\gamma\delta$ subcomplex exhibited a 1:1 subunit stoichiometry (±10%) by densitometric scanning of the SDS−PAGE gel. (Using the hexahistidine-tagged γ was also an effective means of rapidly preparing the $\alpha\gamma\delta$ trimer in high purity.) The $\gamma\delta$ subcomplex exhibited a MW of ∼66000, which is the predicted molecular weight of a $\gamma\delta$ heterodimer (Figure 2).

Specific Activity and pH Dependency. The activity characteristics of the expressed holoenzyme and the $\alpha\gamma\delta$ and

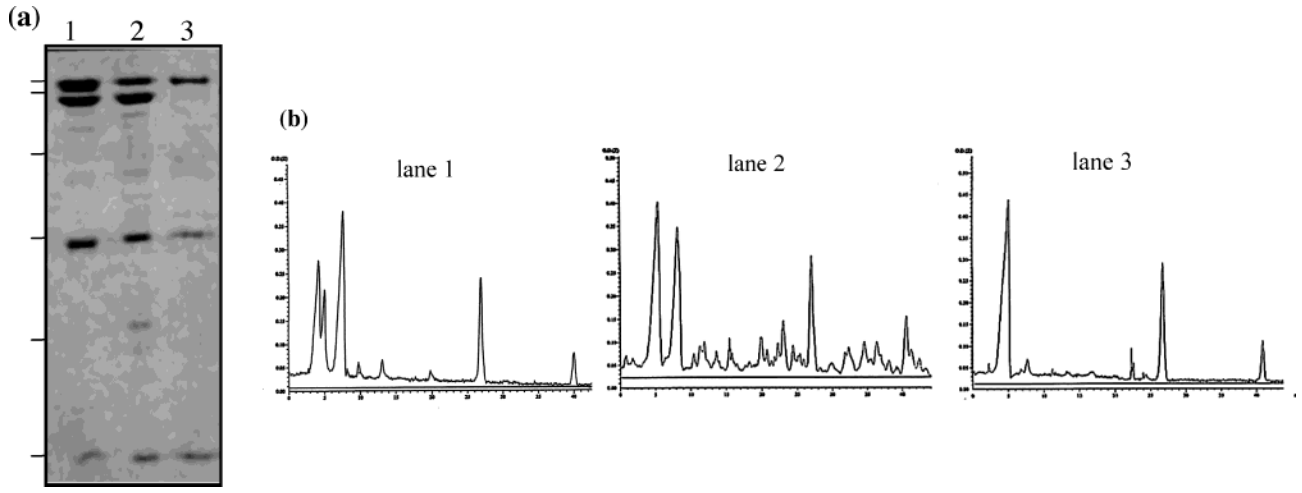

FIGURE 1: SDS−PAGE analysis of Phk subunit complexes expressed in insect cells. (a) Coomassie-stained SDS−PAGE gel of purified skeletal muscle Phk (lane 1), expressed Phk (lane 2), and the $\alpha\gamma\delta$ subcomplex (lane 3). The standards in decreasing molecular weight are β-galactosidase (116250), phosphorylase *b* (97400), serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), and trypsin inhibitor (21500). (b) Representative densitometer tracing of SDS−PAGE gel profiles from panel a. Left to right: lane 1, lane 2, and lane 3.

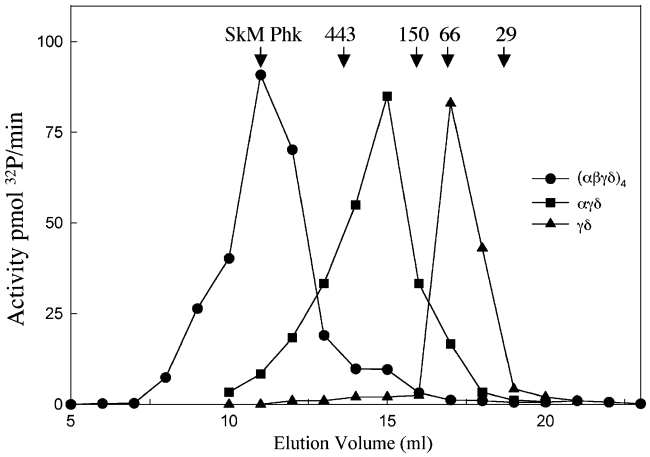

FIGURE 2: Estimation of molecular weight based on analytical gel filtration. Elution profile for analytical gel filtration of expressed Phk, $\alpha\gamma\delta$, and $\gamma\delta$ subcomplexes. The elution volume of standard proteins of known molecular weight and purified SkM Phk (MW 1.3×10^6) are indicated by arrows. Protein standards were obtained in a gel filtration molecular weight marker kit from Sigma Chemical Co. (St. Louis, MO) and included carbonic anhydrase (MW 29000), BSA (MW 66000), alcohol dehydrogenase (MW 150000), and apoferritin (MW 443000).

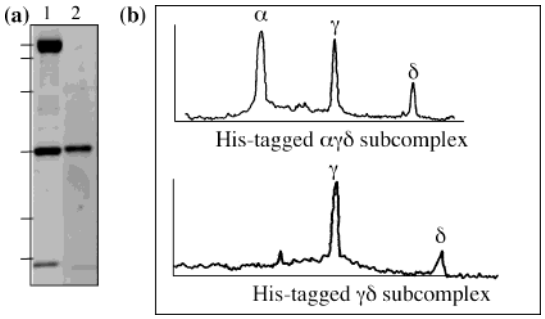

FIGURE 3: SDS−PAGE analysis of His-tagged Phk subunit complexes expressed in insect cells. (a) Coomassie-stained SDS−PAGE gel of purified His-tagged $\alpha\gamma\delta$ (lane 1) and His-tagged $\gamma\delta$ (lane 2) complexes. The standards in decreasing molecular weight are β-galactosidase (116250), phosphorylase *b* (97400), serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), and trypsin inhibitor (21500). (b) Densitometric scans of the purified complexes in panel a.

$\gamma\delta$ subcomplexes are presented in Figure 4 in comparison to Phk isolated from rabbit skeletal muscle (referred to in this paper as SkM Phk). Activities were determined at pH 6.8 and 8.2: the pH 6.8/8.2 activity ratio has been commonly used to evaluate the activation status of the enzyme (*1*). The data presented (Figure 4) are expressed per mole of γ subunit. The Phk obtained by BV-mediated expression exhibited a specific activity at pH 6.8 very similar to that of SkM Phk. Of interest, the pH 8.2 specific activity of Phk obtained by BV-mediated expression was 4-fold lower than that of SkM Phk, and as a consequence the pH 6.8/pH 8.2 activity ratio was 4-fold higher. (Other differences between these forms will also be apparent in characterizations that are presented in subsequent sections of this report.) Such differences are not unexpected since SkM Phk is extensively covalently modified, both by phosphorylation of multiple sites in both the α and β subunits and by farnesylation of both the α and β subunits at the carboxy termini (*1*, *11*). It is not unexpected that there would be differences in the state of covalent modification of the proteins as isolated from adult rabbit skeletal muscle and from BV-infected insect cells (and in fact surprising if they were identical). Such differences might be expected to manifest themselves in the activity characteristics of the expressed enzyme species.

The activity characteristics, expressed per mole of γ subunit, of the BV expressed $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes (Figure 4) agree well with the activity characteristics of subcomplexes isolated by disruption of the Phk enzyme purified from skeletal muscle (*15*, *23*). For the three expressed proteins, $(\alpha\beta\gamma\delta)_4$, $\alpha\gamma\delta$, and $\gamma\delta$, there is an increase in both the pH 6.8 and the pH 8.2 activity as the proteins get less complex. This is consistent with the γ subunit being less inhibited in the absence of the other subunits, an important feature of the protein that has been described in more detail elsewhere (*24*, *25*). Of note, the pH 8.2 activity of the expressed $\gamma\delta$ approached close to that of the holoenzyme Phk enzyme purified from skeletal muscle. This suggests that the difference in the pH 8.2 activity between the Phk obtained by BV-mediated expres-

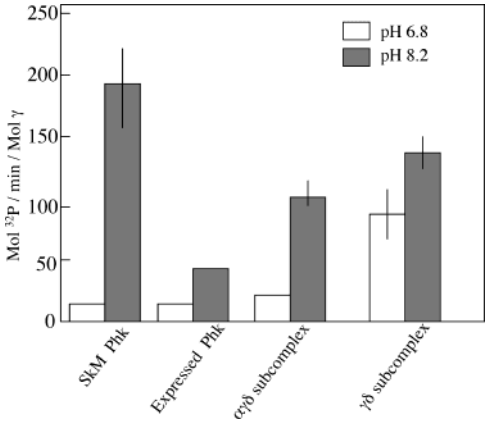

FIGURE 4: pH-dependent specific activity of SkM Phk, expressed Phk, and the expressed $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes. Specific activity was determined as described in Materials and Methods. The reaction mix contained 6 mg/mL phosphorylase *b*, 50 mM Tris, pH 6.8 or 8.2, 50 mM β-glycerophosphate, 600 mM $CaCl_2$, 12 mM magnesium acetate, and 1.2 mM [γ-^{32}P]ATP (~100 dpm/pmol). Activity is expressed per mole of γ subunit to allow direct comparison of all species. The error bars represent the standard deviation of at least three separate activity measurements.

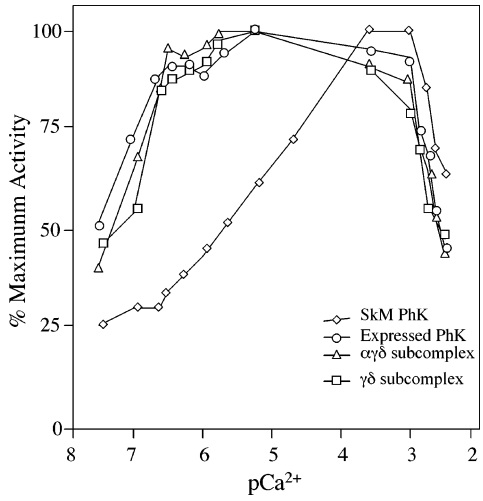

FIGURE 5: Ca^{2+} dependency of SkM Phk, expressed Phk, and the expressed $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes. [Ca^{2+}] was achieved by adjusting the EGTA:Ca^{2+} ratios in the reaction mixture and calculating the final [Ca^{2+}] based on the dissociation constants for Ca^{2+}−EGTA complexes as described (*30*). Activity was measured as described in the legend to Figure 4.

sion and that isolated from skeletal muscle was due to a different (covalent) state of their α and β subunits, with those from the BV-mediated expressed enzyme more strongly inhibiting enzyme activity at that pH.

Ca^{2+} Dependency. Consistent with the presence of an endogenous δ subunit in each, the activity of the expressed $(\alpha\beta\gamma\delta)_4$, $\alpha\gamma\delta$, and $\gamma\delta$ complexes exhibited Ca^{2+} dependency, but it differed somewhat from that observed for SkM Phk (Figure 5). The Ca^{2+} dependency that we measured for SkM Phk (Figure 5) and the general shape of its Ca^{2+} titration curve are similar to what has been reported previously (*26*). The three expressed enzymes exhibited closely similar Ca^{2+} titration curves, but activation of the expressed enzymes occurred at an approximately 10-fold lower Ca^{2+} concentration (EC_{50} of ~0.1 μM) than for SkM Phk. The reason for the differences between the expressed enzymes and SkM Phk is not clear, but similar to the observation on the pH

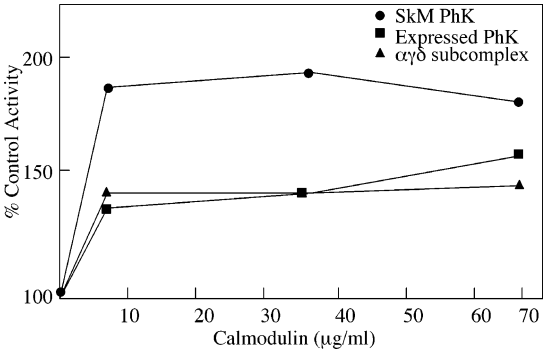

FIGURE 6: Titration with exogenous CaM. The CaM concentration was adjusted in the reaction mixes by addition of a concentrated CaM stock solution. Activity was measured as described in the legend to Figure 4.

dependency of activity (Figure 4), it may well be attributable to differences in the state of covalent modification of the proteins as isolated from adult rabbit skeletal muscle and from BV-infected insect cells. All four proteins were inhibited by millimolar Ca^{2+} due to competition with Mg^{2+} to form the ATP complex essential for phosphotransferase activity.

Activation by Exogenous CaM (δ′). SkM Phk can be activated by δ' (*1*). The expressed Phk holoenzyme and $\alpha\gamma\delta$ subcomplex were activated ~1.5-fold by the addition of CaM (Figure 6). This is consistent with previous observations for the $\alpha\gamma\delta$ subcomplex isolated by disruption of the holoenzyme (*16*).

Autophosphorylation. The autophosphorylation of the skeletal muscle Phk α and β subunits has been extensively documented (*1*, *27*). A comparison of the autophosphorylation of SkM Phk, the expressed holoenzyme, and the $\alpha\gamma\delta$ subcomplex is presented in Figure 7 (as undertaken using the conditions detailed in Materials and Methods). Autophosphorylation of the α and β subunits of SkM Phk and the expressed Phk holoenzymes was generally similar, with both enzymes exhibiting a similar pH dependency and with a higher level of α subunit phosphorylation than β subunit phosphorylation at the higher pH (pH 8.2). Autophosphorylation was inhibited in the absence of Ca^{2+} (data not shown), and no autophosphorylation of the γ and δ subunits was observed. Autophosphorylation of the α subunit in the $\alpha\gamma\delta$ subcomplex occurred with a pattern similar to that of the holoenzyme except it did not exhibit pH dependency (Figure 7); it too was Ca^{2+}-dependent (data not shown).

Autophosphorylation of SkM Phk under the conditions defined in Materials and Methods led to a characteristic activation of the enzyme, which is expressed at pH 6.8 but is not observed at pH 8.2 where the enzyme is already in an activated state (Figure 8). Autophosphorylation of the BV-expressed Phk holoenzyme did not lead to enzyme activation even at pH 6.8 (Figure 8). Thus, like the pH dependency of activity (Figure 4) and the Ca^{2+}-dependent activity profile (Figure 5), autoactivation shows a different profile with the enzyme isolated from skeletal muscle than with that expressed in insect cells. Autophosphorylation of the expressed $\alpha\gamma\delta$ subcomplex also did not change enzyme activity, as has been previously observed with the $\alpha\gamma\delta$ subcomplex isolated by fractionation of skeletal muscle holoenzyme (*15*).

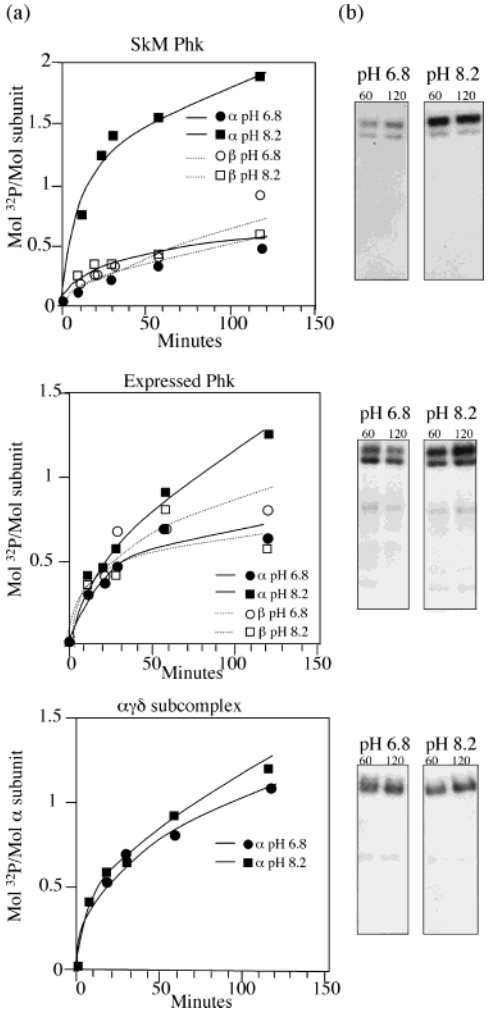

FIGURE 7: Autophosphorylation of the α and β subunits as a function of time and pH. (a) SkM Phk (upper panel), expressed Phk (middle panel), and the expressed $\alpha\gamma\delta$ subcomplex (lower panel) were subjected to autophosphorylation and SDS−PAGE as described in Materials and Methods. The relative intensity of the autophosphorylated α and β subunits in SDS−PAGE gels was determined with a Molecular Dynamics Phosphoimager, and the fraction of the total phosphate incorporated into each subunit was then calculated and plotted as a function of time and pH. (b) Autoradiograph of autophosphorylated SkM Phk, expressed Phk, and the expressed $\alpha\gamma\delta$ subcomplex at 60 and 120 min. The apparent slight diminishment of α subunit phosphorylation in the expressed Phk was not a consistent finding.

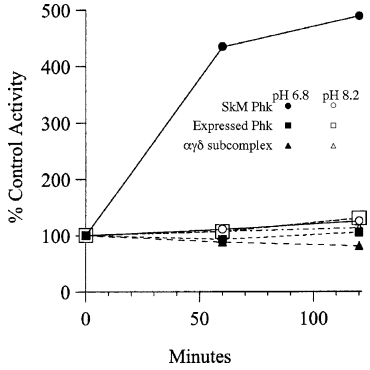

FIGURE 8: Effect of autophosphorylation on the activity of the SkM Phk, the expressed holoenzyme, and the expressed $\alpha\gamma\delta$ subcomplex. Activity was measured as described in the legend to Figure 4. The 100% control activities for SkM Phk, the expressed holoenzyme, and the expressed $\alpha\gamma\delta$ subcomplex were 10, 15, and 20 mol of ^{32}P min^{-1} (mol of γ)$^{-1}$ at pH 6.8, respectively, and 175, 48, and 102 mol of ^{32}P min^{-1} (mol of γ)$^{-1}$ at pH 8.2, respectively.

DISCUSSION

We have created recombinant baculoviruses capable of driving the expression of the rat SkM Phk α, γ, and δ subunits and the rabbit SkM Phk β subunit, leading to the effective production of soluble $(\alpha\beta\gamma\delta)_4$ hexadecamer and $\alpha\gamma\delta$ heterotrimer and $\gamma\delta$ heterodimer subcomplexes. The production of a variety of other forms was also examined using a variety of expression conditions, including different incubation times and the multiplicities of infection. Expressed alone, there is very active synthesis of the intact α subunit, but none that is soluble. Expressed alone, there is moderate expression of the β subunit (∼10−20% of that of the α subunit), but again none that is soluble. Expressed alone, there is active synthesis of the intact γ subunit (about equal to that of the α subunit), but essentially all of the expressed γ is insoluble, and there was insufficient soluble protein for further studies. We have not been able to produce any combination of expressed subunits containing the β subunit in a soluble form except for the holoenzyme, raising doubts as to whether subcomplexes such as $\beta\gamma\delta$,$\alpha\beta$, or $\beta\gamma$ ever exist in any sort of functional form. Likewise, we found no evidence for $\alpha\delta$ or $\alpha\gamma$ subcomplexes. To what extent our inability to produce these subcomplexes in our BV expression system is due to the fact that they cannot exist, their lack of solubility, or their susceptibility to proteolytic degradation is unknown. Failure to obtain these subcomplexes in our expression system suggests that despite their considerable sequence similarity, the α and the β subunits play different roles in the structure and stability of the holoenzyme. Differences between the α and the β subunits are also reflected in their strikingly different patterns of phosphorylation (*11*) and mRNA processing (*28*). Since the α and β subunits share significant sequence homology, it is of interest that the $\alpha\gamma\delta$ heterotrimer was actively produced in our expression system but the $\beta\gamma\delta$ subcomplex was not. Thus, very distinctive and unique roles for the α and β subunits in the holoenzyme structure (and function) are a likely possibility.

Of special note, BV-driven expression resulted in the production of a Phk form which, from its apparent molecular mass, subunit composition, and fundamental enzymological properties, was similar to that of the protein produced in skeletal muscle, despite the obvious molecular complexity and structural intricacy inherent in the holoenzyme. Expressed Phk and the $\alpha\gamma\delta$ and $\gamma\delta$ subcomplexes shared many similarities with SkM Phk purified from rabbit muscle tissue. Their activity was Ca^{2+}- and pH-dependent, and the α and β subunits could be autophosphorylated. It is notable that differences between the BV-expressed Phk and the SkM Phk purified from rabbit muscle tissue were evident primarily in the magnitude, but, importantly, not the direction, of effects. The Ca^{2+} sensitivity and the pH 6.8/8.2 activity ratio, two of the classic criteria of the SkM enzyme's activation state, were different for the expressed enzyme forms and the SkM Phk purified from skeletal muscle. Moreover, whereas the various expressed forms and the SkM Phk purified from skeletal muscle could be autophosphorylated, only the enzyme isolated from muscle exhibited an increase in phosphotransferase activity in response to autophosphoryl-

ation. Differences in the catalytic properties of SkM Phk and the expressed holoenzyme may be attributed to a species-specific difference since our expression system utilized rat α, γ, and δ subunit cDNAs and the rabbit β subunit cDNA. Equally, if not more likely, such differences between the BV-expressed enzyme forms and the enzyme purified from skeletal muscle are to be expected since the Phk expressed in insect cells is almost certain to be different from proteins expressed in rabbit skeletal muscle with respect to the extent and sites of posttranslational modifications. Apart from the sites in Phk regulated by the cAMP-dependent protein kinase, and even for those there remains much uncertainty (*29*), we currently know nothing about what regulates the near myriad of other posttranslationally modified sites known to exist in Phk, which number in excess of 50 sites per holoenzyme molecule. It would indeed be remarkable if the BV-expressed protein and the protein isolated from rabbit skeletal muscle were identical with respect to their posttranslational modifications. Differences in posttranslational modifications might be expected to change some of the activity characteristics of the expressed enzyme species.

The studies presented here suggest that the BV expression system we have developed will be very valuable for future structural analyses of Phk. Use of this system will also allow for a detailed evaluation of subunit-domain function by the production of holoenzyme and subcomplexes with selected mutations. This is a journey that others must pursue since this is the last intended biochemical investigation of the senior author.

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