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Evidence for the Location of the Allosteric Activation Switch in the Multisubunit Phosphorylase Kinase Complex from Mass Spectrometric Identification of Chemically Crosslinked Peptides*

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Abstract

Phosphorylase kinase (PhK), an $(\alpha\beta\gamma\delta)_4$ complex, regulates glycogenolysis. Its activity, catalyzed by the γ subunit, is tightly controlled by phosphorylation and activators acting through allosteric sites on its regulatory α , β and δ subunits. Activation of the catalytic γ subunit in the PhK complex by phosphorylation is known to be predominantly mediated by the regulatory β subunit, which undergoes a conformational change that is structurally linked with the γ subunit and that is characterized by the ability to form β - β dimers using a short chemical crosslinker. To determine potential regions of interaction of the β and γ subunits, we have used chemical crosslinking and 2-hybrid screening. The β and γ subunits were chemically crosslinked to each other in phosphorylated PhK, and crosslinked peptides were identified in digests of the kinase by Fourier transform mass spectrometry in combination with a search engine developed 'in house' that generates a hypothetical list of crosslinked peptides. Such a conjugate between β and γ was identified, verified by MS/MS and shown to correspond to crosslinking between K303 in the C-terminal regulatory domain of γ (γ CRD) and R18 in the N-terminal regulatory region of β (β 1-31), which contains the phosphorylatable serines 11 and 26. A synthetic peptide corresponding to residues 1-22 of β inhibited the crosslinking between β and γ in the complex, and was itself crosslinked to K303 of γ . Through the use of 2-hybrid screening, the β 1-31 region was also shown to control β subunit self-interactions, which were favored by truncation of this region or by mutation of the phosphorylatable serines 11 and 26, thus providing structural evidence for a phosphorylation-dependent subunit communication network in the PhK complex involving at least these two regulatory regions of the β and γ subunits. The sum of our results considered together with previous findings implicates the γ CRD as being an allosteric activation switch in PhK that interacts with all three of the enzyme's regulatory subunits and is proximal to the active site cleft.

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Keywords

phosphorylase kinase; allostery; phosphorylation; subunit interactions; protein-protein interactions

Phosphorylase kinase (PhK) is a 1.3 MDa hexadecameric complex, $(\alpha\beta\gamma\delta)_4$, that in the cascade activation of glycogenolysis in skeletal muscle catalyzes the Ca^{2+} -dependent phosphorylation of glycogen phosphorylase (GP),¹ with that Ca^{2+} -dependence coordinately coupling energy production with muscle contraction. Besides this neural Ca^{2+} signal, the activity of PhK, catalyzed by its catalytic γ subunit, is further markedly enhanced by hormonal (cAMP, in addition to Ca^{2+}) and metabolic (ADP)² stimuli, which are integrated through allosteric ligand-binding and covalent modification sites on its three regulatory α , β and δ subunits.³ Even though only 10% of the mass of PhK is directly devoted to catalysis, little has been reported concerning the mechanisms through which the remaining 90% of its mass regulates the activity of its catalytic core.

The γ subunit (44.7 kDa) contains two major functional domains, one being catalytic with a typical protein kinase fold that accounts for the 10% of mass mentioned above,⁴ and the other being a C-terminal regulatory domain (CRD) that is known to bind the δ and α subunits.⁵⁻⁷ The δ subunit is a molecule of tightly bound calmodulin (CaM, 16.7 kDa),⁸ which binds to the complex independently of Ca^{2+} but confers Ca^{2+} -sensitivity to the activity of γ within the PhK complex.^{9,10} It has been proposed that the Ca^{2+} -independent binding of the δ subunit within the complex arises from multiple contacts between this subunit and two non-contiguous CaM-binding domains (CBD) in the γ CRD, termed the N-CBD (residues 287-331) and C-CBD (residues 332-371).⁵ More recent evidence suggests that the γ CRD also mediates the flow of Ca^{2+} -induced structural changes between the regulatory δ and α subunits within the PhK complex.⁷

Both the large α (138.4 kDa) and β (125.2 kDa) subunits, which undergo phosphorylation by PKA and intramolecular autophosphorylation by γ ,¹¹ are 40% sequence identical and apparently the products of gene duplication.¹² Despite their similarities, each subunit contains unique regions that flank known phosphorylation sites.¹² One such region of β , comprising its N-terminal 31 residues, contains the major regulatory phosphorylation site of PhK (Ser-26), which is targeted by both PKA and autophosphorylation,^{13,14} a second autophosphorylation site (Ser-11),¹² and a variable mRNA splice site that results in the expression of unique N-termini in the skeletal and brain isoforms.¹⁵ The α subunit by contrast has a unique C-terminal phosphorylation domain with at least 7 known phosphorylatable serines¹⁶ clustered in proximity to a region of this subunit that binds the CRD of γ .⁷ The rate of phosphorylation of β exceeds that of α and significant conversion of β is required before measurable phosphorylation of α is detected,¹⁷ presumably the result of a phosphorylation-dependent conformational change in β . Further, phosphorylation of β parallels activation of γ .^{11,17,18} Considered together, these results suggest that activation of γ through phosphorylation of PhK is mediated predominately by β and that these two subunits may be structurally coupled in the PhK complex. Evidence for such a linkage was first demonstrated by Wilkinson et al.,¹⁹ who showed that the solvent accessibility of epitopes for monoclonal antibodies (mAbs) on each of the two subunits increased simultaneously in response to effectors that target either β (ADP, phosphorylation) or γ (Mg^{2+}). Those results are supported by a report showing that phosphorylation of PhK results in differential crosslinking of β and γ .²⁰ A conformational change in β associated with the activation of γ in the PhK complex has also been detected by other methods, including differential proteolysis,²¹ as well as chemical crosslinking by 1,5-difluoro-2,4-dinitrobenzene (DFDNB) to form β - β dimers.²² In the latter study it was demonstrated that the extent formation of the β - β dimers paralleled their extent of

phosphorylation, suggesting possible phosphorylation-dependent interactions between the β subunits.

The results described above clearly show that the β and γ subunits are functionally and structurally linked, undergo structural changes during the activation of PhK by phosphorylation, and interact in the PhK complex, allowing formation of crosslinked conjugates of the two subunits; however, nothing has been reported regarding potential regions of interaction between these subunits. We have employed chemical crosslinking, peptide screening and two-hybrid analyses to determine possible regions of interaction between β and γ . The results herein demonstrating that the N-terminal phosphorylatable region of β (residues 1-31: β 1-31 region) is crosslinked to the γ CRD within the PhK complex suggest that this region of the catalytic subunit is a locus for catalytic and regulatory subunit interactions, providing a plausible mechanism for the coordinate regulation of the γ subunit's activity by all three regulatory subunits. Our observation that the β 1-31 region mediates self-association of the β subunits in response to mutation of phosphorylatable serines 11 and 26 and that this region of β is in proximity to both the CRD and active site of γ provide structural evidence for a phosphorylation-dependent subunit communication network within the PhK complex, with the γ CRD being an allosteric activation switch.

Results

The N-terminal phosphorylatable region of the β subunit is chemically crosslinked to the CRD of the γ subunit in phosphorylated PhK (PhK_A)

To determine potential regions of interaction between the β and γ subunits in the PhK complex, we built on previous findings showing that the affinity crosslinker phenylenedimaleimide (PDM) crosslinked these two subunits in the complex in a reaction that was stimulated by phosphorylation.²⁰ To gain greater amounts of a crosslinked β - γ conjugate for further study, we screened a series of hydrophobic analogs of PDM, based on their affinity for PhK and their selectivity for crosslinking β and γ in the complex. Of the reagents tested, N-[γ -maleimidobutyryloxy]succinimide ester (GMBS) selectively promoted intersubunit crosslinking of β and γ to the greatest extent within single molecules (see Materials and Methods for proof) of the phosphorylated PhK complex (PhK_A), resulting in the formation of a major conjugate containing only β and γ subunits (verified by mass spectrometry and cross-reactivity against subunit-specific mAbs) (Fig. 1)). As judged by its apparent mass on SDS-polyacrylamide gels of 182 kDa, this conjugate is more likely a β - γ dimer ($\text{mass}_{\text{Theor}} = 170$ kDa, a 7.1% error) than a β - γ ₂ trimer ($\text{mass}_{\text{Theor}} = 215$ kDa, a 15.9% error). As was previously observed with PDM,²⁰ which binds rapidly to the kinase complex prior to crosslinking, even the use of extremely low concentrations of GMBS (0.5 mole crosslinker per mole $\alpha\beta\gamma\delta$ protomer) for short time intervals (20 sec) resulted in significant crosslinking of the PhK complex (data not shown). When a 10-fold molar excess of GMBS over $\alpha\beta\gamma\delta$ tetramer was used for a period of 2 min, over 75% of the β and γ subunits were consumed (measured by densitometry) in the PhK_A complex (Fig. 1).

To determine the region(s) of β (schematicized in Fig. 2(a)) and γ that were crosslinked, the band corresponding to the β - γ conjugate was excised from a preparative SDS-PAGE gel and digested 'in gel' with trypsin. The resulting tryptic peptides were extracted, resolved by reverse phase chromatography and analyzed by mass spectrometry (MS) using an FT ICR mass spectrometer. The entire mass output for the tryptic digest of the conjugate was screened for potential crosslinked peptides using a search engine recently developed 'in house' that is capable of generating a hypothetical list of conjugates from such complex reaction mixtures.²³ After culling experimental masses corresponding to those predicted either for non-modified peptides or for side-products of chemical crosslinking, a mass ($m/z_{\text{Theor}} = 1045.591$) predicted for a peptide corresponding to crosslinking between regions of γ (residues 302-305: GKFK)

and β (residues 18-20: RAR) matched that of a peptide mass ($m/z_{\text{Exp}} = 1045.590$; 1.0 ppm error) measured from the tryptic digest of the β - γ conjugate. Mass fragmentation (MS/MS) analysis of this peptide was subsequently carried out to verify the predicted sequence and covalent linkage assignments (Fig. 3).

Both the total ion mass and observed fragmentation pattern confirmed the sequence assignments for the γ (302-305) and β (18-20) subunits and showed the incorporation of one mole of the GMBS reagent, which covalently coupled Lys-303 of γ with Arg-18 of β (Fig. 3 (a)). Modification of these residues is consistent with the inability of trypsin to cleave the amide bonds immediately following each residue.²⁴ The chemistry of crosslinking was deduced from cleavage products resulting from disruption of the crosslinker's amide bonds (Fig. 3(b and c)),²⁵ demonstrating unequivocally that Arg-18 and Lys-303 add, respectively, to the succinimide ester and maleimide functional groups of GMBS to form the expected amide linkage and Michael adduct. At the pH of crosslinking (8.2), the rate of addition of epsilon amines to maleimides approaches that of free thiols and is consistent with the observed modification of Lys-303.²⁶ The novel modification of arginine is consistent with the findings of several recent studies which suggest that the side chain guanidinyll nitrogens may act independently as nucleophiles to initiate the formation of arginine-containing conjugates.²⁷⁻²⁹ The preferential modification of Arg-18 over that of more potent side-chain nucleophiles likely results from the proximity of this residue to sites in the PhK complex that have high affinity for small hydrophobic reagents,^{20,30} thus effectively concentrating the crosslinker in an orientation appropriate for attack by Arg-18. The crosslinking of these particular Arg and Lys residues demonstrates that the N-terminal phosphorylatable region of β and the CRD of γ are proximal ($\leq 10\text{\AA}$)²⁶ and possibly interact in the PhK_A complex.

A peptide corresponding to the N-terminal 22 residues of β inhibits the activity of PhK_A

Since Arg-18 is located centrally within a region of β (β 1-31) that is unique from the highly homologous α subunit and is flanked by serines 11 and 26 (Fig. 2(a)), both of which are autophosphorylation sites in the PhK complex,¹² it is possible that regions of β 1-31 flanking either serine may interact with the regulatory domain of the catalytic γ subunit. To further examine the potential roles of these serine-flanking regions, a series of peptides corresponding to the β 1-31 region was synthesized to determine their effects on the activity of PhK, and ultimately, the crosslinking between its β and γ subunits. Such synthetic peptide analogs have been used widely to compete for intermolecular or intramolecular protein contact sites that are targeted by their counterpart regions in the parent proteins,³¹ including PhK.^{5,32} To localize potential subunit contact sites in the inhibitory β 1-31 domain, two peptides corresponding to residues 1-22 (N β 1) and 15-36 (N β 2) were synthesized with the intent of preserving predicted secondary structural elements flanking phosphorylatable serines 11 and 26 (Fig. 2(c)). Because serines 11 and 26 become autophosphorylated in the PhK complex,¹² both peptides were tested as substrates of the kinase. Under identical conditions, the rates of phosphorylation of both N β 1 (5.7 nmole P incorporated/min/mg PhK) and N β 2 (16.5 nmole P incorporated/min/mg PhK) were roughly comparable to the rate of 20.2 nmole P incorporated/min/mg PhK observed for a synthetic peptide (S-peptide) corresponding to residues 5-18 in the convertible region of glycogen phosphorylase (GP), the known physiological substrate of PhK. Another peptide, a Ser11/Ala analog of N β 1 (N β 1_{S/A}) (Fig. 2(c)), was not phosphorylated by PhK, indicating that neither of the two Thr residues in N β 1 was converted by the kinase.

Under identical conditions, none of these peptides significantly affected the GP conversion activity of nonactivated PhK (data not shown), whereas both N β 1 and N β 1_{S/A}, but not N β 2, inhibited the rate of conversion of GP (1.20 $\mu\text{mole/min/mg PhK}_A$) by PhK_A by 31% (0.83 $\mu\text{mole/min/mg PhK}_A$) and 23% (0.93 $\mu\text{mole/min/mg PhK}_A$), respectively. As observed independently by Newsholme et al.³² for a peptide corresponding to residues 5-28 of the β

subunit, neither N β 1 nor N β 1_{S/A} inhibited PhK_A to an extent that matched the low level of activity of its nonactivated counterpart. The patterns of inhibition of PhK_A by N β 1 and N β 1_{S/A} were analyzed (data not shown), but the data did not discriminate sufficiently between competitive and non-competitive inhibition, because of the limits of solubility of GP and the peptides. The inhibitory effects of each of the peptides on S-peptide conversion by PhK were essentially identical to those using GP as substrate (data not shown), demonstrating that the actions of the β peptides are on PhK and not GP.

An additional finding from the study of Newsholme et al.³² showed that changing Trp-12 to Ala in their β 5-28 peptide abolished its ability to inhibit the activity of phosphorylated PhK, which is consistent with our findings showing that the N β 1 and N β 1_{S/A} peptides, each containing Trp-12, inhibited PhK_A's activity, whereas N β 2 (residues 15-36) had no such effect. Their results and ours together suggest that Trp-12 may be critical for the inhibitory effect of N β 1 and N β 1_{S/A}. Because Ser-11 is adjacent to Trp-12, we utilized a phosphoserine analog of N β 1 (N β 1_{Phos}) to determine whether phosphorylation of Ser-11 perturbed inhibition by N β 1. The activity of PhK_A was unaffected by N β 1_{Phos}, suggesting that introduction of the negatively charged phosphate adjacent to Trp-12 may perturb the interaction observed with the non-phosphorylated analog. The specific inhibition of PhK_A by the N β 1 peptides suggests that a region flanking Trp-12 in the N-terminal 22 residues of β contains structural element(s) that facilitate its binding to the kinase, possibly representing a subunit contact site in this region of the β subunit. To test this possibility, N β 1 and N β 2 were added separately to PhK_A and incubated with the crosslinker GMBS.

N β 1 perturbs GMBS crosslinking of the β and γ subunits in the PhK_A complex and is crosslinked to the identical region of γ as its counterpart region of the intact β subunit

Samples of the PhK_A preparation used above were incubated separately under identical conditions with the N β 1 or N β 2 peptides, followed immediately (5 sec) by addition of GMBS at the same ratio of crosslinker to $\alpha\beta\gamma\delta$ tetramer used above for PhK_A alone. Both N β 1 (Fig. 1(a)) and N β 2 (data not shown) protected β and γ from crosslinking; however, only N β 1 promoted the formation of a new band that migrated just above the γ subunit. The cross-reactivity against the subunit specific mAbs (Fig. 1(b)) and apparent mass on SDS-PAGE of 49 kDa suggested that this new band corresponded to a γ -N β 1 conjugate (mass_{Theor} = 47 kDa; 4.2% error). To verify its identity, the conjugate was blotted onto a PVDF membrane and subjected to gas phase sequencing. The first 10 cycles yielded two sequences that corresponded exactly to the N β 1 peptide and γ , demonstrating unequivocally that the conjugate was γ -N β 1.

To determine the region(s) of γ crosslinked to N β 1, the conjugate band was excised from a single preparative SDS-PAGE gel of PhK_A that had been crosslinked in the presence of N β 1, digested 'in gel' with trypsin and analyzed as described above for the crosslinked β - γ peptides. The predicted mass for a peptide corresponding to crosslinking between γ (residues 302-305: GKFK) and N β 1 (residues 18-22: RARTK), namely $m/z_{Theor} = 1475.808$, matched that of a peptide mass ($m/z_{Exp} = 1475.797$; 7.5 ppm error) measured in the tryptic digest of the N β 1- γ conjugate. The peptide components and corresponding chemical modifications predicted for each ion were subsequently verified by MS/MS (Fig. 4).

Both MS and MS/MS analyses of the γ (302-305)-N β 1(18-22) conjugate confirmed the incorporation of two distinct forms of crosslinker (Fig. 4(a)): one form ($m/z = 165.0504$ Da) covalently linking residues R20 of the N β 1 tryptic fragment and K303 of γ (Fig. 4(b)), and the other form additionally modifying R18 to yield a non-productive monosubstitution product ($m/z = 201.0715$ Da) (Fig. 4(c)), which results from hydrolysis of the active succinimide ester functional group.^{26,33} As was observed with the crosslinking of β and γ , the modification of arginines 18 and 20, as well as Lys-303, corresponded to the observed pattern of proteolysis for the conjugate, in that the amide bonds C-terminal to each residue were refractory to

hydrolysis by trypsin; however, the chemistry of crosslinking of the Arg and Lys residues was reversed, with the Arg residues now adding to the maleimide moiety. The novel Michael addition of arginines 18 and 20 to the maleimido functional group of GMBS, indicated by observed mass additions corresponding exactly to known reaction and hydrolysis products of the crosslinker,²⁶ was evident from the fragmentation patterns resulting from disruption of the amide bonds of these derivatives in the crosslinked peptide (Fig. 4(d)).²⁵ For example, cleavage of the amide bonds (denoted A', B' and C') in the proposed Arg-18 mono-substitution product (Fig. 4(b)) corresponded to mass losses of the butanoic (87.0446 Da), 4-aminobutanoic (102.0555 Da) and carbamoylbutanoic (130.0504 Da) substructures that were apparent in numerous ion fragments (Fig. 4(e)). Cleavage of the intervening crosslink was demonstrated by ion masses corresponding to fragments of the spacer group (Fig. 4(d)) arising from the indicated amide cleavages shown in Figure 4(b).

As discussed previously, the modification of these arginines, rather than other reactive groups on the kinase, suggests that they are proximal to sites on the kinase known to bind small hydrophobic reagents, including the crosslinkers PDM and MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester).^{20,34} Since the binding of all these maleimide-containing reagents, as well as GMBS, by the PhK complex leads to β - γ crosslinking only at alkaline pH values,^{20,34} our results suggest that the binding of both GMBS and N β 1 by the kinase effectively concentrates both the active maleimido reagent and N β 1 in an orientation that facilitates modification of both arginines 18 and 20 at this pH value. Inhibition of β - γ crosslinking in the PhK_A•N β 1 complex by N β 1 and its crosslinking to the same residue of γ as its counterpart region on the β subunit together indicate that N β 1 competes for the same site as β 1-22 in the complex, and in doing so, perturbs interactions between the β and γ subunits. It should be noted, however, that both the regulatory α and δ subunits bind to regions of the regulatory C-terminus of γ ,^{7,35} thus the β 1-22 region could perhaps interact directly with either or both of these subunits, resulting in an orientation that promotes the observed crosslinking between β and γ . Regardless, the phosphorylation and crosslinking data demonstrate that the active site and regulatory C-terminus of γ are proximal to β 1-22 in the PhK complex.

Self-association of PhK β subunits mediated by two domains comprising the N-terminal 31 and C-terminal 177 amino acid residues

Given that the N-terminal region of β is proximal to the γ subunit, that phosphorylation of this region of β leads to the activation of γ ,^{13,14} that proximal epitopes on the β and γ subunits are structurally coupled to each other and with PhK activation,¹⁹ and that activation of PhK promotes formation of β - β dimers by the crosslinker DFDNB,^{22,36} we asked whether the N-terminal region of β might also affect the ability of β subunits to self-associate. This question was first approached by determining whether either N β peptide perturbs crosslinking of the β subunits by DFDNB in the PhK_A complex (40-fold molar excess of crosslinker to PhK_A), and that was indeed found to be the case. Inclusion of N β 1 (200 μ M), but not N β 2, under linear conditions of crosslinking promoted an increase in the crosslinking of β ($17 \pm 6\%$) and the formation of a new β trimer, although in an amount less than the typical β - β dimers formed²² (data not shown). Although these results suggest a role for the β 1-22 region in mediating the interactions between β subunits in the PhK complex, they are of limited use because the specific regions of β that are crosslinked by DFDNB remain unknown. Consequently, we employed two-hybrid screening to more fully analyze the roles of specific regions of β in its homodimeric interactions.

To determine the regions of the β subunits that mediate (either directly or indirectly) their interaction with each other, we screened a series of N- and C-terminal truncation mutants of β against one another in the yeast two-hybrid system. The truncation mutants were engineered to avoid disrupting functional domains, including known covalent modification sites, as well

as potential protein-protein interaction sites on that subunit (Fig. 2). Incremental C-terminal truncations (averaging 146 residues) were made within predicted loop regions, with the intent of preserving predicted secondary structural elements (amphipathic α -helical and high propensity coiled-coil forming stretches) commonly observed at protein interfaces. Using the same principles, mutants with a 31 amino acid N-terminal deletion were also constructed to test for β - β interactions potentially mediated by the β 1-31 region, which contains the phosphorylatable serines 11 and 26 (the latter being the principle regulatory phosphorylation site of PhK¹³) and a variable splice site that is differentially encoded by exon 2 in different tissues¹⁵ (Fig. 2). The expression of all constructs tested was verified by Western blotting to eliminate false negatives arising from the loss of one or both partners of a potentially interacting pair.

When all binary combinations of β constructs were assayed against one another, no positive interactions were observed for either full length β (β FL) or any C-terminal truncations assayed against any constructs having an intact N-terminus (Table 1); however, a high level of β -galactosidase activity (117 units) was induced by the homologous interaction between the two β mutants missing only their N-terminal 31 residues (β 32N). Weaker interactions were observed between heterologous combinations of β 32N and β FL constructs, respectively expressed either as AD and BD (60.08 units) or BD and AD (8.35 units) fusions, with the reciprocal combination of yeast domains resulting in a 7-fold difference in activity. Such domain effects are often observed in two-hybrid screens involving many proteins, including the α and γ subunits of PhK⁷ and the transcriptional regulators Myc and Max.³⁷ Some weak interactions were also observed between β 32N and several C-terminal truncation mutants, most notably β 520C, in one BD/AD orientation; however, no activity was observed for the same mutants in the opposite orientation. That the β FL constructs do not interact with each other, but do in both orientations with the β 32N constructs, considered together with the fact that the highest reading measured is for the homologous interaction between the β 32N constructs, suggests that the β 1-31 region inhibits self-association of the β subunits.

To determine the region(s) responsible for β - β association upon the deletion of β 1-31, a series of double truncation mutants combining the N-terminal deletion (β 32N) with each of the C-terminal truncations shown in Fig. 2 was engineered and tested in all possible binary combinations. In short, all C-terminal truncations resulted in the loss of binding activity (data not shown), suggesting that a region or regions within the C-terminal 177 residues of β (i.e., the shortest dual truncation, namely β 32N,916C) are necessary for self-association. The sum of these two-hybrid results using truncation mutants to evaluate β - β interactions suggests then a positive effect of the C-terminal 177 residues (β 917-1093 region) and a negative effect of the β 1-31 region. Consistent with such a role for the N-terminal region of β , which includes the phosphorylatable residues Ser-11 and Ser-26, in influencing β - β interactions, is the fact that the chemical crosslinker DFDNB forms β - β dimers with the phosphorylated, activated form of PhK, but not with nonactivated forms of the enzyme complex.²²

To evaluate the potential influence of phosphorylation of Ser-11 and Ser-26 on the β - β interaction, point mutations of β FL were constructed in which one or both of the two serine residues were converted to Glu to mimic phosphorylation of the hydroxyl group or to Ala as an isosteric control lacking the hydroxyl group. All possible binary combinations of the single and double point mutants were screened to determine the effect of these serine mutations on the self-association of β . Unlike the negative result when two wt β FL constructs were tested against each other, when a single wt β FL was screened against full-length β constructs having either one or two mutations, interactions occurred, with the number of positives observed with two mutations being greater than with just one (Table 2 and Supplement Table). Similarly, when full-length constructs having mutations in both β chains were screened, there was a progressive increase in the percentage of positive interactions as the total number of serines

that were mutated in the binary combinations increased from 2 to 3 to 4 (Table 2 and Supplement Table; summarized in Table 3). The extent of galactosidase activity induced when the two mutated β constructs were assayed against one another also progressively increased with the total number of serines mutated in the interacting pairs (Table 3), indicating an effect of both serine mutations on β self-association. It is noteworthy that mutation of the N-terminal serine residues to either Glu or Ala allowed β self-association, which was not observed with wt β FL subunits, suggesting a general inhibitory effect of the two serine hydroxyl groups on β - β interaction. Mutation of both serine residues at the N-terminus of β had an effect similar to the truncation of the N-terminus of β in promoting dimerization of the β mutants, as exhibited by the percentage of positive interactions observed in all possible combinations of the constructs containing either type of mutation (75% and 80%, respectively; Table 3). The extent of galactosidase activity induced by truncation of the N-terminal regulatory region of β exceeded that promoted by mutation of its serine residues, which would not be surprising if the wt nonphosphorylated serine residues inhibit dimerization. It should be noted though that assessment of the influence of these serines, or their mutants, on β - β interactions as measured in two-hybrid assays may not fully correspond to their role when they are part of the entire PhK complex, in that the remaining α , γ and δ subunits have all been shown to influence the structure of the β subunits.^{19,34,38,39} Regardless, the overall results from two-hybrid screens of truncation or mutation constructs of the N-terminus of β indicate that this region of the subunit inhibits its self-association and suggest that perturbations in the structure of this region attenuate that inhibition. That N β 1, but not N β 2, inhibits PhK_A and perturbs crosslinking of β - γ and β - β dimers by GMBS and DFDNB, respectively, suggest that the inhibitory effect may be predominantly transmitted through the β 1-22 region.

DISCUSSION

Wilkinson et al.¹⁹ first demonstrated that coupled conformational changes occur in the β and γ subunits in response to the activation of PhK by a variety of mechanisms. In that study it was shown that phosphorylation of PhK simultaneously increased the accessibility of epitopes on β and γ (residues 277-290) for subunit-specific mAbs against those subunits and that the observed structural changes in the two subunits were structurally coupled to each other and with enzyme activation through some unknown structural component(s) of the PhK complex. Further, the flow of structural information between the subunits through this component was shown to be reciprocal (i.e., activators that targeted either β (ADP, phosphorylation) or γ (Mg^{2+}) increased the solvent accessibility of the epitopes on both subunits) and monovalent fragments of the mAbs against either region stimulated the activity of nonactivated PhK. Our current results demonstrating that GMBS selectively crosslinks the β and γ subunits in the PhK_A complex and that a peptide corresponding to the N-terminal 22 residues of β (N β 1) inhibits that crosslinking while at the same time being crosslinked to the same residue on γ (K303) via the ¹⁸RAR motif used by its counterpart β subunit provide a plausible mechanism for the structural coupling of the two epitopes¹⁹ on the β and γ subunits and suggest that the coupling may be through a direct mechanism.

It has been proposed that the β subunit exerts quaternary constraint on the activity of the catalytic γ subunit in the nonactivated PhK complex and that attenuation of the constraining interaction, which results in the activation of γ , occurs concomitantly with a characteristic conformational change(s) induced in β through phosphorylation or occupancy of its allosteric activation sites.^{22,36} This conformational change in β has been detected by a variety of techniques,^{19,21,22} including chemical crosslinking by DFDNB to form β - β dimers within activated PhK.^{22,36} In addition to evaluating the potential relationship between the conformational change in β induced by activators and the apparent self-association of the β subunits in the PhK_A complex detected by DFDNB crosslinking, a corollary goal with respect to the latter was to determine those regions of β involved in its self-association. Two such

regions of the β subunit, β 1-31 and β 977-1032, were identified by two-hybrid screening of deletion and point mutants of β and were shown, respectively, to negatively and positively affect self-interaction of the β subunits. Attenuation of the inhibitory effect of the β 1-31 region on β - β interactions by Ser to Glu mutations to mimic the phosphorylation of serines 11 and 22 suggests a possible mechanism for the observed structural changes induced in the β subunits by their phosphorylation in the PhK complex.

Distinct from the well characterized regulatory phosphorylation site on β (Ser-26),¹³ little is known about the effect of Ser-11 and Ser-700 phosphorylation. A novel aspect of our findings is that Ser-11 also appears to be a regulatory site, as indicated by the increased effect of double Ser-11 and -26 point mutations over that of corresponding single point mutations on promoting β 's self-association in the two-hybrid assay. The increased effect observed with the double serine mutations is consistent with previous work demonstrating that the extent formation of crosslinked β - β dimers by DFDNB in the PhK complex parallels the extent of phosphorylation of β .²² Inasmuch as the β 1-31 region has such a dominant role in mediating β - β interactions, as judged by the negative and positive two-hybrid interactions observed respectively between the wtFL β and β 32N constructs, it is reasonable to hypothesize that phosphorylation of either serine 11 and/or 26 disrupts a constraining interaction formed by contacts between the β 1-31 region and other region(s) of β that inhibits the self-interaction of these subunits. This hypothesis is supported by the fact that the N β 1 peptide perturbs DFDNB crosslinking of the β subunits and that it does so by binding to the same region in the PhK_A complex as does its counterpart region of β .

Because of the binding interactions observed between β constructs in the two-hybrid assay and those between PhK_A and N β 1 described above, we analyzed the sequence of β ¹² for predicted secondary structure motifs that might promote protein-protein interactions. One such motif is a coiled coil domain, which is characterized by a heptad repeat with the first and fourth positions being occupied by hydrophobic residues in amphipathic helical structures.⁴⁰ Several helical stretches with such repeats are observed in different regions of β , including one just C-terminal to the β 1-32 region, namely β 42-62, and two located in the β 917-1093 region (residues 969-1009 and 1032-1047). The β 1032-1047 stretch is predicted to have a coiled coil forming propensity of 0.8, which approaches the theoretical limit of 1 defined in the Coil⁴¹ and Paircoil programs.⁴² In addition to coiled-coil motifs, secondary structure predictions for the region of β spanning residues 1-22 suggest the presence of a basic amphiphilic helix (baa motif),³² a secondary structural element commonly observed in protein-protein interactions, including CaM targeting. In fact, a synthetic peptide corresponding to residues 5-28 of β has been shown to bind CaM with nM affinity³² and a CNBr-fragment of β containing this region was isolated from the digest of a conjugate formed by crosslinking CaM to the isolated β subunit.⁴³ The effects of synthetic peptides on the activity of PhK_A and on the crosslinking of its subunits suggest that the N-terminus of β is a key contact site, based on the fact that only those peptides in which this motif remains intact (N β 1, N β 2 and β 5-28³²) inhibited PhK_A and that positive and negative effects on β - γ and β - β dimer formation were observed respectively for N β 1 and N β 2. Considering that the structural motifs listed above either overlap or are in proximity to the β 1-22 and β 917-1093 regions that mediate the self-association of β in the two-hybrid screens, we constructed a model (Fig. 5) that incorporates the activation of PhK by phosphorylation of β and the β - β and β - γ interactions described herein. Our combined results from two-hybrid screening, the effects of peptides, and chemical crosslinking support a mechanism for β self-association in which the β 917-1093 region is required for the interaction between these subunits and in which inhibition of this binding interaction by the β 1-22 region is attenuated by phosphorylation of serines 11 and 26. Although the β 1-22 region may contact γ and possibly other subunits in the PhK complex, the negative and positive interactions observed respectively between wt β FL and β 32N truncation mutants in the two-hybrid assay suggest that inhibition of the β - β interaction by the non-phosphorylated N-terminus of β

requires no subunits other than β . If this is the case, the β 1-22 region may directly impose steric constraint on the β - β interaction, or alternatively, it could interact with other regions of β to indirectly promote a conformation incapable of self-association. In the former case, phosphorylation of serines 11 and 26 could simply perturb the conformation of the β 1-22 region and thereby weaken the steric constraint on the β - β interaction, and in the latter case, a structural change in the N-terminus could disrupt intramolecular interactions between the inhibitory β 1-22 region and a more C-terminal region of β , resulting in exposure of the β 917-1093 binding region. Our model depicted in Figure 5 is based on the second, indirect scenario because of the behavior of the N β 1 peptide in binding to the same region of the PhK_A complex as the endogenous β subunit, and in doing so, perturbing β - β interactions (as judged by crosslinking with DFDNB).

The PhK complex consists of two bridged ($\alpha\beta\gamma\delta$)₂ octameric lobes, in which $\alpha\beta\gamma\delta$ tetramers pack head-to-head⁴⁴ with an overall D₂ symmetry.^{39,45} Considering that the connecting interlobal bridges are thought to be primarily, if not wholly, composed of β subunits,⁴⁶ the observed crosslinked β - β dimers formed in phosphorylated PhK could arise from either intralobal or interlobal interactions between these subunits. In our model (Fig 5), the non-phosphorylated β subunits are prevented from associating by a constraining hairpin arrangement between the inhibitory β 1-22 region and the postulated amphiphilic α -helical stretch of residues located C-terminal to it, namely β 42-62. The phosphorylation of serines 11 and 26 in the inhibitory region is hypothesized to disrupt the constraining interaction, resulting in the formation of two identical intermolecular contacts between the β 42-62 and β 917-1093 regions of opposing β subunits. The juxtaposition of the subunits in the dimer pairs depicted in Fig. 5 reflects: (1) the known symmetry of the subunits in the PhK complex; (2) the adjacent positions of the β and γ subunits observed in immunoelectron microscopy;¹⁹ and (3) the observed increased effect of serine mutations on β - β interactions measured herein in the two-hybrid screens, with the number of possible contacts between interacting β constructs being dependent upon the extent mutation (or phosphorylation) within the β 1-31 region in either subunit. The proposed contact between β 1-22 and the C-terminus of γ is supported by previous results demonstrating a structural linkage between the β and γ subunits by immunochemical¹⁹ and chemical crosslinking methods,²⁰ by the fact that activation of γ parallels phosphorylation of β in the PhK complex,¹³ and by our current results showing that both β and N β 1 are crosslinked by GMBS to the same region of γ in the N β 1•PhK complex. The self-association of β subunits is consistent with a large body of evidence that these subunits undergo a common conformational change that is associated with crosslinking of β - β dimers in activated, including phosphorylated, forms of the kinase complex.^{19,21,22,36}

It has been noted that the skeletal muscle PhK complex and the actin•troponin (Tn) complex system have evolved similar mechanisms through which their functions are regulated by Ca²⁺ ions.⁴⁷ The heterotrimeric Tn complex is composed of troponin T (TnT), troponin I (TnI) and troponin C (TnC). Not only are TnC and CaM both Ca²⁺-binding homologs,⁴⁸ but TnI and γ share structural and functional similarities as well.^{5,47} Regarding the latter pair, we have demonstrated that the isolated γ subunit of PhK binds CaM and TnC with equal affinity and that a discrete region of the γ CRD (residues 294-309) bears remarkable sequence similarity to the inhibitory region of TnI encoded by exon VII,⁴⁷ a region that has been shown to bind TnC.⁴⁹ Based on our current observation that K303 in the TnI-like region of γ (³⁰²GKFK) is crosslinked to the inhibitory 1-22 terminus of the β subunit in the PhK complex and that in the Tn complex TnI is known to bind TnT in addition to TnC, we screened the sequence of β against those of TnT and TnI for the best ‘non-intersecting’ alignments that might suggest additional structurally similar regions in the two complexes.⁵⁰ The best alignment scores, although not necessarily statistically relevant because of their short length, were observed between the very regions of β discussed herein and known interaction sites of TnI and TnT observed in recent X-ray crystallographic studies (Table 4).^{51,52} Moreover, the secondary

structure predicted for regions of β directly flanking the sequence similar stretches matched those observed in flanking regions in crystal structures of the corresponding Tn proteins.^{51, 52} The gene structure of the human PhK β subunit, which shares 95% amino acid sequence identity with the rabbit muscle β subunit,⁵³ comprises 33 exons, of which 3 are differentially spliced in brain and muscle to encode both alternative internal and N-terminal sequences.¹⁵ In each protein (β , TnI and TnT), the regions of sequence and predicted structural similarity are encoded by a single exon (Table 4). Incorporating these similarities into our model (Fig. 5), disruption of the inhibitory interaction between TnI-like β 1-22 and TnT-like β 42-62 by phosphorylation, leads to the binding interaction between TnT-like β 42-62 and TnI-like β 917-1093, or a flip-flop between TnI-like and TnT-like regions of β . Such a model of flip-flop binding has been previously proposed for the interactions of CaM within the PhK complex.⁵⁴

All the crystal structures of the γ subunit of PhK have been solved for the truncated catalytic core of the protein that was missing its entire C-terminal regulatory domain;^{4,55,56} thus, the positioning of that domain with respect to the rest of the subunit has remained unknown. The crosslinking reported herein of the β 1-22 region to the regulatory domain of γ automatically locates this regulatory region of γ as being proximal to its catalytic cleft, because during PhK's autophosphorylation serines 11 and 26 of β are phosphorylated by γ .¹² That this regulatory domain of γ also contains the binding sites for PhK's endogenous CaM subunit (δ)^{5,6} as well as its regulatory α subunit,⁷ strongly suggests that this regulatory region of the catalytic γ subunit is the focal point, the allosteric switch, for the multiple interactions through which PhK's regulatory α , β and δ subunits coordinately regulate the activity of its catalytic γ subunit.

Materials and Methods

Yeast and bacterial strains

Saccharomyces cerevisiae strain EGY48 (MAT α , his3, trp1, ura3, LexAop-LEU2) (CLONTECH) was used for all two-hybrid analyses.⁵⁷ All plasmid manipulations were performed according to standard protocols for the *Escherichia coli* strain DH5 α .

Proteins and peptides

PhK was purified from the psoas muscle of New Zealand White rabbits,⁵⁸ dialyzed against 50 mM Hepes (pH 6.8), 0.2 mM EDTA and 10% sucrose, and stored at -80 °C. GP was purified as described,⁵⁹ and residual AMP was removed with activated charcoal. The concentrations of PhK and GP were determined spectrophotometrically using their respective absorbance indices.^{60,61} Autophosphorylated PhK_A was prepared from 3 different PhK preparations and the amount of phosphate incorporated into its α and β subunits was determined using ³²P-labeled ATP by the method of King et al.¹¹ The autophosphorylation reactions were terminated so that the final amount of phosphate incorporated into the different PhK_A samples was relatively constant at 0.3 mol P/mol α and 1.0 mol P per mol β . The mAbs against the α , β and γ subunits of PhK were previously described,^{19,44} and the anti-CaM mAb was from Zymed. All other secondary conjugates were from Southern Biotechnology. The N β 1, N β 1_{S/A}, N β 1_{Phos} and N β 2 peptides were purchased from Biopeptide (San Diego, CA), and their purity and composition were verified by both MS and MS/MS analyses using a MALDI 4700 mass spectrometer in the Mass Spectrometry Facility of the University of Kansas Medical Center.

Two-hybrid plasmid construction of fusions containing the PhK β subunit and its deletion mutants

All constructs of full-length PhK β and its N-terminal and C-terminal deletions were generated by PCR, followed by subcloning into yeast two-hybrid vectors of pLexA and pB42AD (CLONTECH). PCR was performed using a PCR SuperMix High Fidelity thermocycler

(GibcoBRL). Rabbit skeletal muscle β cDNA was kindly provided by Dr. Manfred Killiman (Uppsala University, Uppsala, Sweden) and used as the template for the preparation of all β constructs. The cDNA fragments were ligated to either pLexA (a 2- μ *HIS3* plasmid) or to pB42AD (a 2- μ *TRP1* plasmid), both from CLONTECH, to generate fusion proteins consisting of PhK β mutants and the DNA binding domain (BD: amino acids 1-202) of LexA or the B42 activation domain (AD), respectively. Constructs of β FL, β 215C, β 350C, β 520C, β 703C, β 815C and β 916C were generated using primers to yield cDNAs flanked 5' by *NcoI* or 5'-*XhoI* and 3' by *XhoI* restriction sites as indicated: sense-strand

NcoI 5'-CGGCCGCCATGGATGGCGGGGGCGAC-3',
 sense-strand *XhoI* 5'-CGGCCGCTCGAGATGGCGGGGGCGAC-3';
 antisense-strand, (β FL) 5'-CGCCTCTCGAGCTAGCTAACCAGACA-3',
 (β 215C) 5'-ACCAAACCTCGAGCACACGGTAAACTC-3',
 (β 350C) 5'-TTCAGCCTCGAGGTAGTAGCGTCTTT-3',
 (β 520C) 5'-TTCTACCTCGAGAGGCGTTTGAGTTT-3',
 (β 703C) 5'-TTCAGGCTCGAGGGAGGTGCTGCTTT-3',
 (β 815C) 5'-TTCTTCCTCGAGAAAGGCACCCAGCG-3',
 (β 916C) 5'-ATTTTGCTCGAGCTGGAGAATATCCA-3'.

PCR products encoding the full length β subunit (β FL) and the C-terminal serial deletions were generated using sense strand primers flanked 5' by either the *NcoI* or *XhoI* restriction sites for subcloning into the pLexA vector or the pB42AD vector, respectively, and 3' by the *XhoI* restriction site for the corresponding antisense strand primers described above. PCR products encoding truncation mutants missing the first 31 amino acids of the β subunit (β 32N) and double truncation mutants missing both the N-terminal 31 residue region and progressively increasing C-terminal regions (β 32N, $\beta\Delta$ N-215C, $\beta\Delta$ N-350C, $\beta\Delta$ N-520C, $\beta\Delta$ N-703C, $\beta\Delta$ N-815C and $\beta\Delta$ N-916C) were generated using the sense *XhoI* site for the pB42AD vector with the corresponding restriction enzymes (*NcoI/XhoI* for pLexA constructs and *XhoI* for pB42AD constructs), followed by ligation to the *NcoI/XhoI* sites of pLexA and the *XhoI* site of pB42AD:

sense-strand *NcoI* (β 32N) 5'GTTTATCCATGGCTTAAAAGCATTA-3',
 sense-strand *XhoI* (β 32N) 5'GTTTATCTCGAGCTTAAAAGCATTA-3';
 antisense-strand *XhoI* (β 32N) 5'CGCCTCTCGAGCTAGCTAACCAGACA-3',
 ($\beta\Delta$ N-215C) 5'ACCAAACCTCGAGCACACGGTAAACTC-3',
 ($\beta\Delta$ N-350C) 5'TTCAGCCTCGAGGTAGTAGCGTCTTT-3',
 ($\beta\Delta$ N-520C) 5'TTCTACCTCGAGAGGCGTTTGAGTTT-3',
 ($\beta\Delta$ N-703C) 5'TTCAGGCTCGAGGGAGGTGCTGCTTT-3',
 ($\beta\Delta$ N-815C) 5'TTCTTCCTCGAGAAAGGCACCCAGCG-3',
 ($\beta\Delta$ N-916C) 5'ATTTTGCTCGAGCTGGAGAATATCCA-3'.

The pLexA and pB42AD vectors were linearized respectively using either *NcoI* (5') and *XhoI* (3') or *XhoI*, followed by dephosphorylation with shrimp alkaline phosphatase (GibcoBRL). After dephosphorylation and linearization, both vectors were purified using a QIAquick PCR Purification spin column (Qiagen). The orientations and sequences of all LexA and B42 β constructs were verified by restriction mapping, dideoxy sequencing, and Western blot analysis.

Two-hybrid plasmid construction for fusions containing PhK β point mutants

Using a Transformer Site-Directed Mutagenesis Kit (CLONTECH), two point mutations were induced into PhK β at positions in its primary sequence corresponding to serines 11 and 26. The serines at both positions were mutated to either alanine (A) or glutamate (E) on the pB42AD β FL as a parental plasmid. The designed mutagenic primers were as follows:

(β S12A) 5'GGCCGAAGTGGCCTGGAAGGTC-3',

(β S12E) 5'GGCCGAAGTGGAGTGGAAGGTC-3',

(β S26A) 5'GCGCTCAGGCGCAGTTTATGAAC-3',

(β S26E) 5'GCGCTCAGGCGAGTTTATGAAC-3'.

The selection primer was designed to replace the unique restriction enzyme *NotI* site in the pB42AD β FL with 5'GTCGACTAGCAGCCGCTTCGAC-3'. After verifying this sequence in each of the β constructs, the β mutants were subcloned into pLexA at the *XhoI* site and were subsequently checked again for content by both restriction mapping and sequencing.

Yeast two-hybrid transformation

To screen for β - β interactions, all constructs of the β subunit, including full-length, truncation and point mutants, were fused in both LexA and B42 orientations and assayed for interactions in all possible binary combinations. Yeast strain EGY48, possessing the pSH18-34 lacZ reporter plasmid, was transformed by a modified lithium acetate procedure as previously described,⁶² and transformants were grown for 3 days at 30 °C on a synthetic medium lacking histidine, tryptophan, and uracil (SD_His_Trp_Ura). Protein expression of all β constructs was verified by Western analyses, essentially as described,⁶³ using either a DNA BD cross-reactive LexA polyclonal Ab (kindly provided by Dr. Erica Golemis, Fox Chase Cancer Center) or an AD cross-reactive hemagglutinin mAb (Roche Molecular Biochemicals). Positive associations between β constructs were monitored by transcriptional activation of either the LEU2 or lacZ reporter genes by growth on defined media lacking leucine (Leu-) and by the activity of β -galactosidase measured in assays with *o*-nitrophenyl galactopyranoside as substrate, respectively.^{64,65} All negative and positive interactions were quantified using values determined from the β -galactosidase liquid assay.

Yeast β -galactosidase liquid assay

The relative affinities of the β - β protein interactions were assessed using a microplate liquid assay, with only minor modifications of the method described by Serebriiskii et al.⁶⁶ Prior to performing the assays, transformed yeast were grown overnight at 30 °C in 5ml of growth medium (SD/-His/-Trp/-Ura + 40% glucose) until reaching saturation. Sterile 96-well flat bottom plates were then prepared for inducing growth by adding 75 μ L of inducing media (SD/-His/-Trp/-Ura/-Leu + 10X BU salts + 40% Raffinose + 40% Galactose) to each well. Using sterile techniques, 5 μ L aliquots of a yeast suspension from a single overnight growth culture were added to one well column on the microplate for a total of eight replicates; a maximum of ten different sample cultures was added per plate, leaving columns 11 and 12 for replicates of a negative control (empty vectors) and a positive control. The latter consisted of fusions of PhK α and γ in their respective LexA AD and pB42 AD orientations, which have been previously shown to induce high levels of β -galactosidase activity in two-hybrid screens of these subunits.⁷ The microplate was then placed in a sterile humidity chamber (Nalgene container lined with moist paper towels) and incubated for 3-3.5 hr at 30 °C. After the incubation period, the density of the yeast culture was determined by measuring absorbance at 600 nm using a microplate reader with autoshake capabilities. The wavelength was then adjusted to 420 nm and 75 μ L aliquots of an ONPG solution [component final concentrations: 120 mM Na₂HPO₄, 80 mM NaHPO₄, 20 mM KCl, 4mM MgSO₄, 8 mg/ml ONPG and 2X

diluted Y-PER Yeast extraction reagent (Pierce)] was added to the wells, following which, an initial absorbance measurement was recorded. After incubating for 60 min, a final absorbance measurement was made, and activity was calculated using the following equation: Activity = $1000 * [(A_{420t60} - A_{420t0}) / (A_{600} * \Delta t A_{420})]$. The average positive control values determined for each plate were used to normalize all sample activities. Interactions were considered to be positive only for those binary combinations having activities (A_{Exp}) that exceeded the sum of the value for the negative control expressing the maximal extent activity (A_{Cmax}) plus the combined standard errors for the experimental and control measurements, i.e. $A_{Exp} - (A_{Cmax} + SE A_{Exp} + SE A_{Cmax}) > 0$.

Enzymatic assays

Using the filter paper assay of Roskoski,⁶⁷ the activity of PhK was determined at 30 °C at either pH 6.8 or pH 8.2 by phosphate incorporation into GP or a synthetic peptide (S peptide) corresponding to residues 5-18 of its convertible region. Reactions were initiated with PhK and final concentrations in the assay mixture were: PhK [0.7 µg/ml (pH 6.8) or 0.07 µg/ml (pH 8.2)], PhK_A [0.07 µg/ml (pH 6.8)], buffer (50 mM Tris/50 mM β-glycerophosphate, pH 6.8 or 8.2), GP (6.0 mg/ml) or S peptide (50 µM), EGTA (0.1 mM), CaCl₂ (0.2 mM), β-mercaptoethanol (13 mM), [γ -³²P]ATP (Perkin Elmer/NEN; 1.0 mM, 0.17 Ci/mol), Mg (CH₃CO₂)₂ (10 mM) and sucrose (2-3%). When synthetic peptides corresponding to N-terminal regions of the β subunit (Nβ1, Nβ1_{S/A}, Nβ1_{Phos} and Nβ2) were also included in the assays, their concentrations were 200 µM. All assays were performed in triplicate three times using three different preparations of PhK and its corresponding PhK_A.

Chemical crosslinking and preparation of crosslinked peptides

PhK_A was crosslinked with DFDNB as described.²² Crosslinking of PhK_A in the presence of Nβ1 (200 µM) was initiated by adding DFDNB and carried out at 30 °C for 5 min at pH 7.0 in 50 mM Hepes, 0.2 mM EDTA. Final concentrations of PhK [αβγδ protomer] and DFDNB in the reaction were 0.47 and 5.0 µM, respectively. The reaction was terminated by adding an equal volume of SDS buffer [0.125 M Tris (pH 6.8), 20% glycerol, 5% β-mercaptoethanol, 4% SDS], followed by brief vortexing. The PhK subunits were separated on 6-16% linear gradient polyacrylamide gels and stained with Coomassie Blue. Densitometry of gels was carried out using a Fluorochem 8900 analyzer (Alpha Innotech). Western blotting of the proteins was performed on PVDF membranes with subunit-specific mAbs as previously described.³⁵ Crosslinking of PhK_A with GMBS was carried out by the methods described above for the crosslinking of PhK_A with PDM and was determined to be intramolecular as previously described.²⁰ PhK_A was crosslinked with GMBS in the presence and absence of Nβ1 for 2 min at 30 °C at pH 8.2. Final concentrations in the reaction were: PhK_A (0.47 µM αβγδ protomer), GMBS (4.7 µM), Hepes (50 mM, pH 8.2), EDTA (0.2 mM) and Nβ1 (200 µM). The reaction was initiated by adding GMBS and terminated using SDS buffer as described above for DFDNB. To determine the composition of the γ-Nβ1 conjugate, a reaction mix with PhK_A crosslinked in the presence of Nβ1 was subjected to gel electrophoresis, followed by a 5-min equilibration step in Caps buffer (10 mM Caps, pH 11, 20% methanol). The proteins were transferred to PVDF membranes in the Caps buffer as described previously,³⁵ and stained with Ponceau S. The band corresponding to the γ-Nβ1 conjugate by apparent mass and crossreactivity with subunit-specific mAbs was excised from the blot and submitted to Harvard Microchem (William S. Lane, Director) for N-terminal sequencing and amino acid analysis. All crosslinking reactions were performed at least twice using different preparations of PhK_A.

To determine regions of crosslinking in both the γ-β and γ-Nβ1 conjugates, PhK_A was crosslinked in the presence of Nβ1 as described above, and the cross-linked complex was then resolved by preparative SDS-PAGE. Following protein staining with Coomassie blue, the

bands corresponding to each conjugate were excised from the gel, sectioned and exchanged with three aliquots of 50 mM ammonium bicarbonate, 50% acetonitrile to remove SDS. The proteins were then reduced in 10 mM dithiothreitol for one hour at 55 °C, and subsequently carboxymethylated with 50 mM iodoacetic acid for one hr in the dark. The gel pieces were washed as described above with 50 mM ammonium bicarbonate, followed by several exchanges with 50 mM ammonium bicarbonate, 50% acetonitrile. After removing the last wash, the gels were dried in a Speed Vac (Savant) and treated with trypsin (Promega; 12.5 ng/μL) for 24 hours at 30 °C. Peptides were extracted from the gel pieces with 50% acetonitrile, 5% formic acid.

Liquid chromatography, MS and MS/MS of crosslinked peptides

Protein digests were pressure-loaded on a 75 μm i.d. × 10 cm picofrit column (PF360-75-30-N, New Objective Woburn, MA), hand-packed with magic C-18 particles (200Å, 5μ; Michrom Bioresources, Auburn, CA). Reverse phase liquid chromatography was carried out using a Surveyor pump (ThermoFinnigan San Jose, CA). Following off-line desalting for 15 min at a flow rate of 0.250 μl/min with 0.1% formic acid, the effluent was directed in-line to the mass spectrometer. The column was washed for 2 min with 0.1% formic acid in water at a flow rate of 0.250 μl/min, and peptides were then eluted over a 90-min period using a gradient of 0-90 % acetonitrile: (Buffer A = 0.1% formic acid; Buffer B = acetonitrile, 0.1% formic acid). The source was operated at 1.1 kV. LC MS data were obtained in a hybrid linear ion trap FT-ICR MS (LTQ-FT, ThermoFinnigan, San Jose, CA) equipped with a 7 tesla magnet. The mass spectrometer was operated in the data-dependent acquisition mode to automatically switch between MS and MSMS acquisition modes. Survey MS spectra (m/z range of 400-2000) were acquired in the FT-ICR cell in profile mode and the 6 most intense ions in each FT scan were selected for MS/MS in the linear ion trap in centroid mode using a 2 min exclusion list window.

For data analyses, dta files were generated by the TurboSequest Dta algorithm (included in the BioWorks 3.1 package software; ThermoFinnigan, San Jose, CA) using the following constraints: peptide mass range = 500-8000; threshold = 10; group scan = 2; and minimum ion count = 1. The parent masses were extracted from the resulting lcq_dta.txt file, and the correctness of charge assignment was confirmed by manual inspection of the isotope envelope on the corresponding FT-ICR survey scan. Masses corresponding to ions arising from trypsin or keratin contamination were eliminated using the Sequest browser (W.S. Lane, Harvard University). The resulting corrected mass list was then analyzed for potential crosslinked peptides using an 'in-house' search engine, which eliminates both non-modified peptides and known side products of crosslinking.²³ Mass assignments of ions corresponding to crosslinked peptides were verified by MS/MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Glossary

The abbreviations used are:

PhK, phosphorylase kinase

PhK_A, autophosphorylated PhK

Nβ1, peptide corresponding to residues 1-22 of the PhK β subunit

Nβ1_{S/A}, a SER11ALA analog of Nβ1

Nβ1_{Phos}, a phosphoserine 11 analog of Nβ1

Nβ2, peptide corresponding to residues 15-36 of the PhK β subunit

β1-22, region corresponding to the N-terminal 22 residues of the β subunit

β 1-31, region corresponding to the N-terminal 31 residues of the β subunit
 β 42-62, region corresponding to residues 42-62 of the β subunit
 β 917-1093, region corresponding to the C-terminal 177 residues of the β subunit
 β_{FL} , full-length β subunit (residues 1-1093)
 β 32N, β 215C, β 350C, β 520C, β 703C, β 815C, and β 916C: N- and C-terminal truncation mutants of β with first N-terminal and final C-terminal residues indicated
 CRD, C-terminal regulatory domain of the gamma subunit (residues 298-386)
 CaM, calmodulin
 BD, binding domain
 CBD, CaM-BD
 N-CBD, N-terminal CBD (residues 307-331)
 C-CBD, C-terminal CBD (residues 352-371)
 AD, activation domain
 pLexA, a 2- μ *HIS3* plasmid containing the DNA-BD of LexA
 pB42AD, a 2- μ *TRP1* plasmid containing a B42 activation domain
 mAb, monoclonal antibody
 GP, glycogen phosphorylase
 Tn, Troponin
 ONPG, *o*-nitrophenyl galactopyranoside
 MS, mass spectrometry
 MP, monoderivatization product
 DFDNB, 1,5-difluoro-2,4-dinitrobenzene
 GMBS, N-[γ -maleimidobutyryloxy]succinimide ester
 PDM, phenylenedimaleimide
 MBS, *m*-maleimidobenzoyl-N-hydroxysuccinimide ester
 wt, wild-type

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Figure 1.

GMBS crosslinking of the β and γ subunits in the PhK complex: a peptide corresponding to the N-terminal 22 residues of β (N β 1) inhibits $\beta\gamma$ crosslinking, forming an N β 1- γ conjugate. (a) PhK (lane 1) was crosslinked by GMBS in the absence (lane 2) and presence (lane 3) of N β 1 and resolved by SDS-PAGE. (b) Parallel samples were transferred to PVDF membranes and probed with mAbs against all the PhK subunits. All major high molecular weight conjugates crossreacted with anti- β and anti- γ mAbs, but not with anti- α and anti-CaM (δ) mAbs (data not shown). Crosslinking of the PhK complex by GMBS resulted primarily in the formation of a major $\beta\gamma$ cross-reactive conjugate. In the presence of N β 1, a new γ cross-reactive band was observed with an apparent mass of 47 kDa, corresponding by mass and cross-reactivity to an N β 1- γ conjugate, the reacting partners of which were subsequently verified by N-terminal sequencing.

Figure 2.

PhK β domain map, deletion and point mutants. (a) PhK/PKA phosphorylatable serines 11, 26 and 700 in the sequence of β are indicated by P below the subunit schematic. Besides its two phosphorylation sites, the N-terminal 32 residues of β (light grey, termed β32N, designated by **) comprise a variable splice domain (aa's 1-22, designated by pair of white arrowheads) and one of three putative CaM-binding domains (black patterns, aa's 6-28, 767-794 and 919-950). ¹² The dark grey pattern abutting β32N indicates a region with predicted amphiphilic helical secondary structure. A putative glucoamylase-like domain (aa's 69-462, pyramidal pattern) ⁶⁸ contains a region in β (white line, aa's 420-436, designated by *) that corresponds to a peptide known to inhibit PhK activity. ⁶⁹ An additional variable splice domain (aa's 778-809, designated by pair of grey arrowheads) that is differentially spliced in muscle and non-muscle tissues, ¹⁵ precedes a region (aa's 1032-1047, radial pattern) that is predicted to have high propensity for beta coil formation. ⁴¹ The β subunit also contains a C-terminal polyisoprenylation consensus sequence (hash mark pattern, aa's 1090-1093), with known S-farnesyl modification of Cys-1090. ⁷⁰ (b) The β subunit truncation and point mutants (shown in scale with the domain and covalent modification map of β under (a)) were engineered as either transcriptional DNA BD pLexA or AD pB42 fusion proteins as described under Materials and methods. The name of the construct is given, followed by two numbers separated by a dash, indicating in order from left to right, the N-terminal and C-terminal residues of the β subunit that are expressed in the fusion protein. The β32N region is indicated in grey, and

both phosphorylatable serines (11 and 26) and corresponding amino acid substitutions within this domain are indicated in grey shaded boxes in the sequences shown for both full-length native (β FL) and double point mutant constructs, respectively. Four full-length β single serine point mutants were also engineered using the same amino acid substitution patterns shown for the double point mutants, encompassing all possible permutations for a family of constructs containing one intact phosphorylatable serine (11 or 26), with either E or A substituted at the other serine position. (c) Synthetic peptides corresponding to regions flanking phosphorylatable serines 11 (N β 1) and 22 (N β 2) in the N-terminus of β were verified for mass and composition using FT MS.

Figure 3.

MS/MS analysis of the signal at m/z 1045.590 identifying a conjugate comprising residues 302-305 and 18-20 of the γ and β subunits, respectively. (a) Fragmentation pattern of the crosslinked peptide. Small letters denote ions arising from amide bond cleavages of the peptide backbone. (b) Structure of the GMBS crosslink between K303 and R18. The capital letters indicate ions resulting from the observed amide cleavages of the reagent. (c) Fragmentation patterns of ions resulting from cleavage ($\sim$) of the intervening crosslink between K303 and R18. (d) Composition of ions unequivocally identifying the crosslinked peptide. Covalent links formed by the intervening crosslink between the peptides are indicated by the middle dot ($\cdot$). Loss of ammonia (*) or water (0) are denoted as superscripts following the appropriate ion fragment. The β peptide and/or its fragments are listed first, followed by the γ subunit and its fragments. Internal fragments of each peptide are denoted by amino acid codes. Fragments of the crosslink are indicated as above under (a) and (b).

Figure 4.

MS/MS analysis of the signal at m/z 1475.797 identifying a conjugate comprising residues 302-305 and 18-22 of the γ subunit and N β 1 peptide, respectively. (a) Fragmentation pattern of the crosslinked peptide with two forms of GMBS incorporated. Small and capital (prime) letters respectively denote ions arising from amide bond cleavages of the peptide backbone and GMBS monosubstitution product (MP) of R18 shown in (c). (b) Structure of the GMBS crosslink between K303 and R20. The capital letters indicate ions resulting from the observed amide cleavages of the reagent. (c) Structure and fragmentation pattern of the MP of R18 arising from hydrolysis of the succinimide functional group of the crosslinker. (d) Fragmentation patterns of ions resulting from cleavage (~) of the intervening crosslink between K303 and R20. (e) Composition of ions unequivocally identifying the crosslinked peptide. Covalent links formed either by the MP or crosslink between the peptides are indicated by the middle dot (·). Loss of ammonia (*) or water (°) are denoted as superscripts following the appropriate ion fragment. When present, the MP and its fragments are listed first, followed in order by the N β 1 and γ peptides and/or their respective fragment ions. Internal fragments of each peptide are denoted by amino acid codes. Fragments of either the MP or crosslink are indicated as above under (b) and (c).

Figure 5.

Model of phosphorylation-dependent β - β interactions in the PhK complex. The scheme is a simple representation of the interactions between non-modified and fully phosphorylated β subunits within the context of the PhK enzyme complex. The N- and C-terminal residues of each subunit are indicated numerically at the ends of each corresponding line representation. Serines 11 and 26 and their phosphoserine analogs in the N-terminal variable splice region of β are indicated by -OH and -OPO₃, respectively. Zigzag line patterns indicate regions containing predicted amphipathic α -helical secondary structures, including a region predicted to have high propensity β coil-forming potential at the C-termini of the β subunits.⁴¹ These regions also have sequence similarities with TnI (blue) or TnT (red), with these colors corresponding to those used in Table 5. The colored hash marks represent β - β intramolecular (green), β - β intermolecular (orange) and β - γ (purple) protein-protein interactions.

Table 1

Domain Mapping of β - β Interactions

BD	AD	β FL	β 32N	β 215C	Vector encoding pB42			β 815C	β 916C	Empty vector
					β 350C	β 520C	β 703C			
Vector encoding pLexA	β FL	2.93 $\pm$ 0.05	60.08 $\pm$ 0.14	2.50 $\pm$ 0.03	2.09 $\pm$ 0.06	2.32 $\pm$ 0.05	2.29 $\pm$ 0.03	1.77 $\pm$ 0.05	2.55 $\pm$ 0.04	2.06 $\pm$ 0.07
	β 32N	8.35 $\pm$ 0.03	117.64 $\pm$ 0.21	3.10 $\pm$ 0.07	2.55 $\pm$ 0.04	3.12 $\pm$ 0.13	3.63 $\pm$ 0.02	2.10 $\pm$ 0.04	2.50 $\pm$ 0.05	3.16 $\pm$ 0.07
	β 215C	1.41 $\pm$ 0.05	4.56 $\pm$ 0.24	1.86 $\pm$ 0.07	1.24 $\pm$ 0.03	1.54 $\pm$ 0.09	1.68 $\pm$ 0.02	1.66 $\pm$ 0.05	0.80 $\pm$ 0.03	0.02 $\pm$ 0.06
	β 350C	1.28 $\pm$ 0.04	3.90 $\pm$ 0.28	2.49 $\pm$ 0.16	0.89 $\pm$ 0.03	1.78 $\pm$ 0.15	1.58 $\pm$ 0.06	3.00 $\pm$ 0.04	1.02 $\pm$ 0.04	0.10 $\pm$ 0.05
	β 520C	3.20 $\pm$ 0.03	8.97 $\pm$ 0.14	2.84 $\pm$ 0.06	3.32 $\pm$ 0.04	2.99 $\pm$ 0.09	2.47 $\pm$ 0.04	2.76 $\pm$ 0.13	1.06 $\pm$ 0.38	0.46 $\pm$ 0.24
	β 703C	3.09 $\pm$ 0.27	2.21 $\pm$ 0.56	2.18 $\pm$ 0.11	2.43 $\pm$ 0.19	1.07 $\pm$ 1.23	1.97 $\pm$ 0.17	1.05 $\pm$ 0.04	2.95 $\pm$ 0.23	1.61 $\pm$ 0.30
	β 815C	5.77 $\pm$ 0.41	4.29 $\pm$ 0.04	1.31 $\pm$ 0.02	0.52 $\pm$ 0.06	1.05 $\pm$ 0.03	1.40 $\pm$ 0.05	1.27 $\pm$ 0.03	1.72 $\pm$ 0.05	0.34 $\pm$ 0.04
	β 916C	0.73 $\pm$ 0.06	2.77 $\pm$ 0.06	0.95 $\pm$ 0.04	1.43 $\pm$ 0.10	1.07 $\pm$ 0.02	1.23 $\pm$ 0.04	1.12 $\pm$ 0.03	1.24 $\pm$ 0.04	0.13 $\pm$ 0.05
	Empty vector	0.87 $\pm$ 0.02	0.88 $\pm$ 0.03	0.74 $\pm$ 0.04	1.06 $\pm$ 0.04	0.56 $\pm$ 0.03	1.00 $\pm$ 0.06	1.13 $\pm$ 0.04	0.81 $\pm$ 0.03	1.22 $\pm$ 0.05

β -Galactosidase activity from yeast lysates was determined using the substrate β -D-galactopyranoside as described under Experimental Procedures. Activity is expressed in units according to the formula: 1000*[A420₁ - A420₂]/ (A600* Δ A420). Data represent the mean $\pm$ S.E. of eight assays. A positive interaction is determined as being significantly greater than the maximum observed control value. AD and BD indicate activation and binding domains.

Table 2
Point mutation analyses of known PhK/PKA phosphorylatable serines within the 32 residue N-terminus of the PhK β subunit.

BD	AD	β FL	β S11AS26A	Vector encoding pB42		β S11ES26E	β S11ES26A	β S11ES26E	Empty vector
				β S11AS26E	β S11ES26A				
Vector encoding pLexA	β FL	2.93 $\pm$ 0.04	3.14 $\pm$ 0.06	7.98 $\pm$ 0.04	10.48 $\pm$ 0.06	6.81 $\pm$ 0.05	2.06 $\pm$ 0.07		
	β 32N	8.36 $\pm$ 0.14	1.79 $\pm$ 0.02	18.95 $\pm$ 0.17	14.96 $\pm$ 0.06	9.79 $\pm$ 0.06	3.16 $\pm$ 0.07		
	β S11A	6.51 $\pm$ 0.12	1.39 $\pm$ 0.05	6.33 $\pm$ 0.04	8.09 $\pm$ 0.06	6.37 $\pm$ 0.06	2.30 $\pm$ 0.08		
	β S11E	5.04 $\pm$ 0.07	0.67 $\pm$ 0.07	2.15 $\pm$ 0.03	7.07 $\pm$ 0.06	3.86 $\pm$ 0.07	1.41 $\pm$ 0.04		
	β S26A	6.47 $\pm$ 0.07	2.53 $\pm$ 0.06	3.51 $\pm$ 0.06	12.20 $\pm$ 4.96	5.83 $\pm$ 0.06	3.36 $\pm$ 0.06		
	β S26E	5.82 $\pm$ 0.07	3.21 $\pm$ 0.03	5.66 $\pm$ 0.04	12.40 $\pm$ 0.07	4.40 $\pm$ 0.02	3.54 $\pm$ 0.06		
	β S11AS26A	5.98 $\pm$ 0.07	1.81 $\pm$ 0.05	6.14 $\pm$ 0.04	14.89 $\pm$ 0.07	5.04 $\pm$ 0.03	2.90 $\pm$ 0.03		
	β S11AS26E	4.78 $\pm$ 0.08	1.13 $\pm$ 0.04	9.01 $\pm$ 0.06	13.61 $\pm$ 0.12	1.85 $\pm$ 0.03	2.05 $\pm$ 0.07		
	β S11ES26A	5.47 $\pm$ 0.14	1.00 $\pm$ 0.05	7.06 $\pm$ 0.02	7.86 $\pm$ 0.07	3.86 $\pm$ 0.05	2.90 $\pm$ 0.06		
	β S11ES26E	6.48 $\pm$ 0.12	1.07 $\pm$ 0.05	10.20 $\pm$ 0.07	13.99 $\pm$ 0.11	4.22 $\pm$ 0.04	2.89 $\pm$ 0.06		
	Empty vector	0.87 $\pm$ 0.02	0.77 $\pm$ 0.04	0.58 $\pm$ 0.04	0.82 $\pm$ 0.03	0.52 $\pm$ 0.05	1.22 $\pm$ 0.05		

The chemical structure and charge of phosphoserine side-chains are approximated by Ser/Ala mutations serve as negative controls for amino acid substitutions at the indicated positions in the primary sequence of β . β -Galactosidase activity from yeast lysates was determined using the substrate β -o-D-galactopyranoside as described under Experimental Procedures. Activity is expressed in units according to the formula: $1000 \times [(A_{420t_1} - A_{420t_2}) / (A_{600} \times A_{t_2})]$. Data represent the mean $\pm$ S.E. of eight assays. A positive interaction is determined as being significantly greater than the maximum observed control. AD and BD indicate activation and binding domains.

β 32N tested against β_{FL} and all combinations of constructs having mutations in the N-terminus.

Table 3

Interactions	β Constructs	Possible Combinations	Color Development ^d		β -Galactosidase Activity in Microplate Liquid Assay		Average % Increase over Control
			# Positives	# Positives	% Increase over Control		
Single-single		24	14	7 (29%)	9 - 84		44.6
Single-double		32	20	13 (41%)	9 - 250		97.4
Double-double		24	19	18 (75%)	9 - 320		125.5
β 32N-all ^f		20	16	16 (80%)	47 - 3221		1038.3
Total		100	69	54	-		-

β Constructs containing point mutations of either one or both phosphorylatable serines (11 and/or 26) are termed single or double, respectively.

^aYeast colonies containing the indicated constructs of β that showed color development after 48 hours. All interactions were subsequently quantified by the microplate β -Galactosidase liquid assay.

^bAny combination of vectors containing at least one Beta32N construct as a partner. A positive interaction is determined as being significantly greater than the maximum observed control.

Table 4Structural similarities between discrete regions of PhK β/γ and Tn subunit functional domains.

Protein	Exon ^a	Structure	Sequence Similarities within Functional Domains	Functions
PhK β_M	2	Amphipathic α -helical ^b	¹ AGATGLMAEVSWKVLERRARTK ^e	Autophosphorylation site ^h inhibitor of β - β dimerization
TnI _M	5	Amphipathic α -helical, coiled coil ^c	⁵⁵ GSMAEV	TnT-binding ^c
PhK β_B	2	Random ^b	¹ ASSADAVVSSPPA	Unknown
TnT _M	13	Random ^b	¹⁶⁷ LSSMGANYSSYLA	PhK phosphorylation sites II and III ⁱ
PhK β_M	3	Amphipathic α -helical ^b	³⁸ RPDNETLWDKLDY ^{•••••} YKIVK ^e	Unknown
TnT _M	15	Amphipathic α -helical, coiled coil ^c	²¹⁸ RDKAKELWDTL	TnI-Binding ^c
PhK β_M	32	Amphipathic α -helical, coiled coil ^b	¹⁰²⁶ LVKEAFHEFQKDESRLKEIEKQ ^f	β -binding ^j
TnI _M	5	Amphipathic α -helical ^c	²¹ MLQIAATELEKEEGR_REAEKQ	TnC-binding ^c
PhK γ_M	9	Extended ^b	³⁰¹ RGKFKVICL ^g	Region of γ cross-linked to β ^j
TnI _M	7	Extended ^d	¹⁰³ RGKFKRPPL	TnC-binding ^d

All protein sequences were from either rabbit fast skeletal muscle (subscript M) or brain (subscript B) isoforms.

^aExon numbers were derived from sequences of corresponding human proteins.^bSecondary structures of overlined sequences within β functional domains (determined by 2-hybrid analyses) were predicted using the Coils⁴¹ and Parcoil⁴² programs located on the ExPASy web site.^cThe structure and function of Tn domains obtained from crystal structure of the core domain of the Tn complex.⁵¹^dFrom the NMR structure of the TnI inhibitory domain.⁴⁹^eProposed β intramolecular interaction between the β 1-22 TnI-like(blue) and β 42-62 TnT-like (red) regions. LWD is part of an evolutionarily conserved TnT coiled coil, in which the L and W residues are critical for binding TnI.⁷¹^fProposed β TnI-like (blue) dimerization domain.^gTnI-like inhibitory (blue) region of γ .⁴⁷^hPreviously reported phosphorylation sites for PhK and TnT.^{12,72}ⁱPreviously reported phosphorylation sites for PhK and TnT.^{12,72}^jObserved herein.

Table 2 Supplement
Point mutation analyses of known PhK/PhKA phosphorylatable serines within the 32 residue N-terminus of the PhK β subunit.

BD	AD	Vector encoding pB42				β S26E	Empty vector
		β FL	β 32N	β S11A	β S26A		
Vector encoding pLexA	β FL	2.93 $\pm$ 0.04	60.08 $\pm$ 0.62	2.51 $\pm$ 0.06	3.94 $\pm$ 0.04	0.79 $\pm$ 0.05	2.06 $\pm$ 0.07
	β 32N	8.36 $\pm$ 0.14	117.65 $\pm$ 1.17	2.77 $\pm$ 0.07	5.20 $\pm$ 0.08	6.28 $\pm$ 0.05	3.16 $\pm$ 0.07
	β S11A	6.51 $\pm$ 0.12	52.34 $\pm$ 0.40	2.25 $\pm$ 0.16	2.80 $\pm$ 0.05	0.25 $\pm$ 0.05	2.30 $\pm$ 0.08
	β S11E	5.04 $\pm$ 0.07	34.79 $\pm$ 0.18	2.00 $\pm$ 0.03	2.83 $\pm$ 0.03	3.49 $\pm$ 0.06	1.41 $\pm$ 0.04
	β S26A	6.47 $\pm$ 0.07	46.66 $\pm$ 0.19	3.05 $\pm$ 0.04	3.00 $\pm$ 0.07	3.85 $\pm$ 0.05	3.36 $\pm$ 0.06
	β S26E	5.82 $\pm$ 0.07	59.14 $\pm$ 0.36	2.27 $\pm$ 0.02	3.22 $\pm$ 0.25	4.22 $\pm$ 0.03	3.54 $\pm$ 0.06
	β S11A.S26A	5.98 $\pm$ 0.07	49.77 $\pm$ 0.18	2.30 $\pm$ 0.05	3.40 $\pm$ 0.07	6.78 $\pm$ 0.03	2.90 $\pm$ 0.03
	β S11A.S26E	4.78 $\pm$ 0.08	60.27 $\pm$ 0.45	1.83 $\pm$ 0.02	3.58 $\pm$ 0.03	3.05 $\pm$ 1.16	2.05 $\pm$ 0.07
	β S11E.S26A	5.47 $\pm$ 0.14	46.78 $\pm$ 0.45	2.36 $\pm$ 0.03	3.05 $\pm$ 0.02	4.17 $\pm$ 0.06	2.90 $\pm$ 0.06
	β S11E.S26E	6.48 $\pm$ 0.12	54.26 $\pm$ 0.64	2.82 $\pm$ 0.09	2.98 $\pm$ 0.03	7.75 $\pm$ 0.06	2.89 $\pm$ 0.06
	Empty vector	0.87 $\pm$ 0.02	0.88 $\pm$ 0.03	1.15 $\pm$ 0.04	1.13 $\pm$ 0.04	0.99 $\pm$ 0.03	1.22 $\pm$ 0.05

The chemical structure and charge of phosphoserine side-chains are approximated by Ser/Ala mutations serve as negative controls for amino acid substitutions at the indicated positions in the primary sequence of β . β -Galactosidase activity from yeast lysates was determined using the substrate β -D-galactopyranoside as described under Experimental Procedures. Activity is expressed in units according to the formula: $1000 \times [(A_{420})_2 / (A_{600} \times \Delta t \times 20)]$. Data represent the mean $\pm$ S.E. of eight assays. A positive interaction is determined as being significantly greater than the maximum observed control. AD and BD indicate activation and binding domains.