The Calmodulin-binding Domain of the Catalytic γ Subunit of Phosphorylase Kinase Interacts with Its Inhibitory α Subunit

EVIDENCE FOR A Ca\(^{2+}\)-SENSITIVE NETWORK OF QUATERNARY INTERACTIONS

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Chemical cross-linking as a probe of conformation has consistently shown that activators, including Ca\(^{2+}\) ions, of the (αβγδ)\(_4\) phosphorylase kinase holoenzyme (PhK) alter the interactions between its regulatory α and catalytic γ subunits. The γ subunit is also known to interact with the δ subunit, an endogenous molecule of calmodulin that mediates the activation of PhK by Ca\(^{2+}\) ions. In this study, we have used two-hybrid screening and chemical cross-linking to dissect the regulatory quaternary interactions involving these subunits. The yeast two-hybrid system indicated that regions near the C termini of the γ (residues 343–386) and α (residues 1060–1237) subunits interact. The association of this region of α with γ was corroborated by the isolation of a cross-linked fragment of α containing residues 1015–1237 from an α–γ dimer that had been formed within the PhK holoenzyme by formaldehyde, a nearly zero-length cross-linker. Because the region of γ that we found to interact with α has previously been shown to contain a high affinity binding site for calmodulin (Dasgupta, M., Honeycutt, T., and Blumenthal, D. K. (1989) J. Biol. Chem. 264, 17156–17163), we tested the influence of Ca\(^{2+}\) on the conformation of the α subunit and found that the region of α that interacts with γ was, in fact, perturbed by Ca\(^{2+}\). The results herein support the existence of a Ca\(^{2+}\)-sensitive communication network among the δ, γ, and α subunits, with the regulatory domain of γ being the primary mediator. The similarity of such a Ca\(^{2+}\)-dependent network to the interactions among troponin C, troponin I, and actin is discussed in light of the known structural and functional similarities between troponin I and the γ subunit of PhK.

Phosphorylase kinase (PhK)\(^3\), a Ca\(^{2+}\)-dependent enzyme involved in the regulation of glycogenolysis, is among the largest and most complex enzymes known. Structurally, PhK is composed of four copies each of four different subunits, (αβγδ)\(_4\) and has a mass of 1.3 × 10\(^{6}\) Da (for reviews see Refs. 1–3). Of the four subunits, γ is catalytic, whereas the remaining three are regulatory: α and β exert quaternary constraint on the activity of γ, and δ is an intrinsic molecule of calmodulin (CaM). To fully understand how PhK integrates diverse physiological signals to regulate glycogenolytic flux in skeletal muscle, it is first essential to understand how intrasubunit and intersubunit interactions within the hexadecameric holoenzyme change in response to effector ligands, and in so doing, control its catalytic activity. Despite the increased availability of structural information regarding PhK, interactions associated with activation and involving specific regions of individual subunits, in particular the α and β subunits, have largely remained uncharacterized. In this study, we have focused on delineating interacting regions between the large α and catalytic γ subunits to advance our understanding of how structural perturbations correlate with activation of this complex holoenzyme.

By using chemical cross-linkers as structural probes, alterations in the interactions between the regulatory α and catalytic γ subunits of PhK consistently emerge as a common structural marker of enzyme activation by multiple effectors, including Ca\(^{2+}\) ions (4–7). Although there have been many studies on the activation of PhK by Ca\(^{2+}\), it has not been clear how the binding of Ca\(^{2+}\) to the δ subunit (CaM) relays structural information to the remainder of the holoenzyme, especially to the α subunit. It has been shown that Ca\(^{2+}\) increases the accessibility of specific regions of the γ subunit (5), and the C-terminal region of the γ subunit has been shown by a variety of experimental approaches to contain the regulatory domain that binds δ (8–11), thus conferring Ca\(^{2+}\)-sensitivity to the holoenzyme. In fact, truncation of γ to eliminate this regulatory domain renders it constitutively active by itself and Ca\(^{2+}\)/CaM-independent (9, 11). Our findings reported herein suggest that the flow of structural information from δ to α in the holoenzyme is directly mediated by the C-terminal regulatory domain of γ. These are the first results to define a specific region of γ that interacts with any region of either the α or β subunits of PhK. Furthermore, the finding that the region of α–γ interaction includes a portion of the CaM binding domain of γ provides a possible explanation for the previously observed changes in α–γ interactions induced by Ca\(^{2+}\).

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Strains—Saccharomyces cerevisiae strain EGY48 (MATa, his3, trp1, ura3, Leu2-3,112) (CLONTECH) was used for all two-hybrid analyses (12). All plasmid manipulations were

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**Proteins and Enzymes—** Nonactivated PhK was purified from fast-twitch skeletal muscle of New Zealand White rabbits (15), dialyzed against 50 mM Hepes (pH 6.8), 10% sucrose, and 0.2 mM EDTA and either used immediately or stored frozen at −80 °C. SDS-PAGE gels were stained with Coomassie blue. The concentrations of the isolated subunits were determined by the Bio-Rad protein assay with BSA as standard; P-b and PhK concentrations were determined spectrophotometrically by their respective absorbance indices (18, 19). Anti-PhK α, β, and γ subunit-specific mAbs were those previously described (20, 21). Anti-CaM was purchased from Signal Transduction Laboratories; all other detection conjugates were from Southern Biotechnology.

**Two-hybrid Plasmid Construction—** All PhK α constructs were engineered as previously described (22). Rabbit skeletal muscle PhK γ cDNA was kindly provided by Dr. Donald J. Graves (Iowa State University) and used as the template for the preparation of all γ constructs. The cDNA fragments were ligated either to pLexA (CLONTECH), a 2-μm yeast plasmid, to generate a fusion consisting of the DNA BD (aminoc acids 1–203) of LexA fused to PhK γ or to pBAD24 (CLONTECH), a 2-μm TRP1 plasmid, to produce a B42 AD protein fused to PhK γ. Constructs y65C, y110C, and yFL were directionally engineered into the EcoRI-BamHI restriction sites of pLexA by subcloning from previously prepared constructs in plasmid pGAD424. Subsequently, these three constructs were linearized from pLexA by digestion with EcoRI and XhoI restriction enzymes and ligated to pBAD24. Constructs y150C, y205C, y300C, and y342C were generated by PCR using primers to yield cDNAs flanked 5′ and 3′ with EcoRI and BamHI restriction sites, respectively (sense-strand, 5′-TGAATTCACCCGGGACGCGGCA-3′; antisense strand, 5′-ATGGATCCTTAGCCTGGGTGGTTGTC-3′; antisense strand, 5′-ATGGATCCTAGGTTGCTGTTGTTGTTGTC-3′; (p2003) 5′-ATGGATCCCTAGGTTGCTGTTGTTGTTGTC-3′; (p3424) 5′-ATGGATCCCTAGGTTGCTGTTGTTGTTGTC-3′) and ligated to the EcoRI and BamHI sites of pLexA. To prepare the corresponding pBAD24 constructs γ constructs, pLexA-Y150C, pLexA-Y205C, pLexA-Y300C, and pLexA-Y342C were digested with EcoRI and XhoI, and the corresponding linear γ fragments were purified and subcloned into pBAD24. Fragments of γ corresponding to its regulatory tail were generated by PCR using the following primers, each ligated to both pLexA and BamHI sites: sense strands (<pH18–34 lacZ reporter plasmid, was transformed by a modified lithium acetate procedure as previously described (23), and transformants were grown at 30 °C on synthetic medium lacking histidine, tryptophan, and uracil (SD–His–Trp–Ura) for 3 days. Protein expression of all α and γ constructs was verified by Western analysis, with minor modification, as previously described (24) 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 α and γ constructs were monitored by transcriptional activation of the LEU2 gene by growth on defined media lacking leucine (Leu−) and of the lacZ reporter gene by liquid β-galactosidase assays using o-nitrophenyl galactopyranoside as substrate (25, 26).

**Two-hybrid Library Screening—** A rabbit skeletal muscle cDNA library (22) was used to screen for interactors of PhK by using γ342C and yFL constructs (described above) as bait. Yeast library transformants containing either of the DNA BD bait plasmids and the AD cDNA library transformed as previously described (24) were grown at either 30 °C or at 30 °C for 7 days, putative primary LacZ+/Leu+ colonies were restreaked on SD–His−–Trp−–Ura and subjected to secondary and tertiary analyses as previously described (22). Specific interactors were then identified by our subcloning and dye-scan sequencing.

**Renaturation of the His-Trp Complex—** The isolated γ subunit in 8 M urea, 0.1 M HPO₄, 0.1 mM EDTA, and 1 mM DTT was renatured as previously described (26, 27) in the presence of either yeast or bovine brain CaM. Each renaturation, carried out at 4 °C overnight, contained the following final concentrations: 0.125 μM isolated γ, 0.31–3 μM CaCl₂, 0.1 M trisodium BSA, 0.06 M HPO₄, 0.1 mM EDTA, 0.5 mM CaCl₂, 0.3 mM DTT, and 100 mM Hepes (pH 8.0).

**Enzymatic Assays of the γ/CaM Complex—** The activity of the renatured γ/CaM complex was determined by following the incorporation of 32P into P-b at pH 7.0 using the filter paper assay of Reinmann et al. (28). Prior to assaying, each renaturation sample was diluted 10-fold with buffer containing 100 mM Hepes (pH 7.0), 0.2 mM DTT, 0.5 mM CaCl₂, 0.1 mM EDTA, and 1 mM/b. To initiate the reaction, one volume of this dilution solution was added to three volumes of reaction mixtures. Final concentrations in the standard assay were: 3.1 nM isolated γ, 0.67 mM CaCl₂, 10 mM Mg(CH₃COO)₂, 0.5 mM (γ–32P)ATP, 100 mM Hepes (pH 7.0), 7.8–7.5 mM CaM, 0.3 mg/ml BSA, 2.8 mg/ml P-b, 20 μM urea, 25 μM EDTA, 0.75 μM DTT, and 0.25 mM HPO₄⁻. Cross-linking of γ/CaM complex—PhK was cross-linked by formaldehyde (5 mM), prepared by the hydrolysis of paraformaldehyde (29), either alone or in the presence of CaCl₂. The final concentrations in the standard cross-linking reaction were: PhK (0.43 μM), Hepes (35 mM, pH 6.8), CaCl₂ (1.25 mM), and EDTA (1 mM). Following cross-linking, reactions were quenched by an equal volume of SDS buffer (0.125% Triton X-100, 2% SDS, 20% glycerol, 5% β-mercaptoethanol, 4% SDS). Cross-linked proteins were resolved on SDS-PAGE gradient gels (4–20%) and characterized by their apparent mass and cross-reactivity against subunit-specific mAbs (20, 21).

**Partial Proteolysis of Native, CaCl₂-activated, and Cross-linked PhK—** PhK, either in the absence or presence of 1.25 mM CaCl₂, was digested with chymotrypsin under conditions that promoted selective cleavage of the α subunit. The standard proteolysis reaction, containing 0.43 μM PhK, 0.12 μM chymotrypsin, 39 mM Hepes (pH 6.8), and 1 mM EDTA, was carried out for 10 min at 30 °C and subsequently quenched by 2× SDS buffer with brief mixing and heating at 80 °C. Samples were resolved by SDS-PAGE on a 4–20% gradient gel, stained with Coomassie blue, and characterized by their apparent mass. The extent of α subunit digestion, which was linear over the time period used, and of the corresponding formation of proteolytic fragments were determined by optical integrated densitometry of the appropriate protein bands.

To localize the region of α cross-linked to γ by formaldehyde, PhK was cross-linked as described above, but with the reaction quenched by addition of 1 mM Triton (final concentration of 100 μM) and subsequently digested by chymotrypsin (0.5 μg/ml). The subunit composition of cross-linked PhK was determined by Western blotting and peptide sequencing. For sequencing, samples were electrophoretically transferred to polyvinylidene difluoride membranes in 10 mM 3-(cyclohexylamino)propanesulfonic acid (pH 11/10) ethanol and stained with amido black for visualization. Bands of interest were excised and submitted for amino acid analysis and N-terminal sequencing to the Harvard Microchemistry Facility.

**RESULTS**

The C Terminus of PhK α Interacts with the Regulatory Domain of γ in the Yeast Two-hybrid System—Our laboratory has previously demonstrated the formation of α-γ complexes through chemical cross-linking of the PhK holoenzyme (4–7); however, relatively long cross-linkers were used in those studies, and as a result, it was not unequivocally established that the α and γ subunits within the hexadecameric holoenzyme actually interact, as opposed to simply being proximal. To determine whether the observed cross-linking of a γ–γ subunit from the actual association of these subunits, and if so, to define the regions involved in that interaction, we screened a series of C-terminal truncations of α and γ (Fig. 1) against one another in the yeast two-hybrid system. To avoid potential disruption of secondary structural elements, the truncated mutants were designed based upon the known crystal DNA-encoded proteins that interact with either of the two bait proteins. After colonies were grown at 30 °C for 3–7 days, putative primary LacZ+/Leu+ colonies were restreaked on SD–His−–Trp−–Ura and subjected to secondary and tertiary analyses as previously described (22). Specific interactors were then identified by our subcloning and dye-scan sequencing. Protein expression of all α and γ constructs was verified by Western analysis, with minor modification, as previously described (24) 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 α and γ constructs were monitored by transcriptional activation of the LEU2 gene by growth on defined media lacking leucine (Leu−) and of the lacZ reporter gene by liquid β-galactosidase assays using o-nitrophenyl galactopyranoside as substrate (25, 26).

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structure of the catalytic domain of the γ subunit (30) and the predicted secondary structure of the α subunit. When all binary combinations of the α and γ constructs were assayed against each other, no interactions were observed for any C-terminal truncation of either α or γ; a significant interaction did occur, however, when full-length constructs of both subunits were expressed (Table I). High levels of β-galactosidase activity were induced by the interaction of γFL and αFL as either BD or AD fusions, but with the pair BD-αFL/AD-γFL demonstrating β-galactosidase activity 3.4-fold greater than that observed with the reciprocal combination of constructs (133 versus 39.3 Miller units). Such domain effects are not uncommon in two-hybrid screens and have been observed with many proteins, including the transcriptional regulators Myc and Max (31).

The fact that none of the C-terminal deletions, but only the full-length α and γ constructs, interacted suggested that regions near the C termini of both subunits (residues 1060–1237 of α and 343–386 of γ) are those that interact to form the α-γ complex. To test this hypothesis, two constructs comprising either the entire C-terminal tail of γ, amino acids 301–386 (γ301N), or the shorter region therein implicated by deletion analysis to interact with α, amino acids 343–386 (γ343N), were engineered as both DNA BD and AD fusion constructs and screened against both α subunit constructs (Fig. 1). Significant interactions were observed only between the γ343N construct and the full-length α subunit, as either DNA BD or AD fusion proteins (14.0 and 23.6 Miller units, Table I) agreeing with the implied region of association between α and γ from the truncation analyses. The reason why the longer γ construct (γ301N) did not interact with any α construct is unclear. It should be noted, however, that in interpreting two-hybrid data, each BD and AD fusion protein is unique and must be evaluated as such; for instance, it is possible that the longer construct, but not the shorter, resulted in a chimeric protein structure in which the interaction of LexA with its targets was sterically blocked. Similar to the results obtained with γ301N, when a construct expressing only the C terminus of α (residues 1015–1237 (α1015N)) was screened as both a DNA BD and AD fusion protein against all of the γ constructs shown in Fig. 1, no interactions were observed with any γ mutant regardless of the transcriptional domain (data not shown). However, as described below, a slightly shorter α1031N construct was found to interact with γFL.

Additional evidence that the C-terminal CaM-binding regulatory domain of the γ subunit interacts with the C-terminal region of the regulatory α subunit came from screening a rabbit skeletal muscle cDNA library (22) using γFL versus γ300C BD fusions as bait. Of −6 × 10^7 library transformants screened with γFL, 165 primary LacZ+/Leu+ colonies were obtained. Of these initial positives, 63 were selected for further analysis, and 21 library cDNAs were ultimately sequenced. The sequencing results showed every single one of these 21 clones to be overlapping transcripts of the α subunit of PhK containing its entire C terminus, but varying in N-terminal start sites (Table II). Screening the very same cDNA library using γ300C as bait resulted in 137 primary LacZ+/Leu+ library cDNAs, with 23 eventually being sequenced. All but two of these 23 were independent clones, but not a single one of them corresponded to the α subunit of PhK. Thus, elimination of the terminal 86 residues of γ eliminated the α subunit as a target in the cDNA library, further demonstrating that in the two-hybrid system the C-terminal regulatory region of the γ subunit of PhK interacts with the C-terminal region of its regulatory α subunit.

Considering that a CaM-binding regulatory region of γ (8) overlaps the region implicated above to bind α and that α-γ-δ complexes can be isolated following partial dissociation of the rabbit muscle (αβ)4 holoenzyme (32) raised the question of whether, during our two-hybrid screening, endogenous yeast CaM, despite its structural and functional divergence from mammalian CaM (33, 34), may nevertheless interact with γ, giving rise to formation of ternary (α-γ-CaM), as opposed to binary (α-γ) complexes. To evaluate the feasibility of this notion, we determined whether yeast CaM could bind and subsequently activate PhK. When the isolated γ subunit of PhK was renatured in the presence of either purified yeast CaM or bovine brain CaM, the yeast CaM stimulated the P-β conversion activity of γ to a similar extent as that observed with the bovine brain isofrom. The concentrations for half-maximal ac-
A positive interaction is determined as being significantly greater than all control values. NT

**TABLE I**

| Vector encoding LexA     | Empty vector | aFL | γFL | γ843N |
|--------------------------|--------------|-----|-----|-------|
| Empty vector             | 1.7 ± 0.1    | 1.9 ± 0.1 | 1.5 ± 0.2 | 1.7 ± 0.0 |
| aFL                      | 1.3 ± 0.2    | NT  | 133 ± 6.8 | 23.6 ± 5.2 |
| γFL                      | 0.3 ± 0.2    | 39.3 ± 9.7 | NT  | NT   |
| γ343N                    | 0.4 ± 0.1    | 14.0 ± 4.2 | NT  | NT   |

**TABLE II**

**Positive library α clones detected by γFL-BD**

| Clones | Region in PhK α |
|--------|-----------------|
| a40N   | 40–1237         |
| a371N  | 371–1237        |
| a373N  | 373–1237        |
| a426N  | 426–1237        |
| a436N  | 436–1237        |
| a595N  | 595–1237        |
| a620N  | 620–1237        |
| a628N  | 628–1237        |
| a706N  | 706–1237        |
| a708N  | 708–1237        |
| a730N  | 730–1237        |
| a733N  | 733–1237        |
| a757N  | 757–1237        |
| a831N  | 831–1237        |
| a981N  | 981–1237        |
| α1013N | 1013–1237       |
| α1022N | 1022–1237       |
| α1025N | 1025–1237       |
| α1031N | 1031–1237       |

It was determined by mass spectrometry and nuclear magnetic resonance (NMR) analysis that the yeast CaM was capable of binding to γ with high affinity; thus, it is possible that the observed α-γ interactions identified in the two-hybrid system may actually involve yeast CaM also and correspond to interactions occurring within the α-γ-δ trimer, assuming of course that α and CaM do not exclusively compete for binding to the C-terminal region of γ.

**Formaldehyde Cross-links the C Terminus of the α Subunit to the γ Subunit within the PhK Holoenzyme**—To further examine the contact regions between the α and γ subunits, we sought a very short cross-linker that would enable us to substantiate the observed two-hybrid α-γ interactions within the context of the (αβγ)6 holoenzyme and, additionally, to correlate observed conformational changes between the two subunits with the activation of PhK. Formaldehyde was chosen as the cross-linking agent because its reaction with nucleophiles results in the formation of a conjugate, as followed by change in optical density, increased with time along with a corresponding loss in density of both the α and γ subunits. Furthermore, the cross-linked species cross-reacted with only anti-α and anti-γ subunit-specific mAbs by Western analysis; no cross-reactivity was observed with the anti-β or anti-CaM (δ) mAbs (Fig. 4B). Taken together, these data indicate the formation of an α-δ dimer within the PhK holoenzyme by the very short cross-linker formaldehyde.

To localize regions of the α subunit cross-linked to the γ subunit, we relied on the highly selective proteolysis of the α subunit within the holoenzyme by chymotrypsin, which degrades that subunit essentially to completion without significant hydrolysis of the β, γ, or δ subunits (38). Partial digestion of nonactivated PhK generated major fragments of α having apparent masses of 78, 60, 58, 30, and 24 kDa; two fragments of a (58 and 24 kDa, Fig. 4A) have previously been shown to cross-react with an anti-α mAb whose epitope is known to be near the C terminus of that subunit between residues 1192–1237 (7, 20). When cross-linked enzyme was digested with chymotrypsin, two new bands were observed, γ-α1 (102 kDa) and γ-α2 (68 kDa), which cross-reacted with both the anti-α and anti-γ mAbs (Fig. 4B). Based upon mass, these new bands corresponded to the entire γ subunit cross-linked to the 58- and 24-kDa C-terminal fragments of α, respectively. Examination of the proteolytic digestion pattern of cross-linked PhK demonstrated corresponding losses in density of both the 58- and 24-kDa fragments of α in the anti-α blot, as well as a significant loss in density of the γ subunit in the anti-γ blot with respect to uncross-linked proteolysed holoenzyme.

**FIG. 2. Stimulation of isolated γ subunit catalytic activity by CaM.** PhK γ was renatured in the presence of either yeast (○) or bovine brain (□) CaM and assayed for enzymatic activity as described under “Experimental Procedures.” Data points represent the average of triplicate assays with the bars indicating the S.D. Kinetic parameters given in the text were determined by a linear regression analysis of at least three to five separate experiments.

(36, 37). The formation of this 178-kDa conjugate, as followed by change in optical density, increased with time along with a corresponding loss in density of both the α and γ subunits. Furthermore, the cross-linked species cross-reacted with only anti-α and anti-γ subunit-specific mAbs by Western analysis; no cross-reactivity was observed with the anti-β or anti-CaM (δ) mAbs (Fig. 4B). Taken together, these data indicate the formation of an α-δ dimer within the PhK holoenzyme by the very short cross-linker formaldehyde.

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Because γ-α₂ was the better resolved of the two cross-linked α-γ complexes and was present in greater quantities than γ-α₁, it was used for subsequent sequence determination. N-terminal analysis of this conjugate resulted in two sequences being identified, TRDAALPG and RRLSISTE, which correspond exclusively to the N termini of γ and the 24-kDa fragment of α, respectively. The latter is known to result from cleavage at Phe-1014 and correspond to residues 1015–1237 of α (20), which make up the C-terminal one-sixth of the α subunit.

**Effects of Ca²⁺ on the Conformational Stability of the α and γ Subunits—Ca²⁺ is the most fundamental activator of PhK, regulating its activity through the δ subunit, an endogenous molecule of CaM. Because the C terminus of γ, known to bind to δ (8–11), was identified in this study to also associate with α, we investigated the effect of Ca²⁺ on α-γ dimer formation by formaldehyde. The extent of α-γ dimer produced by cross-linking the holoenzyme in the presence of Ca²⁺ increased by 2-fold over nonactivated PhK (Fig. 3). This enhancement of the formation of α-γ by Ca²⁺ corroborates the observed two-hybrid interaction of α with the CaM-binding C terminus of γ and is consistent with other cross-linking studies from our laboratory correlating Ca²⁺-mediated activation of the PhK holoenzyme with perturbations in the interaction between its α and γ subunits (5–7).

Because the δ-mediated effects of Ca²⁺ resulting in the activation of γ are concomitantly transmitted to the C-terminal region of α, we further examined the Ca²⁺-induced perturbations in this region of α by its selective cleavage with chymotrypsin. When the PhK holoenzyme was partially digested in the presence of Ca²⁺, the pattern of cleavage of the α subunit did not change; however, there was a preferential 3-fold increase² in the rate of formation of the 24-kDa immunoreactive fragment of α previously shown to result from cleavage at residue 1014. These cross-linking and partial proteolysis results indicate that the binding of Ca²⁺ ions to the δ subunit of PhK causes a distinct conformational change in the C-terminal region of its α subunit.

**DISCUSSION**

Whereas the catalytic γ subunit of PhK is undoubtedly involved in complex interactions with all three of its regulatory subunits, no specific contact region between γ and either of the large inhibitory α and β subunits has been determined previously. In this study, we have identified a region within the stretch of residues 343–386 at the C terminus of the γ subunit that interacts with a region near the C terminus of the α subunit. This region of γ is of particular importance because it contains one of the two distinct, noncontiguous CaM-binding domains present in the C-terminal regulatory region of the γ subunit. In a thorough study utilizing a series of 18 overlapping 25-mer peptides corresponding to the C-terminal 110-residue sequence (amino acids 277–386) of the γ subunit, Dasgupta et al. (8) identified two domains of γ with nanomolar affinity for CaM: domain N (residues 287–331) and domain C (residues 332–371). Within domain C, the peptide with the highest affinity for CaM corresponded to residues 342–366, which but for one residue are contained entirely within the region of γ shown herein to interact with α.

The fact that the same region within the regulatory domain of the γ subunit potentially interacts with both the δ (CaM) and α subunits provides a plausible mechanism to explain how Ca²⁺ induces tertiary and quaternary structural changes that are associated with activation of the PhK holoenzyme. It has been previously suggested that activation of PhK by various effectors occurs via a hierarchy of tiered conformational changes, largely reflecting differing states of release of quaternary constraint imposed by the regulatory subunits upon the catalytic γ subunit, with the most fundamental change being that induced by Ca²⁺ (discussed in detail in Ref. 5). By cross-linking analysis, it is known that structural perturbations involving the α and γ subunits occur upon activation of PhK by the binding of Ca²⁺ ions to the δ subunit (5–7). Therefore, it is reasonable to hypothesize that the binding of the C-terminal regulatory region of γ to both α and δ may be modulated in a Ca²⁺-dependent manner; that Ca²⁺ affects the conformation of the very region of α found to bind γ (Fig. 4) supports this hypothesis. The evidence indicates that perturbation of the δ-γ interactions caused by the binding of Ca²⁺ to δ occurs concomitant with perturbation of the γ-α interactions, resulting in a δ-γ-α communication network. Recently, three-dimensional structures of the holoenzyme obtained by image reconstruction of PhK particles observed by electron microscopy ≤ Ca²⁺ (39) revealed that Ca²⁺ does indeed induce distinct conformational changes over a region of PhK previously shown to be occupied by portions of its α, γ, and δ subunits (20, 21, 37).

Given that purified yeast CaM activates the isolated γ subunit (Fig. 2), it is possible that endogenous CaM may be binding during our two-hybrid analyses to those γ constructs that have a CaM-binding domain, and as a result, the positive interaction we observe between full-length α and γ may actually represent a trimeric α-γ-δ instead of a dimeric α-γ complex. Typically, yeast CaM is a poor activator of mammalian target enzymes.

² Ca²⁺ causes a modest increase (~20%) in the activity of chymotrypsin.

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**Fig. 3.** Ca²⁺-induced changes of the conformation of PhK. A, PhK (lane 1) was cross-linked by formaldehyde for 30 min in the absence (lane 2) or presence of 250 μM free Ca²⁺ (lane 3) and resolved by SDS-PAGE. B, optical density measurements of the Coomassie-stained α-γ conjugates from lanes 2 and 3 of A were determined on a BioImage whole band analyzer. Bars represent the mean of three experiments ± S.E.
including protein kinases (34, 40), due to its significant divergence both structurally and functionally from mammalian CaMs; it shares only 60% identity in primary structure (33) and does not bind Ca\(^{2+}\) at site IV (41, 42). It is not surprising, however, that yeast CaM is capable of binding to and activating PhK because the subunit seems to be more sensitive to mutations in the second and third domains of CaM than in its first and fourth (43); moreover, the quite dissimilar TnC also activates \(\gamma\), albeit to a lesser extent than CaM (44).

If our two-hybrid interactions do, in fact, involve not only \(\alpha\) and \(\gamma\), but also CaM, then additional issues arise concerning Ca\(^{2+}\)-dependence and the interactions of the \(\delta\) subunit of PhK. Because the three stable species of PhK (the hexadecameric (\(\alpha\beta\gamma\delta\)) \(_2\), holoenzyme, the \(\alpha\-\gamma\-\delta\) trimer, and the \(\gamma\-\delta\) dimer) all differ in the extent to which their activity is dependent on Ca\(^{2+}\) ions, it has been suggested that increasing enzyme complexity, i.e. progressing from \(\gamma\-\delta\) to \(\alpha\-\gamma\-\delta\) to holoenzyme, results in a progressive increase in the Ca\(^{2+}\)-requirement for catalytic activation of the complex (32). Because the \(\gamma\-\delta\) complex readily dissociates in \(\delta\) x urea, whereas \(\alpha\-\gamma\-\delta\) does not, it has been further suggested that extended interactions exist among these three subunits in which \(\delta\), besides binding \(\gamma\), is stabilized from dissociation by the presence of \(\alpha\) (45). The sum of these findings suggests that \(\alpha\) may contribute, at least indirectly, to regulating the effects of Ca\(^{2+}\) on catalysis. Our current finding that the C terminus of \(\alpha\), a regulatory region that undergoes structural perturbations in response to a variety of activating stimuli including Ca\(^{2+}\) (7), interacts with \(\gamma\) within a region where it binds \(\delta\) supports the existence of an intricate, Ca\(^{2+}\)-sensitive communication network among the \(\alpha\), \(\gamma\), and \(\delta\) subunits, an idea also strongly supported by the previously mentioned structures of PhK from electron microscopy (39). Because Ca\(^{2+}\)-triggered communication can flow from the \(\delta\) to the \(\alpha\) subunit, then it is reasonable to expect that some manifestation of the reverse process ought to be present, as well. This expectation may be born out by early reports that activation of the PhK holoenzyme by phosphorylation of its \(\alpha\) and \(\beta\) subunits increases affinity of the \(\delta\) subunit for Ca\(^{2+}\) ions (46, 47).

Cu\(^{2+}\)-dependent structural changes, similar to those described above for PhK, have been observed in the regulation of skeletal muscle troponin, specifically between the inhibitory region of TnI and its protein targets, actin and TnC (a CaM homologue). In reconstituted thin filaments, the inhibitory region of TnI, which shares remarkable sequence similarity with the C-terminal regulatory domain of the \(\gamma\) subunit of PhK (8, 44), interacts preferentially with actin in the absence of Cu\(^{2+}\) but with TnC in the presence of Ca\(^{2+}\) (48). The possibility that the inhibitory domain of TnI and the homologous region within the \(\gamma\) subunit of PhK share a similar mechanism in their interactions with their respective protein targets (TnC and actin versus the \(\delta\) and \(\alpha\) subunits) is supported by the following striking structural and functional similarities. (a) The regions of greatest sequence identity between \(\gamma\) and TnI (amino acids 301–325 and 103–115, respectively) contain in the case of TnI those very residues most critical for its specific interactions with actin and TnC (discussed in Ref. 44) and in the case of \(\gamma\), one of its two adjacent CaM-binding domains (8). (b) TnC activates PhK \(\gamma\) (44). (c) Actin inhibits \(\gamma\)-CaM and \(\gamma\)-TnC complexes (44). (d) \(\gamma\) inhibits actomyosin ATPase (44). The \(\gamma\) subunit of PhK is thought to have evolved from the fusion of a protein kinase protogene with a progenitor of exon VII of the TnI gene (44), which encodes its inhibitory domain. The probable evolutionary link between TnI and PhK \(\gamma\), the homologues that exist between them, and our current findings suggest that in skeletal muscle the troponin complex and the PhK holoenzyme may share related Ca\(^{2+}\)-dependent alterations in quaternary structure, all leading to the simultaneous stimulation by Ca\(^{2+}\) ions of contraction and energy production, respectively.
REFERENCES

1. Pickett-Gies, C. R., and Walsh, D. A. (1986) in The Enzymes (Boyer, P. D., and Krebs, E. G., eds) 3rd Ed., Vol. 17, pp. 395–459, Academic Press, Inc., Orlando, FL.

2. Heilmeyer, L. M. G., Jr. (1991) Biochim. Biophys. Acta 1094, 168–174.

3. Brushia, R. J., and Walsh, D. A. (1999) Front. Biosci. 4, D618–D641.

4. Nadeau, O. W., Sacks, D. B., and Carlson, G. M. (1997) J. Biol. Chem. 272, 26196–26201.

5. Nadeau, O. W., Sacks, D. B., and Carlson, G. M. (1997) J. Biol. Chem. 272, 26202–26209.

6. Ayers, N. A., Nadeau, O. W., Read, M. W., Ray, P., and Carlson, G. M. (1998) Biochem. J. 331, 137–141.

7. Nadeau, O. W., Traxler, K. W., Fee, L. R., Baldwin, B. A., and Carlson, G. M. (1999) Biochemistry 38, 2551–2559.

8. Dasgupta, M., Honeyrutt, T., and Blumenthal, D. K. (1989) J. Biol. Chem. 264, 17156–17163.

9. Harris, W. R., Malenick, D. A., Johnson, C. M., Carr, S. A., Roberts, G. D., Byles, C. A., Anderson, S. R., Heilmeyer, L. M. G., Jr., Fischer, E. H., and Crabh, J. W. (1990) J. Biol. Chem. 265, 11740–11745.

10. James, P., Cohen, P., and Carafoli, E. (1991) J. Biol. Chem. 266, 7087–7091.

11. Cox, S., and Johnson, L. N. (1992) Protein Eng. 5, 811–819.

12. Gyuris, J., Golemis, E. A., Cheretkov, H., and Brent, R. (1993) Cell 75, 791–803.

13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

14. Ausembel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1996) Current Protocols in Molecular Biology, John Wiley and Sons, New York, NY.

15. King, M. M., and Carlson, G. M. (1981) J. Biol. Chem. 256, 11058–11064.

16. Paudel, H. K., and Carlson, G. M. (1987) J. Biol. Chem. 262, 11912–11915.

17. Fischer, E. H., and Krebs, E. G. (1958) J. Biol. Chem. 231, 65–71.

18. Kastenschmidt, L. L., Kastenschmidt, J., and Helnreich, E. (1968) Biochemistry 7, 3590–3608.

19. Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.

20. Wilkinson, D. A., Marion, T. N., Tillman, D. M., Norcum, M. T., Hainfeld, J. F., Seyer, J. M., and Carlson, G. M. (1994) J. Mol. Biol. 235, 974–982.

21. Wilkinson, D. A., Norcum, M. T., Fitzgerald, T. J., Marion, T. N., Tillman, D. M., and Carlson, G. M. (1997) J. Mol. Biol. 265, 319–329.

22. Ayers, N. A., Wilkinson, D. A., Fitzgerald, T. J., and Carlson, G. M. (1999) J. Biol. Chem. 274, 35583–35590.

23. Geitz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425.

24. Law, D. J., Allen, D. L., and Tidball, J. G. (1994) J. Cell Sci. 107, 1477–1483.

25. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

26. Kee, S. M., and Graves, D. J. (1986) J. Biol. Chem. 261, 4732–4737.

27. Farrar, Y. J. K., and Carlson, G. M. (1991) Biochemistry 30, 10274–10279.

28. Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1986–1995.

29. Jentoft, N., and Dearborn, D. G. (1979) J. Biol. Chem. 254, 4359–4365.

30. Owen, D. J., Noble, M. E. M., Garman, E. F., Papageorgiou, A. C., and Johnson, L. N. (1995) Structure 3, 467–482.

31. Estojak, J., Brent, R., and Golemis, E. A. (1995) Mol. Cell. Biol. 15, 5820–5829.

32. Chan, J. K.-F., and Graves, D. J. (1982) J. Biol. Chem. 257, 5948–5955.

33. Davis, T. N., Urdea, M. S., Masiarz, F. R., and Thorner, J. (1986) Cell 47, 423–431.

34. Matsuura, I., Kimura, E., Tai, K., and Yazawa, M. (1993) J. Biol. Chem. 268, 13267–13273.

35. Pine, H. K., Hendrickson, J. B., Cram, D. J., and Hammond, G. S. (1980) in Organic Chemistry, 4th Ed., p. 86, McGraw Hill, New York, NY.

36. Nadeau, O. W., Traxler, K. W., and Carlson, G. M. (1998) Biochem. Biophys. Res. Commun. 251, 637–641.

37. Traxler, K. W., Norcum, M. T., Hainfeld, J. F., and Carlson, G. M. (2001) J. Struct. Biol. 135, 231–238.

38. Trempe, M. R., and Carlson, G. M. (1987) J. Biol. Chem. 262, 4333–4340.

39. Nadeau, O. W., Carlson, G. M., and Gogol, E. P. (2002) Structure 10, 25–32.

40. Lukas, T. J., Collinge, M., Haiech, J., and Walters, D. M. (1994) Biochim. Biophys. Acta 1223, 341–347.

41. Luan, Y., Matsuura, I., Yazawa, M., Nakamura, T., and Yagi, K. (1987) J. Biol. Chem. 262, 1531–1537.

42. Staehelin, M. A., Davis, T. N., and Klemit, R. E. (1993) Biochemistry 32, 2361–2370.

43. Farrar, Y. J. K., Lukas, T. J., Craig, T. A., Watterson, D. M., and Carlson, G. M. (1993) J. Biol. Chem. 268, 4120–4125.

44. Paudel, H. K., and Carlson, G. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7285–7289.

45. Chan, J. K.-F., and Graves, D. J. (1982) J. Biol. Chem. 257, 5956–5961.

46. Brostrom, C. O., Hankeler, F. L., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1961–1967.

47. Cohen, P. (1980) Eur. J. Biochem. 111, 563–574.

48. Kobayashi, T., Kobayashi, M., Gryczynski, Z., Lakowitz, J. R., and Collins, J. H. (2000) Biochemistry 39, 86–91.

49. Wu, F. C., and Laskowski, M. (1956) Biochim. Biophys. Acta 19, 110–115.

50. Harmann, B., Zander, N. F., and Kiliman, M. W. (1991) J. Biol. Chem. 266, 15631–15637.

51. Kiliman, M. W., Zander, N. F., Kuhn, C. C., Crab, J. W., Meyer, H. E., and Heilmeyer, L. M. G., Jr. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9381–9385.

52. Herrera, J. E. (1986) The Activity of Skeletal Muscle Phosphorylase B Kinase at Physiological Temperature. M. S. thesis, University of Mississippi Medical Center.