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PHOSPHORYLASE KINASE: THE COMPLEXITY OF ITS REGULATION IS REFLECTED IN THE COMPLEXITY OF ITS STRUCTURE

Robert J. Brushia [1] and Donal A. Walsh [2]

[1] Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Rd., MS1-260, Berkeley, CA 94720, [2] Department of Med. Biol. Chem., University of California, Davis, 95616

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1. ABSTRACT

Intracellular glycogen stores are used to maintain blood-glucose homeostasis during fasting, are a source of energy for muscle contraction, and are used to support a broad range of cellular activities in most tissues. A diversity of signals accelerate glycogen degradation that are mediated by phosphorylase b kinase (Phk), which phosphorylates and thereby activates glycogen phosphorylase. Phk is among the most complex of the protein kinases so far elucidated. It has one catalytic (gamma) subunit and three different regulatory (alpha, beta, and delta) subunits, a molecular mass of 1.3 X 10^6 daltons, and each holoenzyme molecule is presumed to contain four molecules of each subunit. The three regulatory subunits inhibit the phosphotransferase activity of the gamma subunit. Ca^{2+} relieves inhibition via the delta subunit, which is identical to calmodulin but remains an integral component of the holoenzyme even when the [Ca^{2+}] is lowered to nanomolar levels. Phosphorylation of the alpha and beta subunits by the 3',5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) also relieves inhibition of the gamma subunit and thereby activates the enzyme. The stimulatory effects of Ca^{2+} and phosphorylation appear to be structurally coupled and are cooperative. In addition, Phk is activated *in vitro* by autophosphorylation, limited proteolysis of the regulatory subunits, and various allosteric effectors and these may also be mechanisms of physiological importance. The molecular mechanisms of regulation are currently poorly understood, but new insights are beginning to emerge. This review discusses current knowledge and concepts of the structure, function and regulation of Phk.

2. INTRODUCTION

Although not the first protein kinase to be observed, phosphorylase b kinase (Phk; EC 2.7.1.38) was the first to be purified, have its specific function determined, and have its physicochemical properties characterized [for review see references 1 and 2, especially for the early literature citations]. Phk catalyzes the phosphorylation and activation of glycogen phosphorylase,

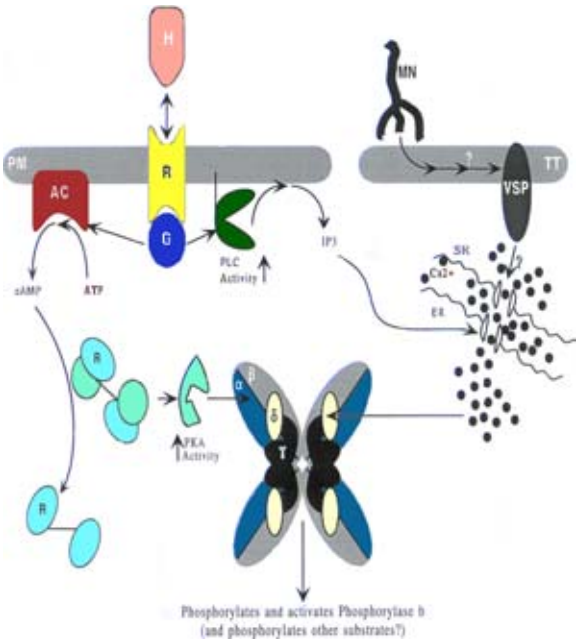

Figure 1. Schematic depicting major signaling pathways that converge at Phk activation. Hormones (H) that bind to plasma membrane (PM)-bound receptors (R) on cells, or motor nerve (MN) impulses that impinge upon the t-tubule membrane (TT) of skeletal muscle cells, can trigger different pathways. In somatic cells, hormone binding can trigger the activation of heterotrimeric G-proteins (G) that can, in turn, activate membrane-bound adenylate cyclase (AC) or phospholipase C (PLC), or both. AC produces 3'5'-cyclic adenosine monophosphate (cAMP) and PLC produces inositol trisphosphate (IP_3). cAMP activates the catalytic subunit (light green) of the cAMP-dependent protein kinase (PKA) by releasing its regulatory (R) subunits. IP_3 opens Ca^{2+} channels in the endoplasmic reticulum, thereby elevating the cytosolic Ca^{2+} concentration. PKA activates Phk by phosphorylating its α and β subunits and Ca^{2+} activates Phk via its integral δ subunit (and exogenous calmodulin, not shown for simplicity). Alternatively, a MN impulse can depolarize the TT membrane of skeletal muscle cells via activation of a voltage-sensitive protein (VSP). The VSP triggers the opening of Ca^{2+} channels in the sarcoplasmic reticulum (SR). The released Ca^{2+} activates Phk via the δ subunit. Thus, all extracellular signals that elevate the intracellular cAMP and Ca^{2+} levels are potentially capable of activating Phk. Note that in the figure, PhK is depicted schematically as an (alpha, beta, gamma, delta)$_4$ complex in a "butterfly-like" orientation. Although there is evidence to support the overall butterfly shape, the arrangement of Phk subunits as shown in this schematic is arbitrary.

which consequentially promotes glycogenolysis (figure 1). Phosphorylase b (dephospho form) is converted to phosphorylasea in the reaction catalyzed by phosphorylase b kinase. The regulation of glycogenolysis is the only physiological function that has so far been established for Phk, albeit other key roles for this enzyme may well emerge. The Phk holoenzyme contains three distinct regulatory subunits, alpha, beta and delta, and one catalytic subunit, gamma (figures 1 and 2). As components of the holoenzyme, the alpha and beta regulatory subunits suppress the catalytic activity of the gamma subunit. Activation is a consequence of these inhibitory effects being reversed, leading to increased enzyme activity. In Phk this deinhibition results, at least in part, from progressive serial modification at multiple sites within the regulatory subunits and the coordinated effects of these modifications on the catalytic subunit. This type of regulation (i.e., phosphotransferase activity being inhibited until a turn-on signal is received) is common in many protein kinases.

The regulation of Phk activity exhibits remarkable complexity (figure 1). *In vivo* it is phosphorylated by the cAMP-dependent protein kinase (PKA) on at least two, perhaps more, distinct sites. There is one well-characterized phosphorylation site on each of the alpha and beta regulatory subunits (1). Phosphorylation by PKA increases the specific activity of Phk. The activity of both unactivated Phk and Phk activated by PKA-catalyzed phosphorylation are Ca^{2+}-dependent. Ca^{2+} likely enhances phosphotransferase activity via at least two separate physiological mechanisms. Phosphorylation and Ca^{2+} act cooperatively to enhance Phk activity and thus Phk is a point of cross-talk between two of the most prominent eucaryotic signal transduction pathways (3). Phk can also be activated *in vitro* by ↑pH, autophosphorylation, limited proteolysis, and ADP (adenosine-5'-diphosphate). ADP acts allosterically by binding to a site(s) separate from the catalytic site (4). The physiological relevance of these *in vitro* demonstrated activation mechanisms can so far only be speculated.

Phk has a wide-spread tissue-distribution and multiple tissue-specific isoforms exist with muscle and liver containing relatively high levels (1). It is estimated that Phk comprises 0.5 % - 1 % of the soluble protein in skeletal muscle (1,5). Phosphorylase comprises ~ 2 % of soluble skeletal muscle protein (1,5). The actual activity of fully unactivated Phk remains somewhat in doubt, however, the very high ratio of Phk / phosphorylase in muscle raises the issue of just how much activation of Phk is necessary to promptly phosphorylate and activate all of the phosphorylase present. There appears to be sufficient unactivated Phk present in skeletal muscle to fully activate glycogen phosphorylase in possibly as short a period as one-tenth of a second, (1,6). The activation of Phk in response to hormonal or neuronal stimuli further increases the rate of phosphorylase activation.

Phk is one of the largest and most structurally complex protein kinases known but its only well-established physiological function is relatively simple, its regulation is exceedingly complex with respect to its function, and more Phk appears to be present in skeletal muscle than is needed for the regulation of glycogenolysis, even under the most extreme circumstances. These observations suggest that Phk may well be involved in other biological processes and have other major physiological function(s) yet to be resolved (1). Although

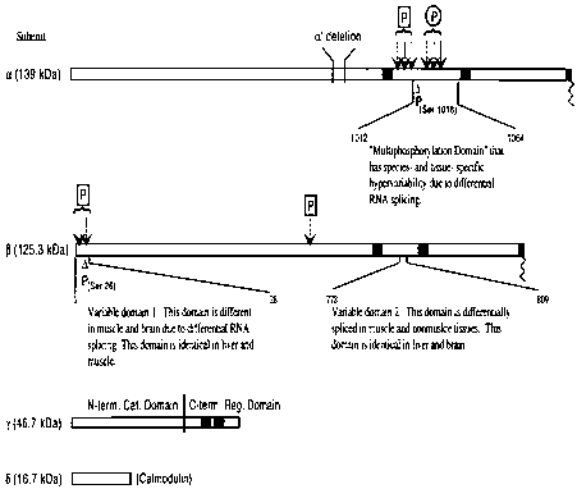

Figure 2. Schematic structure of the Phk subunits showing known post-translational modifications, putative calmodulin-binding domains, and the catalytic domain. The relative linear sizes of the subunits as shown are approximately to scale. The well-characterized sites of cAMP-dependent phosphorylation in the alpha and beta subunits are indicated as Ser^{1018} and Ser^{26}, respectively (numbering relative to the isoforms from rabbit skeletal muscle Phk); other sites known to be autophosphorylated are indicate by a circled P, sites of endogenous phosphate incorporation are indicated by a boxed P. Sites of differential mRNA processing are indicated for both the alpha and beta subunits, and the sequence segment that is deleted from alpha to give the alpha prime isoform is indicated. Putative calmodulin-binding domains are indicated as black boxes. Sites of farnesylation are indicated as gray boxes at the C-termini of the α and β subunits, and the farnesyl group is depicted by a wavy line. This figure was constructed with information compiled from numerous references discussed in the text.

Phk was the first protein kinase to be characterized, and despite its having been the focus of considerable investigation for more than 40 years, much remains to be learned about its structure, the molecular mechanisms by which its catalytic activity is regulated, and its physiological function.

3. SUBUNIT STRUCTURE-FUNCTION, SUBUNIT-SUBUNIT INTERACTIONS, SUBUNIT ISOFORMS

The Phk holoenzyme is most generally presumed to have an $alpha_4$-$beta_4$-$gamma_4$-$delta_4$ stoichiometry, and a corresponding molecular mass of ~1.3 X 10^6 Da (1). (The precise molecular mass of the holoenzyme depends on subunit isozyme composition.) The four different Phk subunits appear to associate as alpha-beta-gamma-delta heterotetramers, with four heterotetramers comprising the holoenzyme. Subunit stoichiometry has not been as firmly established as might be desirable and there remains a good possibility that the subunit stiochiometery may be altered under different physiological conditions or with the enzyme isolated from different tissue sources (e.g., with different isozyme forms). Heterogeneity of subunit composition, with individual enzyme molecules composed of more than one isozyme form of an individual subunit, is also a possibility.

3.1.1. The gamma subunit

The gamma subunit is the catalytic subunit of Phk (figure 2) and the molecular mass of the skeletal muscle isoform, gamma-M (M denoting the skeletal,muscle form), is ~45 kDa (1, 7-12). The gamma-M proteins from rat, rabbit, and mouse have >93% identical amino acid sequences (8,9, 13,14). Rabbit gamma-M has been the most extensively characterized and most discussion of the gamma subunit in this review is based on what has been learned with respect to that protein.

The rabbit gamma-M protein contains 386 amino acids. The N-terminal domain of gamma-M (amino acids 1-298) comprises the catalytic core (figure 2). This domain shares extensive amino acid sequence homology with the catalytic domains of other protein kinases (15, 16). The remaining C-terminal one-third of gamma-M is a regulatory domain. The structure of the N-terminal catalytic domain has been solved to ~3Å resolution by the Johnson laboratory (17,18), and is depicted in figures 3 and 4. The catalytic domain has the classic bilobal protein kinase structure (18), first identified with the catalytic subunit of PKA (cPKA), which has served as the prototype for the general structure of protein kinase catalytic domains (19,20). The catalytic core of gamma-$M_{(1-298)}$ and cPKA proteins share a very high degree of structural homology and only a 5° rotation of one globular lobe of gamma-$M_{(1-298)}$ is needed for direct superposition of its C-alpha backbone trace upon that of the PKA catalytic subunit (figure 3, (18). The degree of rotation between the two lobes is one of the most variable features found in the protein kinase structures and lobe-lobe rotation is part of the mechanism of both enzyme activation and regulatory subunit/regulatory domain regulation. X-ray crystallography has provided much insight into gamma subunit structure (17,18). Yet to be ascertained, however, is how the C-terminal one third of the gamma subunit (residues 299-386), that was deleted in order to obtain a crystal suitable for X-ray analysis and which apparently contains the major site of delta subunit , interacts with the catalytic core. Also of great interest is how the presence of the other Phk subunits might modify the structure of gamma (1).

The C-terminal one-third of the gamma-M subunit (amino acid residues 299-386) does not share any significant amino acid sequence homology with other protein kinases, suggesting that it has a function that is unique to PhK and the gamma subunit. The C-terminal domain contains two calmodulin-binding domains (amino acid residues 301-327 and 332-371; figure 2). Since the delta subunit is identical to calmodulin, these two calmodulin-binding domains appear to comprise the primary delta subunit interaction site. As will be discussed in further detail, two high-affinity calmodulin-binding peptides with the sequences of gamma-M subunit amino acid residues 301-327 and 342-367 have provided valuable

Figure 3. Ribbon diagrams depicting similarity between the structure of catalytic domain of Phk gamma-M (residues 1-298) (left panel) and the catalytic subunit of PKA (right panel). The two structures are colored the same for comparison. The N-terminal small lobe of the catalytic domain is colored yellow and the C-terminal large lobe is colored magenta. The catalytic cleft is situated between the two lobes. This figure was created using data taken from references (18,19).

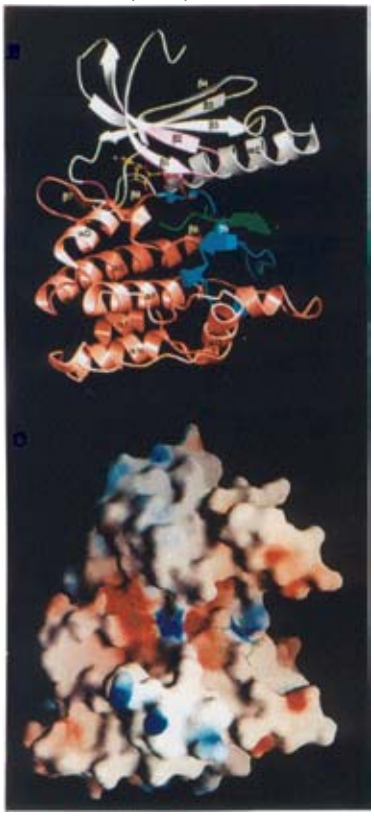

Figure 4. Structure of the catalytic domain of the gamma subunit. The upper figure represents the ribbon structure of Phk gamma-$M_{(1-298)}$ complexed with Mg^{2+}-AMPPNP and a peptide substrate. The N-terminal small lobe is silver, the C-terminal large lobe is orange, the P-loop is pink, the "activation segment" is blue, the hinge region is dark pink and bound peptide substrate is green. The lower figure shows the peptide substrate as a green stick figure bound to the surface of gamma-$M_{(1-298)}$. Colors on the surface represent electrostatic potential with blue representing positive charge and red representing negative charge. This figure was reproduced with permission from reference (94).

insights into the nature of the gamma subunit-delta subunit interaction. (21).

There is currently no direct structural information for this C-terminal component of the gamma-M subunit, either as an isolated fragment or as a part of the intact gamma subunit. This C-terminal region acts as an autoinhibitor of catalytic activity, presumably reflecting some aspect of delta subunit function with respect to the Ca^{2+}-dependence of Phk phosphotransferase activity, but there remains some ambiguity as to the nature of the autoinhibitory interaction (22-25). It has been proposed that the autoinhibitory domain and the two delta subunit-binding domains functions as a Ca^{2+}-dependent molecular switch (22). The delta subunit-binding domains are unusual in that most calmodulin target enzymes have single calmodulin-binding domains that span less than 30 amino acid residues; in gamma there are two nearly contiguous domains spanning approximately 70 residues and the two sites appear capable of simultaneously interacting with an individual delta subunit/calmodulin molecule (26). Possibly as a consequence of the presence of the two domains and their distinctive nature, the gamma subunit can remain associated with the delta subunit even when the $[Ca^{2+}]$ is lowered to nanomolar levels. The nature of the interactions involved remains to be clarified (21-24). The calmodulin-binding domains have been evolutionarily conserved in the liver/testis-specific gamma subunit isoform, gamma-LT (27).

3.1.2. The delta subunit

The delta subunit, as noted, is identical to calmodulin, the Ca^{2+}-dependent modulator protein originally isolated from bovine brain (28-31). The rabbit skeletal muscle delta subunit has 148 amino acids and a molecular mass of ~16.5 kDa [29]. The amino terminus of delta is commonly blocked with an acetyl group and amino acid residue 115 is trimethyllysine (29). Calmodulin is a dumbbell-shaped molecule with a central alpha-helix that connects two globular Ca^{2+}-binding domains. Recently, a calmodulin from rat liver was isolated and shown to be phosphorylated at three sites (32). Two of the residues found to be phosphorylated are situated within the central alpha-helix and the third is located in one of the Ca^{2+}-binding domains. The phosphorylation of these residues was shown to decrease the affinity of calmodulin for its target site in myosin light-chain kinase (32,33). There is tentative evidence that the delta subunit of rabbit skeletal muscle Phk might contain ~1 mol of phosphate (15). The delta subunit is responsible for the Ca^{2+}-dependent activity of Phk, although there are noted differences in how calmodulin is involved in the structure and regulation of Phk compared to how it participates in the regulation of other Ca^{2+}-dependent enzymes (1). Calmodulin most commonly binds to target proteins in a Ca^{2+}-dependent manner and dissociates when the $[Ca^{2+}]$ is lowered to sub-micromolar levels. The delta subunit is unusual in that it remains an integral component of the Phk holoenzyme even when the $[Ca^{2+}]$ is lowered to nanamolar levels 91,340.

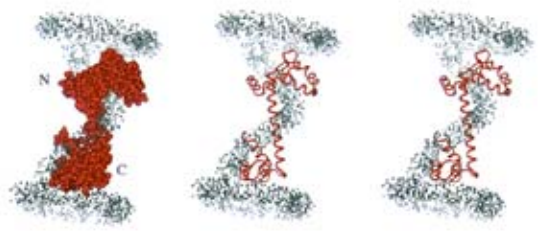

Figure 5. Model for the structure of the troponin C-I complex derived from small-angle X-ray and neutron scattering measurements. The panel on the left shows a space-filling representation of troponin C bound to troponin I. The panels in the center and on the right is a stereoview of the ribbon representation of troponin C bound to troponin I. The N and C termini of troponin C are indicated. In both figures, the structure of troponin I is represented by green dots that were generated by a Monte Carlo modeling program as derived from the scattering data. The figure was reproduced from reference (36) with permission.

Not much is known regarding the molecular nature of the subunit-subunit interactions involved in the gamma-delta association, but a good possibility is that they may be similar to the interactions of troponin C in the troponin complex [26, 28]. The troponin complex interacts with tropomyosin to regulate the Ca^{2+}-dependent contraction of skeletal muscle. The troponin complex contains three subunits, troponins C, I, and T. Troponin C and calmodulin share a high degree of structural and functional homology, but each recognizes and regulates its own unique set of target proteins (1,35). Interestingly, the troponin C-binding domain in troponin I and the calmodulin-binding domains in the gamma-M subunit share significant structural homology (21). As is the case for the delta subunit of Phk, troponin C remains bound to troponin I when the [Ca^{2+}] is lowered to nanomolar levels. Based on small-angle X-ray (SAXS) and neutron (SANS) scattering measurements, a structural model for the troponin C-I complex in solution has been proposed as shown in figure 5 (36,37). In this model troponin C is folded into a rather extended conformation. It has been suggested that in the gamma-delta complex the delta subunit likewise might assume an extended conformation and resemble that of troponin C in the C-I complex (26, 38). This is somewhat unusual in that the extended conformation of calmodulin typically becomes more compact and globular upon Ca^{2+}-dependent binding to its target proteins (33, 42).

The two calmodulin-binding sites in the gamma-M subunit were first identified by peptide studies. Two synthetic peptides, denoted in the original literature as PhK5 (same amino acid sequence as residues 301-327 in gamma-M) and PhK13 (same amino acid sequence as residues 342-367 in gamma-M), have been used extensively to study potential gamma-delta interactions (21, 38,43). PhK5 and PhK13 have the same amino acid sequences as the two calmodulin-binding domains in gamma-M. Calmodulin (delta subunit) is capable of binding these two peptides simultaneously, but only in the presence of Ca^{2+} (21). Calmodulin becomes more compact and globular when it binds to the PhK5 peptide, and so the interactions involved appear to share many of the general features of Ca^{2+}-dependent calmodulin-binding to other target enzymes (21, 38, 43). In contrast, calmodulin assumes an extended conformation when it binds to the PhK13 peptide. The calmodulin-binding domain represented by PhK5 is predicted to fold into an amphipathic alpha helix, as, it is believed, do the calmodulin-binding domains in most other calmodulin target proteins (21,26). The PhK13 peptide, on the other hand, is predicted to fold into a beta-turn / beta-sheet, which could account for the more extended structure of calmodulin when bound to this peptide. When the PhK5 and PhK13 peptides bind simultaneously with calmodulin, calmodulin assumed an extended conformation [38, 43]. it would therefore appear that the effects of the PhK13 peptide predominate over the effects of the PhK5 peptide and, by inference, that the delta subunit is more likely to be in an extended conformation when bound to the gamma subunit (21, 36, 38). It has been suggested that an extended conformation of the delta subunit in the Phk holoenzyme might allow for more extensive intersubunit contacts as required for the optimal regulation of gamma subunit activity (26). It is important to note that calmodulin does not bind to either PhK5 or PhK13 when the [Ca^{2+}] is lowered to nanomolar levels which suggests that these two peptides do not fully reflect the delta-gamma interaction in Phk (21). It has been suggested that synergism resulting from the proper juxtaposition of the two gamma subunit calmodulin-binding domains in the full-length protein might provide gamma with extremely high-affinity for calmodulin, even when the [Ca^{2+}] is lowered to nanomolar levels even though the individual domains can only bind to calmodulin in a Ca^{2+}-dependent manner (21). The predominant alpha-helix of calmodulin wraps around its binding site in target proteins (26 ,39, 40, 42). The ability of calmodulin to adopt different conformations is facilitated by flexibility in this alpha-helix and this flexibility is thought to play an important role in facilitating different types of calmodulin-target enzyme interactions [26, 39, 42, 44]. The gamma-delta subunit interaction probably requires the central alpha helix of the delta subunit to have a certain minimum length (26). The binding of Ca^{2+} to calmodulin triggers a conformational change that exposes hydrophobic surfaces in its globular Ca^{2+}-binding domains. Similar hydrophobic surfaces become exposed when Ca^{2+} binds to troponin C (45, 46). These hydrophobic surfaces interact with complementary sites in target enzymes and provides the driving energy for calmodulin (or troponin C)-target protein association. These hydrophobic interactions are a general feature of the Ca^{2+}-dependent association of calmodulin with its target enzymes. Similar hydrophobic surfaces are likely to be involved in some of the delta-gamma interactions. Calmodulin binding domains typically fold into amphipathic alpha-helices with hydrophobic residues that are spaced 8 to 12 amino acids apart in the primary sequence (44). They also typically contain an aromatic amino acid residue near their N-terminal boundary (25). PhK5 possesses these classical calmodulin binding elements. The PhK13 peptide also

contains an aromatic (phenylalanine) amino acid residue near the amino terminal boundary of its calmodulin-binding site (residue 304), however, when that phenylalanine was replaced with an alanine, the affinity of PhK13 for calmodulin-binding increased instead of decreased (25). This suggests that the calmodulin-binding domain corresponding to gamma-$M_{302-326}$ is somehow different from the classical calmodulin-binding domain found in other proteins, and this supports that idea that PhK13 might adopt a secondary structure that differs from the classic amphipathic alpha helix. Indeed, it has been suggested that the calmodulin-binding domain represented by PhK13 forms a beta-turn / beta-sheet (21, 26).

The gamma-delta interaction exhibits pH-dependency, which suggests that ionic interactions are involved (24). Chan and Graves have provided evidence that Ca^{2+} strengthens the interaction between delta and the gamma-M subunit, as well as the interaction between delta and the alpha and / or beta subunits, (47). In the absence of the alpha subunit, and in either the presence or absence of Ca^{2+}, mild denaturants such as urea can dissociate the delta subunit from the gamma-M -delta dimeric complex (47). However, urea does not dissociate the delta subunit from either the ternary alpha- gamma-M delta complex or the Phk holoenzyme when Ca^{2+} is present. These observations would seem to suggest that the alpha subunit influences delta subunit-gamma subunit interactions, possibly by providing a scaffold upon which the trimeric complex is built (28).

The interactions between delta and the other Phk subunits are complex and have unique characteristics. The interactions between alpha, beta, gamma and delta must change upon enzyme activation but there is presently no specific data that describe the possible changes. Enzyme activation by most means likely involves changes in all or most of the subunit interactions, and as discussed in further detail in a later section of this review, the intersubunit interactions involving the delta subunit might switch between different subunits upon enzyme activation. Most structural studies undertaken to date have utilized isolated delta subunit and peptides that represent the delta subunit-binding domains from gamma-M. There is no basic structural information regarding the delta-gamma-M complex. In spite of its relatively small molecular mass, calmodulin has been shown to form 185 contacts with a calmodulin-binding peptide isolated from myosin light chain kinase (39). Thus the delta subunit has the potential to make a large number of contacts with the other Phk subunits, and the structural flexibility of its central alpha-helical segment would allow its globular Ca^{2+}-binding domains to assume any one of a number of possible relative orientations.

3.1.3. The alpha and beta regulatory subunits

The alpha and beta regulatory subunits are large proteins that together account for 81% of the mass of the Phk holoenzyme. The two subunits are homologous and likely arose from a common ancestral gene (48). Together the alpha and beta subunits inhibit the catalytic activity of gamma, but the regulatory properties unique to each subunit remain to be elucidated (47, 49, 50, 51). The alpha subunit protein (figure 2) isolated from rabbit fast-twitch skeletal muscle, referred to as alpha-FM (FM denotes the fast-twitch skeletal muscle isoform), contains 1237 amino acids and has a molecular mass of 138 kDa (52). The rabbit, rat, and human alpha-FM proteins share 95% identical amino acid sequences (53). The beta subunit protein (figure 2) isolated from rabbit skeletal muscle, denoted beta-M (M denotes the muscle isoform) contains 1093 amino acids and has a molecular mass of 125 kDa (48). The rabbit and human beta-M proteins share >92% identical amino acid sequences. The N-terminus of the alpha-FM protein does not appear to be post-translationally blocked. The N-terminus of recombinant alpha-FM protein expressed in baculovirus-infected insect cells is also unblocked (Brushia and Walsh, unpublished observations). The N-terminus of the beta-M subunit is blocked by acetylation (48). Since the alpha and beta subunits do not share significant sequence homology with any other known proteins, including the regulatory subunits and/or domains of other protein kinases, their primary sequences have not yet provided much insight into their function.

The alpha and beta proteins contain a polyisoprenylation consensus sequence at their C-termini (48). Heilmeyer *et al.* have shown that the rabbit alpha-FM and beta-M proteins are farnesylated (48, 54-56) but the function of the farnesyl group in Phk is unknown. Farnesylation has been shown to promote the membrane association of Ras proteins and there have been various reports of membrane-localized Phk suggesting the possibility that the farnesylation of Phk may be directing its cellular localization (55-58). A second possible role for farnesylation is that it might stabilize the structure of the alpha and beta subunits in the Phk holoenzyme in a manner similar to how the structure of the catalytic subunit of PKA appears to be stabilized by N-terminal myristylation (59). Farnesylation might also promote specific interactions between Phk and other proteins (56). Available data suggests that the muscle holoenzyme contains only fully farnesylated alpha and beta subunits (54). The question as to whether farnesylated and unfarnesylated subunits ever combine to give rise to hybrid structural/functional holoenzyme variants *in vivo* merits investigation. All of the alpha and beta subunit isoforms for which we have cDNA-inferred sequence information contain a polyisoprenylation consensus sequence (60).

The alpha and beta proteins as isolated from mammalian tissues contain covalently-linked phosphate. The alpha-FM protein contains at least seven sites of phosphorylation (48). All seven sites are serine residues and all seven are clustered in a relatively small region of the protein (amino acids 972 to 1030 in rabbit alpha-FM). This region is referred to as the "multiphosphorylation domain" but constitutes only a very small part of the alpha subunit protein (~5 %; figure 6) (52). Ser^{1018} within this multiphosphorylation domain is phosphorylated by PKA and is a major site of regulatory phosphorylation *in vivo* (1). Ser^{1018} and at least three of the other six serine residues (Ser^{972}, -^{985}, and -^{1007}) can be phosphorylated *in vitro* by Phk itself (autophosphorylation); the kinase(s) responsible

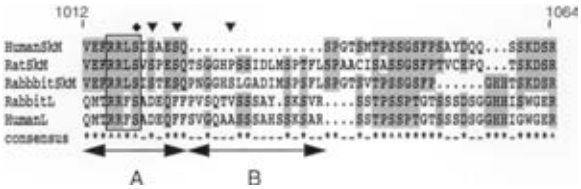

Figure 6. The α subunit multiphosphorylation domain from different species and tissues. Sequences are aligned for maximum homology. N is with respect to thealpha subunit from rabbit skeletal muscle. The PKA recognition site (RRXS) is boxed and the serine phosphorylated by PKA *in vivo* is indicated by a black diamond (Ser^{1018} in the rabbit skeletal muscle isoform). Sites of endogenous phosphate incorporation in the rabbit alpha-FMsubunit are indicated by black triangles. Segments A and B that can be deleted individually or in combination, as discussed in the text, are also indicated. Dots in the alignments represent gaps introduced to achieve maximum alignment. The line labeled "consensus" denotes amino acids that are totally conserved (arrow head) or fairly well conserved (asterisk) in all a variants shown. This data used to construct this figure was taken from references (48,53,60).

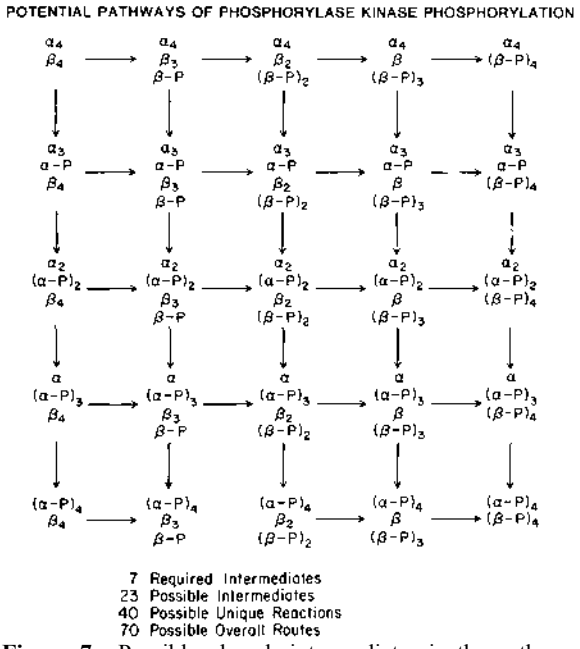

Figure 7. Possible phosphointermediates in the pathway leading to fully phosphorylated Phk by PKA. The Phk holoenzyme is depicted as $alpha_4beta_4$ and the gamma and delta subunits are not indicated for simplicity. As indicated at the bottom of the figure, at least 7 intermediates are required in going from unphosphorylated to fully phosphorylated Phk. Along the way to fully phosphorylated enzyme, there are 23 possible distinct intermediates, 40 possible unique reactions, and 70 possible overall routes. This figure was reproduced from reference (88) with permission.

for phosphorylating the other three sites (Ser^{1020}, -^{1023}, and -^{1030}) has not been identified (61). The existence of more than one PKA-dependent phosphorylation site in the alpha subunit has been demonstrated for Phk phosphorylated *in vitro*, but the additional site(s) have not been identified. Ser^{434} is a possible candidate for a second PKA site since it is in a strong consensus sequence for PKA-catalyzed phosphorylation. The clustering of phosphorylation sites in the multiphosphorylation domain of the alpha subunit suggests a regulatory function for this domain. A similar clustering of sites is an important regulatory feature of glycogen synthase (62). Glycogen synthase is a substrate for both casein kinase II (CK-II) and glycogen synthase kinase 3 (GSK-3) in a regulatory process termed "Hierachal Protein Phosphorylation" (62). Phosphorylation by GSK-3 only occurs after the phosphorylation of a specific serine residue by CK-II with the serine phosphate created by CK-II acting as a primary recognition determinant for GSK-3. GSK-3 subsequently phosphorylates four additional residues with the four sites phosphorylated in a specific order because each subsequent addition of phosphate by GSK-3 creates a new GSK-3 recognition site. Phosphate might be incorporated into the multiphosphorylation domain of the Phk alpha subunit in a similar ordered manner by more than one kinase. A lag in the time-course of glycogen synthase phosphorylation was what initially suggested that phosphate incorporation was ordered (62). A the lag in Phk "autophosphorylation" is clearly evident under some circumstances and might be accounted for by a similar ordered phosphorylation mechanism (63).

The beta-M subunit contains numerous phosphorylation sites,, not all of which have been identified. Three sites (Ser^{11}, Ser^{26}, and Ser^{700}) have been shown to be autophosphorylated *in vitro*. Ser^{26} is phosphorylated by PKA *in vivo* and is a major site of regulatory phosphorylation (48). Ser^{700} lies within a "consensus sequence" for PKA-catalyzed phosphorylation and might be potentially phosphorylated by PKA under some unique regulatory situation. Whether or not autophosphorylation of the alpha and beta subunits occurs *in vivo* is not known (48). The phosphorylation of Phk *in vivo* as so far examined has not been Ca^{2+} sensitive, as would be characteristic of autophosphorylation. It is intriguing that the phosphorylation sites thus far identified in alpha and beta are situated in domains that are unique to each subunit (i.e., where the two proteins do not share any significant sequence homology) (48). These unique domains are also hydrophilic and therefore likely to be surface exposed. With the exception of the well-characterized PKA-target sites, little is known regarding the relationship between the phosphorylation of specific sites in the alpha and beta subunits and the corresponding effects on the structure/activity of the holoenzyme. Phosphorylation-dependent activation does appear to influence some subunit-subunit interactions involving the delta subunit, possibly causing it to change its site of interaction between gamma and the alpha and/or beta subunits (25). The PKA-dependent phosphorylation of the alpha and beta subunits results in the deinhibition of the phosphotransferase activity of the gamma subunit, thereby resulting in increased catalytic activity (1). Since each alpha and beta subunit in the holoenzyme can be phosphorylated, and since there are four of each subunit, there are a large number of possible phosphorylated intermediates and potential pathways by which to achieve fully phosphorylated enzyme (figure 7). The

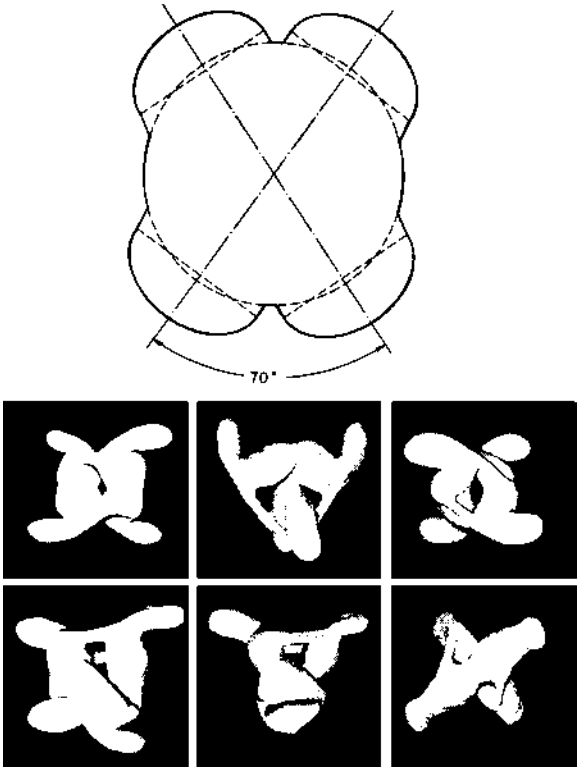

Figure 8. Structural representations of Phk. Upper Panel: Model of Phk derived from x-ray scattering. Details are provided in (69). Resolution of the scattering data modeled Phk as one central ellipsoid of semi-axes dimensions a = 105 Å, b= 105 Å, c= 40 Å and plus four semi-ellipsoids located at the periphery of semiaxes a= 60 Å, b= 54 Å, c= 42.5 Å, with the center of the major ellipsoid located at x = 0, y = 0, z = 0, and the centers of the semi-ellipsoids all on the z = 0 Å plane at x = 60 Å, y = 80 Å; x = 60 Å, y = -80 Å; x = -60 Å, y = 80 Å; and x= -60 Å, y = -80 Å. Lower panel: Model of the Phk holoenzyme structure derived from electron microscopy. The model is shown in several views that represent the different holoenzyme orientations observed in EM. These figures were reproduced with permission from (70).

phosphorylation of Phk must in some manner promote conformational changes and/or changes in subunit-subunit interactions but there are no direct insights into the specific mechanism(s).

To date, insights regarding how phosphorylation of a protein might induce structural and hence functional changes has come primarily from the elegant structural studies performed on glycogen phosphorylase (GP), the substrate of Phk (64-66). The high-resolution structures of the phosphorylated and unphosphorylated forms of GP have revealed some general principles regarding the structural effects of phosphorylation and show the far-reaching possible consequences to the entire protein structure. With the introduction of a single phosphate per molecule of the dimer, multiple and extensive changes occur. In the immediate vicinity of the introduced phosphate both hydrophobic and hydrophilic interactions occur including the direct interaction of the introduced phosphate with specific amino acid side chains. GP is thereby converted into an active conformation that has a catalytic cleft that is more accessible to substrate. Substantial spatial change occurs in a large percentage of the protein. Similar types of changes are likely in the phosphorylation of Phk that leads to its activation. Some limited supporting data for this exists. Detectable changes are apparent by electron microscopy (1). Phosphorylated Phk is also more prone to aggregation as evidenced by SAXS experiments, suggesting that phosphorylation promotes a conformational change that exposes new surfaces on the holoenzyme molecule.

In addition to the effects mediated by the endogenous delta subunit, the Phk holoenzyme can also be activated by exogenous calmodulin (referred to as delta' subunit). The binding of delta' to the Phk holoenzyme is Ca^{2+}-dependent (1, 34). Domains in the alpha-FM (corresponding to amino acids 1070-1093) and beta-M (corresponding to amino acids 5-28 and 770-794) subunits have been identified as likely exogenous calmodulin-binding sites (52, 67). These domains have high calmodulin-binding affinities as ascertained in binding studies that utilized peptides to represent the respective domains, and indeed beta peptide 770-790 has the highest calmodulin affinity yet observed for any peptide. The rabbit alpha-$FM_{1070-1093}$ and beta-M_{5-28} domains lie immediately adjacent to major sites of regulatory phosphorylation in those respective subunits. Such a structural relationship could provide the structural basis for regulatory interplay between phosphorylation and Ca^{2+} [25, 48]. In addition to its regulation by extrinsic calmodulin, Phk can be activated by skeletal muscle troponin C (1). Although troponin C and calmodulin are homologous proteins with very similar structures, much higher levels of troponin C than calmodulin are required for Phk. Nevertheless, the high level of troponin C in skeletal muscle has led to the suggestion that troponin C rather than calmodulin might be the physiological activator of Phk in muscle. Of note, cardiac troponin C does not substitute for skeletal muscle troponin C in the activation of skeletal muscle Phk, and neither skeletal muscle nor cardiac troponin C, nor calmodulin, can activate cardiac Phk.

3.1.4. The structure and subcellular distribution of the holoenzyme

Structural information for the Phk holoenzyme has come from a combination of subunit cross-linking experiments, EM studies and SAXS studies of the holoenzyme in solution. SAXS can be used to estimate the overall shapes of monodisperse particles in solution, and to investigate the nature of conformational changes accompanying activation (68). SAXS studies indicated that the Phk holoenzyme is prone to aggregation when it becomes activated by phosphorylation, Ca^{2+} addition, or Ca^{2+} and delta´ addition (69). Aggregation precluded structural analysis of the activated enzyme. The unactivated Phk enzyme was not prone to aggregation and its overall dimensions in solution were determined(69). A model based on these dimensions was constructed that is shown in figure 8, upper panel. The model depicts a large central disk with four ellipsoids at its periphery and a maximum planar dimension of ~285Å. The model has a

very small third-dimension (i.e., the disk is extremely thin). Phk is presumed to be a tetramer of tetramers and in accord the optimum model was found to have a 4-fold symmetry.

Cross-linking and EM studies have provided the major base of information for what is currently known of possible subunit topology in the Phk holoenzyme; the early studies are reviewed in (1). From the total set of EM observations a three-dimensional model for the Phk holoenzyme structure has been proposed that is depicted in figure 8, lower panel (70). Phk visualized by EM has been described as two opposing parentheses held together by short cross-bridges (this structure is represented schematically in figure 1). This has classically been termed the "butterfly" orientation (1). In the EM-based model, four identical alpha-beta-gamma-delta tetramers associate with D_2 symmetry to form the two major lobes of the Phk holoenzyme. Two tetramers associate in a head-to-head arrangement with one tetramer comprising each lobe of the "butterfly". The lobes are connected by bridges that appear to be formed, at least in large part, by the beta subunits. This important role for the beta subunit in forming the quaternary structure of Phk is supported by the set of observations that (i) an epitope in the beta subunit is localized to an interior position on the lobes near the interconnecting cross-bridges, (ii) that proteolytic cleavage of the beta (and the alpha) subunit disrupts the cross-bridges, and (iii) that an alpha-gamma-delta trimer is produced by LiBr-mediated precipitation of the beta subunit from the holoenzyme (1,50,71). The molecular dimensions of the EM-based model are consistent with those determined by small-angle X-ray scattering measurements (69). Fruitful avenues need to be found by which to further elaborate the details of Phk structure. This problem has been made particularly difficult because the full-length subunits have not been available individually as soluble proteins and that yield of partial complexes prepared by the dissociation of holoenzyme are very poor. Recently, however, partial complexes have been obtained in excellent yields by expression in cultured insect cells (Brushia and Walsh, in preparation).

The subcellular distribution of the Phk holoenyzme has only been partially characterized. There have been several reports that Phk associates with membranes in a variety of tissues, including skeletal muscle (1, 57, 58, 73, 74). "Glycogen particles" associate with sarcoplasmic reticulum in muscle and this association might account for the presence of Phk in preparations of sarcoplasmic reticulum membrane. Conversely, sarcoplasmic reticulum is often a contaminant of "glycogen particle" preparations. ("Sarcoplasmic reticulum preparations" and "glycogen particle preparations" likely more reflect the investigators perspective than distinct subcellular entities.) Defining the amount of Phk that is specifically associated with the sarcoplasmic reticulum is therefore of ambiguous physiological significance. (74). A protein referred to as PTG (acronym for protein targeting to glycogen) has been identified and cloned (72) that appears to act as a molecular scaffold for the assembly into a "metabolic glycogen particle module" of glycogen phosphorylase, glycogen synthase, related enzymes of glycogen metabolism and glycolysis, and various phosphatases and kinases including Phk (72). This molecular scaffold appears to be the structural basis of the glycogen particle. Evidence from immunofluorescent localization studies suggests that a significant fraction of skeletal and cardiac muscle Phk is located at transverse tubule and terminal cisternae membranes. Such a localization has led to the speculation that Phk might have a direct role in Ca^{2+} mobilization in muscle tissues, possibly via a role in the regulation of a Ca^{2+} transport ATPase (73, 74). The Phk subunits are presumed to be present in equimolar amounts in the holoenzyme, however some sarcoplasmic reticulum fractions appears to contain a molar excess of the alpha and beta subunits relative to the gamma. An association of Phk with membranes in tissues other than skeletal muscle has also been observed (1, 58). Paudel reported that Phk is associated with microtubules in rat brain homogenates and speculated that Phk might function in the regulation of microtubule structure in brain (75). Jennissen et al. observed Phk in the basal lamina, although it was unclear if the enzyme was truly extracellular, or associated with membranes at the periphery of cells, thereby giving the appearance of being in the basal lamina (73). These observations raise questions about a possible more extensive physiological function for Phk beyond the regulation of glycogen phosphorylase.

3.2. Subunit isoforms and their tissue distributions

A listing of the Phk subunit isoforms is presented in table 1.

3.2.1. The gamma subunit

To date three isoforms of the Phk gamma subunit have been elucidated. A homologous (probably identical) gamma subunit (gamma-M) is expressed in both cardiac and skeletal muscle tissues (13). A testis/liver-specific isoform, referred to as gamma-LT, shares ~70 % amino acid sequence identity with gamma-M (27, 76, 77). Knowledge of liver Phk and its regulation remains rudimentary; the liver enzyme has been difficult to study because it is extremely susceptible to proteolysis and activation. Whether gamma-LT is the major liver subunit isoform remains unknown. The expression of gamma-LT mRNA in rat testis correlates with postnatal testicular development suggesting a possible association with maturing germ cells. A *Drosophila* gamma subunit (DPhK-gamma) has been reported that shares ~60% amino acid sequence homology with gamma-M. DPhK-gamma has domains homologous to both the N-terminal catalytic domain and C-terminal calmodulin-binding domains of gamma-M, but in contrast to gamma-M, where these two regions are linked by a stretch of just 11 amino acids DPhK-gamma has a unique 179 amino acid linker domain that is extremely glutamine-rich. The function of this extended linker region in DPhK-gamma is unknown. Whether or not DPhK-gamma is regulated by *Drosophila* homologues of the alpha, beta, and delta subunits is not known. Of note, DPhK-gamma knock-out mutants failed to fully develop leg muscles (78).

3.2.2. The delta subunit

The delta subunit is identical to calmodulin. Calmodulin isolated from different species and tissues are identical (29). Three human genes code

Table 1. PHK subunit isoforms

Subunit Isoform	Function	Tissue Distribution[a]	Gene Location[b]	Approx. Mass (Da)
Alpha-FM	Regulatory	Fast Twitch Skeletal Muscle, Brain	Xq13	138,000
Alpha-prime	Regulatory	Slow Twitch Skeletal Muscle, Heart	Xq13	132,000
Alpha-L	Regulatory	Liver, Brain	Xp22	138,000
Beta-M	Regulatory	Fast and Slow Twitch Skeletal Muscle	16q12-q13	125,000
Beta-B	Regulatory	Brain, Intestine, Uterus, testis	16q12-q13	125,000
Beta-L	Regulatory	Liver, Heart, Intestine, Brain	16q12-q13	125,000
Gamma-M	Catalytic	Skeletal Muscle, heart	7p2	45,000
Gamma-LT	Catalytic	Liver, testis, Lung	16p11,2-12.1	45,000
Delta	Regulatory	All tissues	16q24-q31, 2p21, 19q13	16,500

[a] Not an exclusive list. In addition to those listed, low to trace levels of mRNA expression have been observed in other tissues for various isoforms. The isoform expression has not yet been t investigated in many tissues. [b] The chromosomal loci of structural genes for the different subunits were obtained from the following references: Muscle α subunit, (122,123); liver α subunit, (82,100); β subunit, (85, 122); muscle γ subunit, (80); testis γ subunit, (124); δ subunit, (79). Other references are provided in the text.

for identical calmodulin proteins (79, 80). It is likely that all three genes are used to maintain a calmodulin pool which serves as the source of the Phk delta subunit (79, 80).

3.2.3. The alpha and beta subunits

Many structural variants of the alpha and beta subunit exist, those characterized to date are summarized in table 1. The different forms appear to be a consequence of a combination of gene duplication and divergence and differential mRNA splicing. The alpha and beta subunits undergo extensive post-translational modifications that create structural diversity. Each of the alpha and beta subunit isoforms has a unique tissue distribution, and some tissues contain two or more isozyme forms (53, 60). Thus a single cell might contain more than one form of Phk with each form having distinctive functional properties. (60). Phk Isoforms could also arise from individual holoenzyme molecules being assembled with more than one species of a given subunit isoform. Such diversity could contribute to extensive fine-tuning of Phk's regulatory properties with respect to the specific metabolic requirements of various tissues (82).

The alpha-prime isoform was originally observed as a minor protein in pure skeletal muscle Phk that migrated faster than alpha-FM in SDS-PAGE. The alpha-prime isoform was subsequently shown to also be the predominant alpha subunit isoform in slow-twitch skeletal muscle and heart. Kilimann et al. demonstrated that the alpha-prime isoform arises from differential mRNA splicing of the alpha-FM mRNA (60), with an internal deletion of 59 amino acids (amino acid residues 654-712) from alpha-FM giving rise to alpha-prime. The deletion results in the loss of 6418 Da of mass from alpha-FM (figure 9). Phk holoenzyme that contains the alpha-FM isoform can be activated by exogenous calmodulin (delta′), but Phk containing the alpha-prime isoform cannot be (1). Since the alpha-FM and alpha-prime isoforms each has their own unique tissue distribution (table 1), the alpha/alpha-prime deletion has tissue-specific regulatory implications. The cDNA for a liver-specific alpha isoform (alpha-L) has been cloned and sequenced [81, 82]. The alpha-L and alpha-FM isoforms share 68% amino acid sequence identity and 15% conservative amino acid substitutions. The alpha-L and alpha-FM isoform sequences diverge in regions where the alpha-FM and beta subunit sequences are also most dissimilar (82). The alpha-L and alpha-FM isoforms are encoded by two different genes with both genes located on the human X-chromosome (table 1 and reference (82). The sequence that is deleted from alpha-FM to create alpha′ is retained in alpha-L. The existence of a neonatal alpha subunit isoform has been suggested but not confirmed (83, 84).

At least three beta subunit isoforms exist; the muscle beta-M, the liver beta-L, and the brain beta-B (table 1 and reference (60). There is evidence for only a single beta subunit gene that is located on human chromosome 16, and all beta subunit isoforms appear to arise from differential splicing of the same mRNA transcript (85). The beta-M and beta-L isoforms share identical N-terminal amino acid sequences (amino acid residues 1-25); this sequence is replaced with 18 different amino acids in the beta-B isoform (figure 10). Phosphorylation of Ser26 in beta-M by PKA is a major mechanism for the activation of Phk in vivo. Although Ser26 is conserved in all three beta subunit isoforms, the change in residues in the brain (beta-B) isoform (figure 10) might alter its capacity to act as a PKA substrate. Alternative splicing also leads to the replacement of amino acid residues 779-806 in the beta-M isoform sequence with an identical alternative sequence in the beta-B and beta-L isoforms (figure 10 and reference (60). This differential splicing of the beta subunit mRNA effects two putative calmodulin-binding sites. The N-terminal calmodulin-binding site in beta-M and beta-L (residues 5-28) is missing from the beta-B isoform, and the internal very high affinity calmodulin site in beta-M (residues 767-794) is changed significantly in the beta-L and beta-B isoforms (1, 60). Thus, differential mRNA splicing could lead to an altered response of Phk to both of its major physiological regulators in a tissue-specific context. These possible differences remain to be evaluated. No other beta subunit isoforms have been characterized to date, but other isoforms likely exist [60]. These additional isoforms would likely arise from differential mRNA splicing as there is presently no evidence for additional beta subunit genes (80, 86).

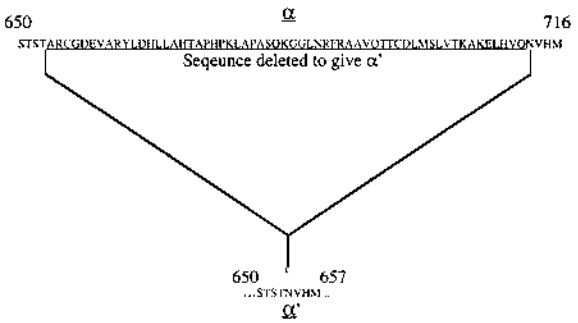

Figure 9. The amino acid segment that is deleted from the rabbit skeletal muscle alpha protein to give the alpha prime isoform.

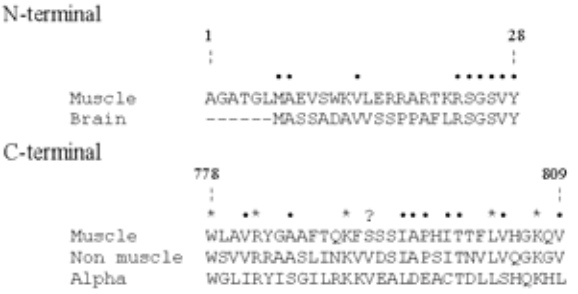

Figure 10. Alternate forms of Phk beta subunit Top: Alignment of two alternative N-terminal beta sequences. The muscle-specific sequence corresponds to amino acids 1-25 of the rabbit muscle protein. The dashes in the brain-specific sequence denote the absence of amino acids in these positions. Thus, the brain-type sequence starts at methionine and there is no evidence that this methionine is post-translationally removed. Lower: Alignment of an internal segment of the beta-M isoform (muscle-specific sequence, amino acid residues 779-806 in the rabbit protein) with the alternative "nonmuscle sequence" present both the beta-L and beta-B isoforms. The mutually exclusive muscle and nonmuscle internal sequence shares 43% identity and is in a region in which the alpha-FM and beta subunits also share homology. (The sequence segment corresponding to amino acids 823-854 in the rabbit alpha-FM are also shown.) Interestingly, the "nonmuscle sequence" is nearly as homologous to its counterpart in the alpha-FM subunit isoform as it is to its counterpart in the beta-M sequence. The data used to construct this figure was obtained from reference (60).

Phosphorylation sites are clustered in a small "multiphosphorylation domain" within the alpha subunit (figure 6) (53) and the primary structure of this domain has extensive species- and tissue- specific variability as a result of differential mRNA splicing (53). Segment A of the multiphosphorylation domain (corresponding to amino acids 1012-1024, figure 6) contains the major site of PKA phosphorylation. Segment B (corresponding to amino acids 1025-1041) contains sites of endogenous phosphate incorporation. These sites are deleted in various isoforms either individually or in combination (53). The full-length alpha-FM protein, containing both segments A and B is present in rabbit muscle, liver, heart, intestine, uterus, brain, kidney and testis. A structural variant lacking segment A is coexpressed along with the full-length alpha-FM in liver, intestine, uterus, brain, lung and kidney. A structural variant that lacked both segments A and B or only segment B was not observed in any of the rabbit tissues that were examined. In contrast, the full-length alpha-FM protein (i.e., containing both segments A and B) was not observed in a range of human tissues examined. Human tissues contain either a variant that have segment A but lack segment B or one in which both segments A and B (of the rabbit alpha-FM protein) are missing. This latter form would lack both the main site of PKA phosphorylation (Ser^{1018}) and several sites of autophosphorylation and would certainly give rise to a very distinctive Phk holoenzyme. Similar analysis of the multiphosphorylation domain in the liver-specific alpha subunit isoform (alpha-L) revealed an equally complex pattern of splice variant expression (53). In rabbit, only the full-length alpha-L isoform containing both segments A and B was observed in all tissues examined, whereas an alpha-L structural variant lacking either segment B or both segments A and B was observed in human muscle, heart, and lymphocytes. The sequence of the multiphosphorylation domain diverges between the liver and muscle alpha isoforms as shown in figure 6 (82). Three sites that contain endogenous phosphate in the alpha-FM isoform (Ser^{1018}, -^{1023}, and -^{1030}) are not conserved in the alpha-L isoform (82). An implication of these observations is that the Phk from different tissues and different species are likely to have quite different regulatory properties (53). These data indicate that there is likely to be major differences in the consequences of PKA-dependent phosphorylation in different tissues. Alternative mRNA splicing appears to be primarily directed to modification of either phosphorylation sites or putative calmodulin-binding sites (85) suggesting a broad variation in physiological control patterns. Differential splicing appears to reflect a general evolutionary mechanism that has led to the adaptation of the Phk response to various regulatory signals in specific tissues.

4. THE REGULATION OF ENZYMATIC ACTIVITY

In the absence of the other subunits, free Phk gamma subunit exhibits a greater phosphotransferase activity than the holenzyme, even when the latter is maximally activated by any of a variety of processes. It is now well evident that the regulatory subunits act as an inhibitor of gamma catalytic activity and that activation occurs as a consequence of these inhibitory effects being reversed. This type of "deinhibition-activation" mechanism is evident in many protein kinases. In those best characterized, inhibition is a consequence of an autoinhibitory domain acting as a pseudosubstrate and in consequence blocking the access of protein substrate and the closure of the catalytic cleft. There are several suggested autoinhibitory domains in the phosphorylase kinase regulatory subunits including a domain with homology to glycogen phosphorylase on the beta subunit (amino acid residues 420-436) (51) and the two PKA phosphorylation sites on the beta (Ser^{27}) and alpha (Ser^{1018}) subunits that are also major sites where

autophosphorylation occurs. It is likely that a "pseudosubstrate inhibitory mechanism" explains how Phk is regulated but there as yet is no direct evidence that would indicate which if any of these three sites might be the critical catalytic subunit regulator.

4.1. Phosphorylation

The regulation of Phk phosphotransferase activity by phosphorylation has been thoroughly reviewed elsewhere (1, 61, 87, 88). Briefly, it appears that PKA-catalyzed phosphorylation of the beta subunit leads directly to an increase in the specific activity of Phk, and that alpha subunit phosphorylation amplifies this effect but alone does not directly activate (61, 88). Autophosphorylation also significantly increases the specific activity of Phk *in vitro* (87*)*. The correlation between the autophosphorylation of specific amino acids and Phk activation is not well characterized. Autophosphoryation results in the phosphorylation of many sites including those phosphorylated by PKA. The early preliminary data had suggested that Phk activation caused a change in the K_m for the substrate phosphorylase (1), but a subsequent more detailed analysis has shown that the effect of PKA-catalyzed phosphorylation of Phk is predominantly to promote a change the V_{max} (89). Physiologically such an activation mechanism is the most appropriate. Glycogen phosphorylase is at nearly saturating concentration in muscle with respect to Phk and in vast excess of its K_m value, a circumstance that is defined as "zero-order ultrasensitivity" (89). Thus a change in the Km value for phosphorylase upon Phk activation would give rise to little to no change in the rate of muscle glycogenolysis, whereas the system will be exquisitely sensitive to a change in the catalytic capacity (i.e. V_{max}), as indeed occurs.

Since both the alpha and beta subunits are phosphorylated by PKA, and since there are four of each subunit, there are many potential phosphorylated intermediates in the cAMP-dependent activation pathway of Phk (figure 7). Almost certainly major amounts of these intermediates are produced in the cell in response to cAMP, and indeed physiologically the fully phosphorylated species may only be produced rarely, if at all. The physiological implications of the existence of these intermediates is an important area that needs to be explored. Each phosphorylation event produces a unique protein species whose catalytic activity, sensitivity to allosteric activators, and efficacy as a substrate for further phosphorylation or for dephosphorylation, will also possibly be unique for that species. Thus the possible complexity of the phosphorylation-dependent activation and dephosphorylation-dependent inactivation is enormous. The two sites in Phk that are phosphorylated by PKA are dephosphorylated by different protein phosphatases (61). As one consequence it is to be expected that the pathway of intermediates that occurs with phosphorylation will likely be different from the pathway of dephosphorylation, thus leading to a different set of intermediates being produced during the activation and inactivation phases. Such a hysteresis may well be of considerable physiological consequence. As reviewed elsewhere (120), the consequences of sequential phosphorylation of either single sites in a multivalent protein or distinct sites adds a whole new layer of regulatory complexity not yet investigated for any of the myriad of proteins to which this would apply. Protein phosphorylation is far more than a simple on/off switch but rather takes a protein through a sequence of intermediates each with their own unique characteristics. Such a programming simply inherent from the nature of the protein allows for exquisite physiological regulation.

4.2. ΔPH-induced changes in activity

The phosphotransferase activity of Phk exhibits pronounced pH-dependency (1). Phk specific activity increases significantly as the pH is raised from 6.8 to 8.2. Activity remains Ca^{2+}-dependent at pH 8.2. The ↑pH apparently activates Phk via both a direct effect on the catalytic mechanism resulting in a small (2-3 fold) increase in catalytic competency, and also by relieving the inhibitory effect of the alpha and beta subunits resulting in a large (10-20 fold) increase in specific activity (25). The latter effect probably arises from a ΔpH-induced alteration in the conformation and interactions of the alpha and beta subunits that mimics the effects of PKA-catalyzed phosphorylation (25, 50). This is supported by the observation that the enzymatic activity of the isolated gamma-delta complex is significantly less pH-sensitive than that of the Phk holoenyzme (50). Currently very little is known regarding the molecular mechanisms by which inhibition is relieved by ↑pH. Of note, Phk activity would be very sensitive to the range of intracellular pH changes that can occur *in vivo*.

4.3. Calcium and autoinhibition

The phosphotransferase activity of Phk is Ca^{2+}-dependent and the effect of Ca^{2+} is mediated by the delta subunit (1). As discussed in a previous section, amino acid residues 301-327 and 342-367 in the gamma subunit comprise two calmodulin-binding domains that overlap with a putative autoinhibitory domain (23, 90). The PhK13 peptide (discussed in an earlier section of this review) competitively inhibited ***activated*** phosphorylase kinase, and the addition of Ca^{2+}/calmodulin relieved inhibition (91). Of note, PhK13 stimulates, whereas the PhK5 peptide (also discussed earlier) slightly inhibits ***unactivated*** Phk at pH 6.8 (25). When two amino acid residues in a recombinant and bacterially-expressed gamma subunit were changed, the K_m for phosphorylase and the K_i for competitive inhibition by PhK13 increased several fold, possibly indicating that the PhK13 peptide binds directly to the phosphorylase binding site on gamma-M (91). From these observations it has been proposed that the C-terminal domain of gamma comprises a Ca^{2+}-dependent molecular switch [91]. When this switch is in the "off" position (absence of Ca^{2+}), the autoinhibitory domain adopts a conformation that excludes glycogen phosphorylase from the catalytic cleft. In the presence of Ca^{2+} the delta subunit binds to the C-terminus of gamma and induces a conformational change in the autoinhibitory domain that "flips" the switch to the "on" position, thereby rendering the catalytic cleft more accessible and effectively relieving autoinhibition. This switch provides a simple mechanism for possible activation but it is likely that in the holoenzyme the activation also involves changing interactions with the other subunits.

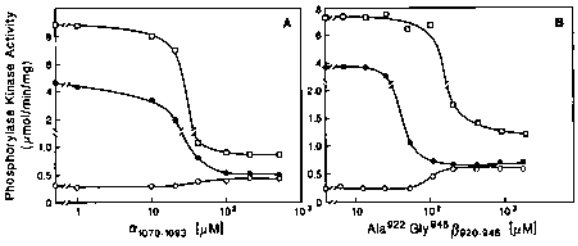

Figure 11. Titration of Phk with two synthetic peptides representing calmodulin-binding domains of the alpha and beta subunits (panel A and B, respectively). The effect of the two synthetic peptides on phosphotransferase activity are shown. This data is representative of the results obtained for several peptides representing potential calmodulin-binding domains from the α and β subunits. The effect on Phk activated by increased pH (open squares), PKA-catalyzed phosphorylation (closed circles), and unactivated Phk assayed at pH 6.8 (open circles) are shown. Both peptides slightly stimulated unactivated enzyme, and neither peptide could fully inhibit activated enzyme but could only lower its specific activity down to a level that was still two- or three- fold higher than the unacitvated enzyme. Both the unactivated and activated enzymes remained fully Ca^{2+}-dependent (data not shown). These data show that delta subunit interactions are likely a key component of the mode of signal transduction that arises from alpha and beta subunit phosphorylation leading to activation of the gamma subunit cataltic activity. A model consistent with this data was proposed and is shown in Figure 12. Figure 11 was reproduced with permission from reference (25).

A second mechanism for Ca^{2+}- dependent activation occurs with exogeneous calmodulin (or TNC), often termed the delta-prime subunit A 2-3-fold increase in the specific activity of unactivated Phk can be achieved with exogenous calmodulin (delta-prime) or troponin C, which bind to Phk in a Ca^{2+}-dependent manner (1). Interestingly, delta-prime has no effect on Phk activated by phosphorylation (1). Thus suggests that delta-prime activates Phk by somehow influencing the inhibitory properties of the alpha and/or beta subunits. A number of sites in the alpha and beta subunits have been identified as candidate delta-prime binding sites. An alpha-gamma-delta complex obtained by heterologous coexpression of Phk subunits in insect cells exhibits Ca^{2+}-dependent binding to a calmodulin affinity column and can be activated by exogenous Ca^{2+} -calmodulin, which suggests that the alpha subunit indeed possesses a delta-prime binding site (Brushia and Walsh, manuscript in preparation). Available data do not clearly distinguish between potential sites on alpha and beta subunits as favoring interaction with endogenous delta subunit or with exogenous delta-prime or troponin C (25). Available information suggests that the interactions between delta and the other Phk subunits are not static, but rather are quite dynamic and change depending on the state of the enzyme (i.e., phosphorylated or not).

4.4. Coordination of activation of PHK by calcium and phosphorylation

The calmodulin-binding domains in the alpha and beta subunits were identified based on synthesized peptides and these peptides have been used as probes to examine the nature of Phk activation by either PKA-catalyzed phosphorylation or ↑pH. The peptides were potent inhibitors of activated Phk with a close correlation between the calmodulin-binding affinity of the peptides and their IC_{50} for Phk inhibition. These peptides did not completely inhibit activated Phk, but only reduced its specific activity to the level of the unactivated enzyme (figure 11). This suggested that the peptides inhibited Phk by specifically reversing the activating effects of PKA-catalyzed phosphorylation. (25). The peptides also inhibited enzyme activated by ↑pH (25). The small remaining stimulatory effect of ↑pH in the presence of peptides likely represents a pH dependency of catalysis. The peptides had no effect on the Ca^{2+}-dependency of either activated or unactivated enzyme, which suggests that they do not disrupt gamma-delta interactions. The calmodulin-binding peptides appear to act by disrupting regulatory interactions between the alpha and beta subunits and the gamma subunit. This suggests that the delta subunit may be intimately involved in the interactions of the alpha and/or beta subunits with gamma and in the mediation of phosphorylation-dependent activation. (25,50). Of particular note, the beta-M 770-794 domain has higher calmodulin affinity than the calmodulin binding sites on the gamma subunit suggesting that it might well interact with the endogenous delta subunit under some circumstances. Other experimental evidence supports this idea. For example, the activity of the Phk holoenzyme is largely Ca^{2+}-dependent, whereas the isolated gamma-delta complex exhibits markedly reduced Ca^{2+}-dependency. In the presence of Ca^{2+}, both species have identical specific activities as measured per mole of gamma subunit (47, 50, 92). Also, the activation of Phk by limited tryptic digestion of the alpha and beta subunits promotes a large increase in Ca^{2+} affinity (1).

A model (figure 12) for subunit-subunit interactions has been proposed (25) that is consistent with all of the experimental observations so far available for Phk, taking into account both delta and delta´ interactions, the effects of phosphorylation and Ca^{2+}-stimulation and the properties of all the subcomplexes of Phk that have so far been characterized. The model suggests that the activation of Phk by any of a variety of routes involves a complex reorganization of subunit-subunit interactions. The interaction of the alpha and/or beta subunits with the delta subunit appears to plays a critical role in the regulation of the gamma subunit. This set of interactions will clearly be altered by the subunit isoform composition of the holoenzyme, especially since the high affinity beta-M 770-794 calmodulin site is deleted in both the beta-B and beta-L isoforms. This could result in major differences in Phk activation in different tissues (1).

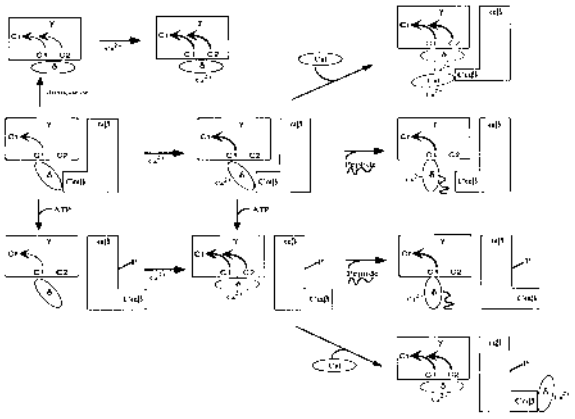

Figure 12. Schematic Model for the regulation of phosphorylase kinase by Ca^{2+}, cAMP-dependent phosphorylation, and extrinsic calmodulin. *Ct* = catalytic site on the gamma subunit; *C1 and C2,* depict the calmodulin/δ binding sites on gamma, and *C* C-alpha/beta depict calmodulin (or delta subunit) binding sites on alpha or beta. The intensity of the *arrows* within gamma symbolizes the magnitude of an activating effect, *-P* denotes the phosphorylation of the alpha and beta subunits by PKA. For simplicity, no distinction is made between the separate roles of the alpha and beta subunits. Both are regulated by phosphorylation and are sites of calmodulin binding but clearly each does have separate functions. The center model on the left-hand side is that of unactivated phosphory-lase kinase. Arrows to other forms depict the effects of dissociation, Ca^{2+}-stimulation, cAMP-dependent phosphorylation, exogenous calmodulin (denoted *Cal*), and the effect of high-affinity calmodulin-binding peptides (denoted ~~) Cross-linking studies, the ability to isolate a gamma-delta complex, and the identification of calmodulin binding sites on the γ subunit, would all indicate that the gamma subunit is bound to the delta subunit in the holoenzyme. However, since the Ca^{2+} sensitivity of the isolated gamma-delta complex is markedly different from that of the holoenzyme, there must be a difference in the interaction of delta with gamma when the two are part ot the holoenzyme. To account for this the model suggests that in the inactive, nonphosphorylated protein the delta subunit is bound to both the gamma subunit and to alpha/beta. This is also compatible with the observed inhibitory effects of high-affinity calmodulin-binding peptides on activated holoenzyme. The calmodulin binding site designated C-alpha/beta may represent one of several potential binding sites on alpha or beta that have been identified; however, because of its high affinity beta770-794 would appear to be the most likely candidate. Of note, extrinsic calmodulin binding to the beta770-794 site was only detected with isolated subunits and not with the intact holoenzyme, suggesting that this site is unavailable when the intrinsic deltąsubunit is present. To account for the difference seen in the activity characteristics of the holoenzyme and the gamm-delta complex the model suggests that when the holoenzyme structure is disrupted, delta subunit binding is altered, so that it is now bound to both the C1 and C2 sites on the gamma subunit . Both holoenzyme and gamma-delta complex have a very similar specific activity (per mol) when measured in the presence of Ca2+. The gamma-delta complex, however, has a much higher Ca2+-independent activity so its activity is stimulated much less by Ca^{2+} than occurs with the holoenzyme (2-fold versus 40-fold). This difference could be a consequence of the delta subunit binding to only one site on the gamma subunit in the holoenzyme but to both the C1 and C2 sites in the gamma-delta complex, leading to differences in the magnitude of the Ca^{2+} response. Peptides from both the gamma302-326 and gamma342-366 regions can bind calmodulin simultaneously thus it is reasonable to suggest that calmodulin can be bound to both C1 and C2 simultaneously or alternatively to C1 and C-alpha/beta. The different characteristics in gamma 302-326 and gamma342-366, also suggest that interaction of the delta subunit with the C1 and C2 sites might have different roles. The addition of Ca^{2+} to nonactivated holoenzyme is proposed to alter the binding characteristic of delta to gamma, resulting in an increase in activity. In the model the phosphorylation of alpha/beta is proposed to cause a conformational change that prevents the binding of the delta subunit to alpha/beta and results in its transfer so that it now occupies both the C1 and C2 sites on the gamma subunit. The model proposes that when the delta subunit is bound to both the C1 and C2 sites, the gamma subunit has a greater activity than when delta subunit is bound to only a single site. This would explain why phosphorylated enzyme is activated. It is also compatible with the gamma-delta complex having a greater activity than the nonphosphorylated holoenzyme. Isolated gamma-delta complex has an activity quite similar to that of maximally phosphorylated enzyme. High-affinity calmodulin-binding peptides inhibit the activity of phosphorylated enzyme down to the level of the nonphosphorylated protein but do not inhibit the activity of the nonphosphorylated enzyme. Further, in the presence of these peptides both the nonphosphorylated protein and the peptide-inhibited phosphorylation-activated kinase retain Ca^{2+}-independent activity. To account for these data it is suggested that these calmodulin-binding peptides only interact at the delta binding site that binds C-alpha/beta in the nonphosphorylated enzyme or C2 in the phosphorylated enzyme, but do not disrupt δ-C1 binding. With nonphosphorylated enzyme the calmodulin-binding peptides, in this model, likely would disrupt the delta-C-alpha/beta interaction but the delta-C1 interaction would be retained, resulting in no change in either activity or Ca^{2+} sensitivity. In contrast, with phosphorylated enzyme calmodulin-binding peptides would disrupt the delta-C2 interaction dropping the activity to the level of nonphosphorylated enzyme; but this decreased activity would still be Ca^{2+}-dependent. This model would predict that in the presence of peptide both nonphosphorylated and phosphorylated protein would have similar gamma-delta interactions with only one of the two calmodulin binding sites on the γ subunit being occupied. Thus, under these conditions, both forms of the protein would have similar activities, as is observed. The proposed model could also nicely account for the observed effects of extrinsic calmodulin which has been shown to partially activate nonphosphorylated protein but be without effect on enzyme that was activated by phosphorylation. This exogenous calmodulin has been shown by cross-linking studies to bind to alpha/beta. If extrinsic calmodulin were to bind to the C-alpha/beta calmodulin binding site in the nonphosphorylated enzyme, it would release the endogenous δ subunit, allowing it to bind to the gamma subunit C2 site, resulting in activation. In contrast, a similar binding of exogenous calmodulin to phosphorylated alpha/beta would not alter the delta-gamma subunit interaction which is already occurring at both the C1 and C2 sites. Hence exogenous calmodulin would not modify the activity of enzyme already activated by phosphorylation. References in support of the above observation in this figure legend are in (25).

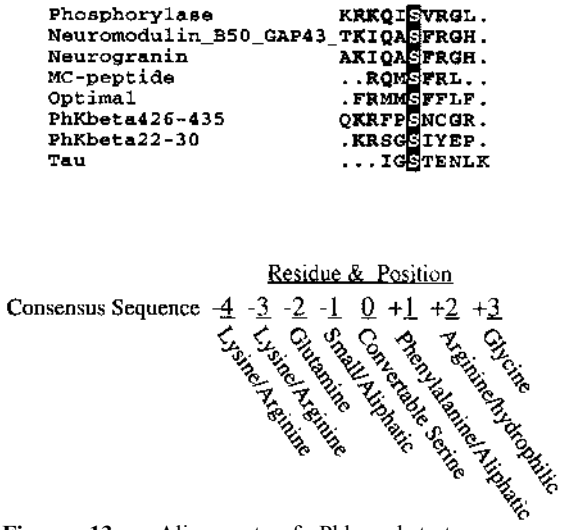

Figure 13. Alignment of Phk substrate sequences surrounding the phosphorylated serine residue. The substrate consensus sequence is indicated. The numbering of residues with respect to the convertable serine (position 0) is indicated and follows current convention for the numbering of residues that comprise protein kinase recognition sites. The data used to construct this figure are referenced in the text.

4.5. ADP Is an allosteric effector

Adenosine-5'-diphosphate (ADP) activates rabbit skeletal muscle Phk by binding to a site that is distinct from the catalytic site (4). There appear to be ~8 ADP binding sites per holoenzyme molecule likely located on the beta subunit. The K_d values for ADP binding range from 0.26 to 17 micromolar depending on whether or not the enzyme is phosphorylated. PKA-catalyzed phosphorylation increases the affinity of Phk for ADP but abolishes its allosteric effect on catalytic activity. ADP binding is pH-dependent and affinity is higher at pH 6.8 than at pH 8.2 (4). ADP stimulates the rate of both autophosphorylation and glycogen phosphorylase phosphorylation by ~2-fold at pH 6.8 but no stimulation is observed at pH 8.2. Thus, it appears that ADP stimulates unactivated Phk but has no effect on activated enzyme. The allosteric effect of ADP is masked by beta-glycerophosphate, which has historically been a buffer component for the *in vitro* assay of Phk enzymatic activity, but the reason for the beta-glycerophosphate effect is not known (1,4). ADP concentrations above 25 micromolar abolish the lag that is normally observed in the time-course of Phk autophosphorylation

5. SUBSTRATE SPECIFICITY

5.1. Protein substrate specificity

Substrate specificity is crucial to ensure the fidelity of signaling events mediated by protein kinases (93) with each protein kinase likely having a set of unique spectrum of protein substrates. Our knowledge of the substrate specificity of Phk remains fragmentary. Early studies on substrate specificity are described in (1) Based upon phosphorylase and glycogen synthase as models a preference is apparent in the linear peptide sequence for positively charged residues at the (P^{-3}) and (P^{-4}), a hydrophobic residue at the (P^{+1}) and a basic residue at the (P^{+2}) positions relative to the phosphorylatable serine P^0 (figure 13) (91). An oriented degenerate oligopeptide library has been used to identify Phk substrate parameters and the sequence of an "optimal" Phk substrate was elucidated., Noteably, the "optimum" peptide differs in sequence from the Phk phosphorylation site in glycogen phosphorylase (93). From this array of data are derived a set of possible consensus/desirable residues arround the phosphorylation site (figure 13), although it seems clearly evident that linear sequence alone is not all that determines the substrate specificity of Phk and there is some ambiguity as to what provides for substrate recognition in Phk. Glycogen phosphorylase is a much more efficient Phk substrate than the "idealized" peptide and is also a much better substrate than the tetradecapeptide derived from its phosphorylation site. The tetradecapeptide exhibits a five-fold lower V_{max} and five-fold higher K_m than glycogen phosphorylase. This suggests that, in addition to the linear amino acid sequence surrounding the phosphorylated serine, the tertiary and/or quaternary structure of glycogen phosphorylase contributes to its capacity to act as a substrate. It is well established that phosphorylase a and phosphorylase b differ significantly in structure, especially in the region of Ser-14, the Phk phosphorylation site. With such a structural difference the off-rate of phosphorylase during its phosphorylation should be expected to differ significantly from that of the phosphorylase-based tetradecapeptide substrate. This could be the cause of the noted decreases in "catalytic competency" with peptides compared to the native substrate phosphorylase.

The "MC" peptide (figure 13) was derived, at least in part, upon the studies of what creates an optimum Phk substrate sequence and is the peptide that has been used for crystal-structure studies. Many insights into the structural basis for Phk protein substrate specificity have come from the crystal structure of a complex formed between gamma-$M_{(1-298)}$ and the MC peptide substrate [94]. The binding of the MC peptide to the active site groove in the gamma subunit has features similar to the binding of the pseudosubstrate PKA inhibitor protein (PKI) to the PKA catalytic subunit (19, 20). An ionic interaction was observed between the substrate arginine at the (P^{-3}) and Glu^{110} in the gamma subunit, which might explain the apparent preference of Phk for substrates with basic residues to the N-terminal side of the phosphorylatable serine (91, 94). A similar ionic interaction has been shown to occur between PKA and PKI. The substrate glutamine at position (P^{-2}) forms a hydrogen bond with Ser^{188} in gamma. Glycogen phosphorylase has a glutamine in an identical position relative to its phosphorylatable serine (figure 13). No hydrogen bonds were observed between the substrate methionine at position (P^{-1}) and the catalytic subunit, but this methionine did form an apolar interaction with the glycine-rich P-loop. A hydrophobic interaction was observed between a phenylalanine in the substrate peptide and a hydrophobic pocket in the gamma subunit. This was the most extensive apolar contact observed and an analogous interaction between PKA and PKI occurs (19, 20, 95).

The crystal structure revealed an interesting feature of the catalytic subunit-peptide substrate interaction that has not been observed in other protein kinase-substrate interactions. The C-terminus of the substrate peptide formed a short stretch of anti-parallel beta-sheet with a segment of gamma-$M_{(1-298)}$ that is quite different from the alpha-helical structure of PKI bound to cPKA. The beta-sheet was stabilized by interactions between the leucine and phenylalanine residues in the substrate peptide and Val^{183} and Gly^{185} in the gamma subunit. The beta-sheet interaction fixes the position of the phosphorylatable serine relative to the gamma-phosphate of ATP and limits the length of the side chain of the phosphorylatable residue, which as Johnson *et al.* suggested might explain the selectivity of Phk for serine over threonine and tyrosine (1, 94). Interestingly, a naturally occurring transition mutation that changes Gly^{185} in gamma-LT to a glutamate residue is associated with autosomal liver glycogenosis in humans (77, 94).

Glu^{182} is situated in a region of the gamma subunit that is homologous to the so-called activation segment found in some other protein kinases, including cPKA and the cyclin-dependent kinase 2 (CDK2). Amino acid residues in the activation segments of cPKA and CDK2 are subject to phosphorylation that modulate active site geometry and influence phosphotransferase activity (96). An arginine at position (P^{+2}) in the MC peptide interacts with the side chain of Glu^{182} in the gamma subunit. In gamma the "activation segment" does not contain residues (i.e., serines or threonines) that are capable of being phosphorylated and an "activating phosphorylation" of gamma to our knowledge has never been observed. Glu^{182}, however, is similar in charge and structure to a phosphoserine. It may well contribute in an analogous manner to the phosphorylated residues in the activation segments in PKA (phosphorylated on residue Thr^{197}) and CDK2 (phosphorylated on residue Thr^{160})and be involved in properly orienting substrate with respect to the catalytic cleft. Thus, as stated by Johnson *et al*. the "...interaction between the gamma activation segment and its peptide substrate identifies another mechanism by which the detailed conformation of the activation segment can influence kinase activity" (94).

Some additional *in vitro* substrates for Phk have been identified, including the alpha and beta subunits of Phk (autophosphorylation), glycogen synthase, troponin I, troponin T, casein, myosin light chain, the regulatory serine of microtubule-associated protein Tau, neuronal tissue-specific proteins B-50 (also known as GAP-43 and neuromodulin) and neurogranin (also known as RC3 or Bicks) (1, 58, 75, 97). Some are phosphorylated on a single site, others are phosphorylated on multiple sites. Analysis of sequences surrounding the sites of phosphorylation show some similarities with phosphorylase (figure 13**).** Of all the alternate Phk substrates that have been identified *in vitro*, there is no evidence that any of them are phosphorylated by Phk under physiological conditions. The neuronal tissue-specific proteins B-50 and neurogranin might be of particular interest if their Phk-dependent phosphorylation were found to occur physiologically. B-50 and neurogranin bind to calmodulin in a Ca^{2+}-independent manner (58). The Phk phosphorylation site is situated within the respective calmodulin-binding domains of B-50 and neurogranin and its phosphorylation reduces their affinity for calmodulin. Phk activity has been observed in synaptic membrane preparations where B-50 and neurogranin are localized (58). Paudel has speculated that B-50 and neurogranin sequester a cellular calmodulin pool until the proper stimulus (possibly phosphorylation) triggers its release into the cytosol (58).

5.2. Nucleoside triphosphate and divalent cation specificity

A nucleoside triphosphate coordinated with a Mg^{2+} ion serves as the phosphate donor in the Phk-catalyzed phosphotransfer reaction. The divalent metal ion and nucleoside triphosphate-dependency of Phk has been investigated and is reviewed in (1). Phk has a strong preference for Mg^{2+} as the divalent metal ion, and for adenosine-5'-triphosphate (ATP) as the nucleoside triphosphate. A highly conserved component of the ATP binding site in protein kinases is the P-loop motif, Gly-X-Gly-X-X-Gly-X-Val. Indeed, this structural motif is a landmark feature by which newly discovered enzymes can be classified as members of the protein kinase family (9, 10, 98). Phk gamma subunit was the first protein kinase in which the role of the amino acids of the P-loop motif was investigated (98). The P-loop sequence in the rat gamma-M subunit is ^{26}Gly-Arg-Gly-Val-Ser-Ser-Val-Val^{33}. This sequence is conserved in the rat, rabbit, mouse and human gamma subunits. In this sequence residues 26 and 28 mimic structurally equivalent glycines that are conserved in 96 and 100 % , respectively, of the known protein kinases. The P-loop sequence in Phk is unusual with Ser^{31} in place of the much more typical glycine, which is present in >85 % of known protein kinases. In the remainder of the known protein kinases it is predominantly conservatively substituted by serine, as in Phk. Substitution of either Gly^{26} or Gly^{28} in Phk with serine was not especially well tolerated and led to a large decrease in catalytic activity. Replacement of Gly^{26} with a serine diminished ATP and ADP affinity and reduced V_{max} by 75%. Although substitution of Gly^{26} did compromise activity, given the very high degree of its conservation in the protein kinase family (<96%), it is somewhat surprising that its substitution could be so well tolerated. Replacement of Gly^{28} with serine, in contrast, very severely diminished gamma subunit phosphotransferase activity with an estimated decrease in the apparent V_{max} of the mutant by ~98% relative to the wild-type enzyme. The extremely low phosphotransferase activity of the Gly28Ser substitution precluded any further biochemical analysis. This is as might be expected given the nearly 100% conservation of a glycine structurally equivalent to Gly^{28}in the protein kinase family. As noted, the Phk gamma subunit P-loop differs from the "perfect" P-loop consensus sequence in the third position of the glycine trio (i.e., Ser^{31}). Replacement of Ser^{31} with a glycine only changed the enzyme activity modestly, with slightly diminished ATP affinity and increased ADP affinity (98). These changes were offset by effects on other kinetic parameters so that no net effect was

observed on the overall rate of glycogen phosphorylase phosphorylation. The evolutionary substitution of this P-loop residue by serine in Phk did not produce an enzyme of modified catalytic potential suggesting that its substitution might provide some more subtle regulatory advantage. Replacement of Val^{33} with glycine was not well tolerated and led to a ~98% decrease in Phk activity. The equivalent of Val^{33} is conserved in 95 % of protein kinases.

X-ray studies of the Phk gamma subunit have confirmed the involvement of the P-loop in nucleoside triphosphate binding (18), as had also been previously shown from the X-ray structure of cPKA (19). The contacts between the gamma P-loop and bound nucleoside triphosphate appear qualitatively similar to those observed for cPKA, but differ in several noted aspects. The P-loop in Phk gamma is displaced from the nucleoside triphosphate binding site by several Å as compared to the P-loop in cPKA (some of the detailed interactions involved in nucleoside triphosphate binding can be seen in figure 4). As a result of this structural shift, the main-chain amide groups of the gamma P-loop do not appear to be directly involved in hydrogen bonds with the nucleoside triphosphate, as in the case of cPKA. Johnson *et al.* suggested that the side chain of Ser^{31}, which replaces the third glycine of the P-loop consensus that is found in cPKA, causes some displacement of the gamma P-loop and that binding of substrates might shift the P-loop and "tighten the binding site", as observed for cPKA-substrate complexes. As noted, the mutation of Ser^{31} to glycine had no significant overall effect on the catalytic activity of gamma (98).

In the crystal structure of gamma-$M_{(1-298)}$ complexed with AMPPNP, the purine base is completely buried in a hydrophobic pocket in the catalytic cleft of gamma. N6 and N1 in the purine form hydrogen bonds with the carbonyl oxygen of Asp^{104} and the main-chain amide of Met^{106}, respectively. The P-loop Val^{33} forms a van der Waals interaction with the purine base. Extensive van der Waals interactions are also observed between the purine and several amino acid residues in both the N-terminal and C-terminal globular domains of gamma. Ionic interactions between the triphosphate group and the protein appear to be mediated by the bound Mg^{2+} ion. The ribose of AMPPNP is bound in a C3'-*endo* conformation through hydrogen bonds from the O2'-hydroxyl of the sugar to Glu^{110} and Glu^{127} and from the O3'-hydroxyl to Glu^{153} (18).

6. GENETICS AND ENZYMATIC REGULATION

6.1. Genetic deficiencies

A variety of heritable human glycogen storage diseases have been described (80). The most common of these are attributable to Phk deficiency and in combination occur with a frequency of ~1 in 100,000 births (77,80). The symptomology, tissue-specificity, and modes of inheritance vary considerably. X-linked liver glycogenosis type I (XLG-I) is the most frequent type of PhK deficiency. The symptoms include decreased hepatic Phk activity, hepatomegaly, growth retardation, hypercholesterolemia, hypertriglyceridemia, and, in some cases, hypoglycemia and metabolic acidosis (80). Diagnosis of XLG-I is based on hepatomegaly coupled with low Phk activity in the erythrocytes and leukocytes of afflicted individuals. The symptoms of XLG-I usually disappear in adulthood, although in some rare cases the symptoms do not disappear and progress to liver cirrhosis (99). Mutations in alpha-L have been linked to XLG-I (100-102). XLG-II a second class of XLG and much rarer than XLG-I. XLG-II manifests symptoms that are very similar to those of XLG-I, but individuals with XLG-II have normal Phk activity in their erythrocytes and leukocytes (102, 103). XLG-II has been linked to mutations in alpha-L (102-105). In addition to the major XLG's that have been described, a variety of rarer glycogenoses have also been observed. For example, X-linked muscle-specific Phk deficiency has been linked to mutations in the alpha-M gene (106, 107). There is an extremely rare autosomally inherited liver PhK deficiency in which patients exhibit hepatomegaly and growth retardation, and occasionally the disease progresses to liver cirrhosis (80, 105, 108). The only genetic defects that have been linked to autosomally inherited liver Phk deficiency are in the gamma-LT gene (77, 108). A combined liver and muscle PhK deficiency has been described in which afflicted individuals exhibit muscular hypotonia and fasting hypoglycemia, in addition to all of the symptoms that are characteristic of XLG-I The combined liver and muscle genetic deficiency has been linked to the beta subunit gene (86, 109, 110). An autosomally inherited skeletal muscle-specific deficiency has been reported in which patients suffered severe muscle weakness and cramps following exercise and their symptoms remained into adulthood (80). Only two cases of cardiac muscle PhK deficiency have been reported, and both of the afflicted individuals suffered from severe cardiomegaly and died at an early age (111, 112). The genetic defect(s) linked to cardiac muscle Phk deficiency has not been identified. The molecular basis for many heritable Phk deficiencies are being identified at a rapid pace. The symptomology and modes of inheritance appear to reflect tissue-specific isoform expression and the multifaceted modes of Phk regulation (13, 80). There are many Phk deficiencies for which the modes of inheritance and underlying genetic defects have not yet been identified, and this has led some investigators to suspect the existence of additional subunit isoforms (85).

6.2. Genetic disorders provide insights into regulation

Mutations linked to Phk deficiency can be divided into two classes, and both provide unique insights into the regulation of Phk activity *in vivo*. The first class includes mutations that disrupt protein expression altogether (i.e., frameshift mutations). Mutations that cause premature translation termination of the gamma subunit lead to a complete loss of enzymatic activity in affected tissues, which is not surprising since gamma is the catalytic subunit (77, 105). Mutations that disrupt alpha subunit expression lead to decreased levels of the beta and gamma subunit proteins and a significant loss of Phk enzymatic activity (102, 106, 107). This suggests that the uncoupling of Phk activity from the regulatory control of the cAMP pathway is not tolerated. Walsh *et al.* have observed that the *in vivo* expression of the alpha, beta, and gamma subunit genes in reinnervating rat skeletal muscle appears

to be coordinated so that unregulated gamma subunit does not accumulate to significant levels (113). The gamma subunit gene appears to be the major site of transcriptional regulation of Phk . (O'Mahony, A and Walsh, D.A., manuscript in preparation)

Translation-disrupting mutations in the beta subunit are unique in that many are associated with mild clinical symptoms, and significant Phk activity remains in affected tissues (86). One patient with mutations in the beta subunit and total loss of Phk activity in liver has been described (109). This patient had normal Phk activity in erythrocytes (86). Kilimann *et al.* suggested two reasons for the normal Phk activity observed in erythrocytes [86]. First, the alpha subunit might partially substitute for the missing homologous beta subunit protein in the holoenzyme in erythrocytes. Alternatively, an alpha-gamma-delta complex might form in the absence of the beta subunit. There is some experimental evidence to support this latter possibility. Chan and Graves have isolated an active alpha-gamma-delta complex from purified holoenzyme, and we have isolated an active alpha-gamma-delta subcomplex that was formed as a result of heterologous coexpression of the alpha, gamma, and delta subunits in baculovirus-infected insect cells (50, Brushia and Walsh, manuscript in preparation). The expressed alpha-gamma-delta complex isolated from insect cells possesses many of the same regulatory properties as the Phk holoenzyme, and the complex appears to be relatively stable.

A second class of mutations are more subtle in that they do not cause premature translation termination. Such mutations can have significant effects on Phk activity. For example, Willems *et al.* have identified three different mutations in the liver-specific alpha-L subunit gene. One mutation is a missense that changes Arg^{186} into a cysteine, another is an in-frame insertion of six-nucleotides that causes the insertion of one threonine and one arginine between Arg^{1111} and Glu^{1112}, and the third is an in-frame deletion of three nucleotides that results in the deletion of Thr^{251} (114). Patients harboring any one of these mutations exhibit symptoms characteristic of classical XLG-I except that the Phk activity in their erythrocytes and leukocytes is normal, and sometimes even elevated, when measured *in vitro* using muscle phosphorylase as the substrate (103, 114). In classical XLG-I the Phk activity in erythrocytes and leukocytes is significantly lower than in nonpatient controls. Thus, the phenotype of this XLG has similarities to the phenotypes of both XLG-I and XLG-II. Willems *et al.* suggested two possible explanations to resolve the apparent paradox. First, all of the mutations identified in this study altered an RXX(X)T amino acid sequence motif (114). This sequence motif is a potential site of regulatory phosphorylation and it is possible that the mutations influence the *in vivo* regulation of liver Phk activity. Second, the mutations might influence the interaction of liver Phk with its cognate substrate, liver phosphorylase, and the classical clinical assay that uses the muscle isoform of phosphorylase might mask this effect (103, 114). One patient with a missense mutation in the beta subunit did exhibit low Phk activity in erythrocytes when measured with endogenous liver phosphorylase, but normal activity when measured with muscle phosphorylase (110). In other case studies of XLG-II patients, Kilimann *et al.* identified several mutations that were clustered in a fairly small region of the alpha-L gene that (i) changed His^{132} to a proline, (ii) changed His^{132} to tyrosine, (iii) changed Arg^{186} to a histidine, (iv) changed Lys^{189} to a glutamate, or (v) changed Asp^{299} to glycine (102, 104). All of these mutations alter amino acid residues that are highly conserved in the alpha-L, alpha-M and beta subunits of Phk, and all were associated with the XLG-II phenotype. None of these other XLG-II mutations, however, occurred in an RXX(X)T motif, and so such a motif might not be involved in the regulation of Phk *in vivo* as Willems *et al.* had suggested. However, based on the limited data currently available that possibility cannot be excluded. As an interesting hypothesis to explain the tissue specificity of some XLG's, it has been proposed that that the missense mutations linked to XLG-II might in some manner selectively destabilize the Phk enzyme in a cell-type-specific manner (110).

Kilimann *et al.* described two patients with Phk deficiency accompanied by unusual clinical symptoms (102). One displayed dysfunction of the kidneys and the other dysfunction of the nervous system. Two mutations were identified in the alpha-L gene from the patient that exhibited kidney dysfunction. One was a missense mutation that replaced Pro^{399} with a serine residue and the other was a trinucleotide deletion that resulted in the replacement of two amino acids (Arg^{953} and $Leu^{954)}$ with a single isoleucine residue. The patient with neurological symptoms had a missense mutation in the alpha-L gene that substituted Gly^{1207} with a tryptophan residue. These were the first reports of tissues other than liver or muscle that appear to be affected by subunit-specific mutations in one of the Phk subunits. The Phk holoenzymes harboring these mutations await biochemical characterization.

7. PERSPECTIVES

The protein kinases comprise a huge family of regulatory enzymes (10, 20). Structural information is now available for several hundred distinct members of this enzyme family that are currently listed in the protein kinase database (Internet address: http://www.sdsc.edu/kinases). Protein kinases share a homologous catalytic domain that probably evolved from a common ancestral gene (10, 20, 115). The catalytic domain has a characteristic bilobal architecture in which the catalytic site is juxtaposed between small N-terminal and large C-terminal globular domains (20). Despite the highly conserved structure of the catalytic domain, different protein kinases have unique and diverse physiological functions. Many are quite promiscuous (the *multisubstrate protein kinases*) and it is not yet known what constitutes a "good" physiological substrate for many of them. Protein kinases are found in many different species, from simple single-celled creatures to the most complex multicellular organisms. This high degree of evolutionary conservation suggests that protein kinases are especially well-suited for performing their respective regulatory functions. Protein kinases are

involved in regulating virtually all aspects of cell physiology, from progression through the cell cycle to programmed cell death and most cellular events in between. Indeed, the reversible, covalent attachment of phosphate to target proteins is perhaps the most common mechanism utilized for regulating protein function in multicellular animals (20, 116-118). The "typical" cell may devote as much as 20 % of its energy store to the regulation of protein function via covalent modification (119) and an estimated one-third of the proteins in a "typical" mammalian cell contain covalently bound phosphate (1170. It has been estimated that the human genome encodes at least 1000 different protein kinases (115).

Kinases catalyze the transfer of the gamma-phosphate from a nucleoside triphosphate donor to a substrate. Two different but related catalytic mechanisms have been proposed, but the exact mechanism remains to be established (94). The protein kinases transfer phosphate specifically to the side-chain hydroxyl of a serine, threonine, tyrosine, or in some cases a histidine, residue in substrate proteins. Though this reaction is relatively simple, considerable complexity is evident in the regulation of kinase activity. The activity of the protein kinases is generally subject to strict regulatory control (i.e., unregulated kinases are usually detrimental and are the underlying cause of some serious human diseases, such as cancer). The *in vivo* activity of a kinase is determined by various signals that impinge on its regulatory domain. Some kinases have more than one type of regulatory subunit or domain and their phosphotransfer activity can be affected by more than one type of signal. Conversely, some signals can activate numerous kinases. Most enzymes catalyze a single reaction involving well-defined substrates. Protein kinases are somewhat unique in that many have multiple substrates. These properties make the task of identifying the physiological functions of a given protein kinase incredibly difficult (120). Some kinases have very simple structures and modes of regulation, whereas others are exceedingly complex. The protein kinases have evolved into such a large and functionally diverse enzyme family because they perform so exquisitely their respective regulatory functions.

Of all the protein kinases characterized to date, Phk is the most complex in structure. Coupled to its complexity in structure is its complexity of regulation. Yet its complexity seems to belie its function since the only characterized substrate to date is phosphorylase, and even though it has multiple mechanisms of control it is not apparent why the protein should be so large and have a structure so complex. It would seem certain that there remains much to be uncovered about its physiological function. Phosphorylase kinase has proven difficult to characterize, and particular the inability to break it down easily into its component parts has markedly hampered its investigation. It is now apparent as to why it has been difficult to dissect. Free soluble gamma subunit cannot be isolated following synthesis in bacterial, yeast, insect or mammalian expression systems and free soluble alpha or beta subunit cannot be isolated from synthesis in insect cells (98, 121, Brushia and Walsh, in preparation). [To our knowledge no one has attempted to express the individual alpha and beta subunits in other expression systems.] This appears to indicate that the alpha, beta and gamma proteins are not sufficiently hydophilic to be soluble as isolated proteins. We have now been able to synthesize the holoenzyme and alpha-gamma-delta and gamma-delta subcomplexes using a baculoviral expression system [Brushia and Walsh, in preparation] and this should open up an array of investigative possibilities to probe the structure and function of Phk.

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Send correspondence to: Dr Donal Walsh, Department of. Biol. Chem., School of Medicine, University of California, Davis, California, 95616, Tel:530-752-3399, Fax: 530-752-3516, E-mail: dawalsh@ucdavis.edu

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