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The α and β subunits of phosphorylase kinase are homologous: cDNA cloning and primary structure of the β subunit

(phosphoproteins/glycogen metabolism/isoenzymes/calmodulin/gene expression)

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ABSTRACT **We have cloned cDNA molecules encoding the β subunit of phosphorylase kinase (ATP:phosphorylase-*b* phosphotransferase; EC 2.7.1.38) from rabbit fast-twitch skeletal muscle and have determined the complete primary structure of the polypeptide by a combination of peptide and DNA sequencing. In the mature β subunit, the initial methionine is replaced by an acetyl group. The subunit is composed of 1092 amino acids and has a calculated molecular mass of 125,205 Da. Alignment of its sequence with the α subunit of phosphorylase kinase reveals extensive regions of homology, but each molecule also possesses unique sequences. Two of the three phosphorylation sites known for the β subunit and all seven phosphorylation sites known for the α subunit are located in these unique domains.**

Protein phosphorylation was discovered during the last three decades to be a major mechanism of signal transduction in eukaryotic cells. Nearly 100 protein kinases have been described, and their total number may be much higher (for review, see ref. 1). During the last few years, features of the primary structure have emerged that are shared by all protein kinases and additional ones that discriminate the serine/threonine kinases from the tyrosine kinases. It appears that small catalytic subunits of ≈350 amino acids, like those of cAMP-dependent protein kinase (subunit C) or phosphorylase kinase (ATP: phosphorylase-*b* phosphotransferase; EC 2.7.1.38) (subunit γ), represent the catalytic core of a serine kinase essentially stripped of regulatory domains. It may possess substrate recognition elements that allow the targeting of catalytic activity to short peptide segments with certain primary structural features. These core domains are homologous in all protein kinases studied. In many protein kinases the catalytic core is equipped with additional regulatory domains that block its activity, to release it only in response to the action of allosteric (e.g., Ca^{2+} or cAMP) or covalent ligands (e.g., phosphorylation). Regulatory domains may also define further the kinase's protein substrate specificity.

These domains can be contiguous extensions of the polypeptide chain (as in cGMP-dependent protein kinase) or separate subunits that may either bind reversibly (as in cAMP-dependent protein kinase) or stably (as in phosphorylase kinase) to the catalytic subunit (for review, see ref. 2). For the enzymologist, it is important to understand how they transform the signal of a ligand and transmit it to the catalytic center. For the molecular biologist, it is of interest to learn how the diversity of protein kinases arose in the course of evolution.

Among the protein kinases, phosphorylase kinase shows particular structural complexity. In muscle, the enzyme integrates the regulatory pathways that lead from depolarization (via Ca^{2+}) or β-adrenergic hormones (via cAMP) to the activation of glycogen breakdown. Correspondingly, the catalytic γ subunit is combined with several regulatory subunits that act as receptors for these signals: calmodulin as the integral δ subunit transmits the Ca^{2+} signal, and the two large subunits, α and β, modulate activity in response to phosphorylation. Together, they form a hexadekameric complex $(\alpha\beta\gamma\delta)_4$ of 1.3×10^6 Da (for review, see ref. 3). Although the free γ subunit is active *in vitro* (4), its activity is suppressed almost completely in the holoenzyme to be released stepwise by Ca^{2+} saturation of the δ subunit and phosphorylation of the subunits α and β (5).

To understand how the α and β subunits control the catalytic activity of the γ subunit and how this is modulated by phosphorylation, it is necessary to know their primary structures and their domain organization and to learn what functions these domains serve. The isolation and sequencing of cDNA will also open up ways to characterize the structures of the cell-type-specific isoenzymes and the molecular basis of the various hereditary deficiencies of phosphorylase kinase (cf. ref. 6).

Recently, we have published the primary structure of the α subunit of fast-twitch skeletal muscle phosphorylase kinase (6). We have now cloned a nearly full-copy cDNA coding for the β subunit (βPhk) and present its sequence and the complete primary structure of the polypeptide.§ Structural comparison of the subunits α and β reveals a distinct pattern of domains. Both subunits share extensive regions of sequence similarity but also possess unique domains. Nine of the 10 known phosphorylation sites are located in the domains that are unique to either the α or the β subunit.

MATERIALS AND METHODS

Methods were essentially as described in ref. 6 with the following exceptions. The isolated β subunit was cleaved with cyanogen bromide or endoprotease Glu-C or Lys-C (Boehringer Mannheim). For oligonucleotide hybridization, a temperature gradient from 65°C to 32°C was employed; filters were washed in 0.9 M NaCl/90 mM sodium citrate/0.1% $NaDodSO_4$, pH 7.0, at 47°C. For the sequencing of (G + C)-rich DNA regions, 7-deaza-2′-deoxyguanosine 5′-triphosphate (Boehringer Mannheim) or dITP and modified T7 DNA polymerase (Sequenase; United States Biochemical, Cleveland) were employed. Fast-atom bombardment

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Abbreviations: βPhk, β subunit of phosphorylase kinase; nt, nucleotides.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04120).

mass spectra of the N-terminal peptides were obtained on a Kratos MS 50 RF instrument.

RESULTS

Peptide Sequencing and cDNA Isolation. Partial amino acid sequences of the βPhk from rabbit skeletal muscle were determined essentially as described in ref. 6. A detailed account of these experiments will be published separately together with characterization of the N terminus and identification of phosphorylation sites (see below). A size-selected cDNA library [inserts larger than 2300 nucleotides (nt)] from rabbit psoas muscle mRNA was constructed in pBR322 and transformed into *Escherichia coli* DH5. The library of ≈5000 recombinants was screened with the 96-fold degenerate 20-mer oligonucleotide AC(A,G,T)AT(C,T)TT(A,G)TA-(A,G)TA(A,G)TA(A,G)TC derived from the amino acid sequence Asp-Tyr-Tyr-Tyr-Lys-Ile-Val. The screening yielded three positive clones that were colinear according to restriction analysis. The clone with the longest insert, pβPhk-D1N, was selected for sequencing.

Nucleotide and Amino Acid Sequence of the βPhk. The βPhk-D1N cDNA has a sequence of 56 5′ flanking, 3279 coding, and 764 3′ flanking nt (Fig. 1). The 5′ flanking sequence is extremely (G + C)-rich (41 G, 14 C, and 2 A). The 3′ flanking sequence is (A + T)-rich (63%) and contains many in-frame stop codons (underlined) and six inverted repeats (8–10 nt). The cDNA does not contain a poly(dA) tract, but

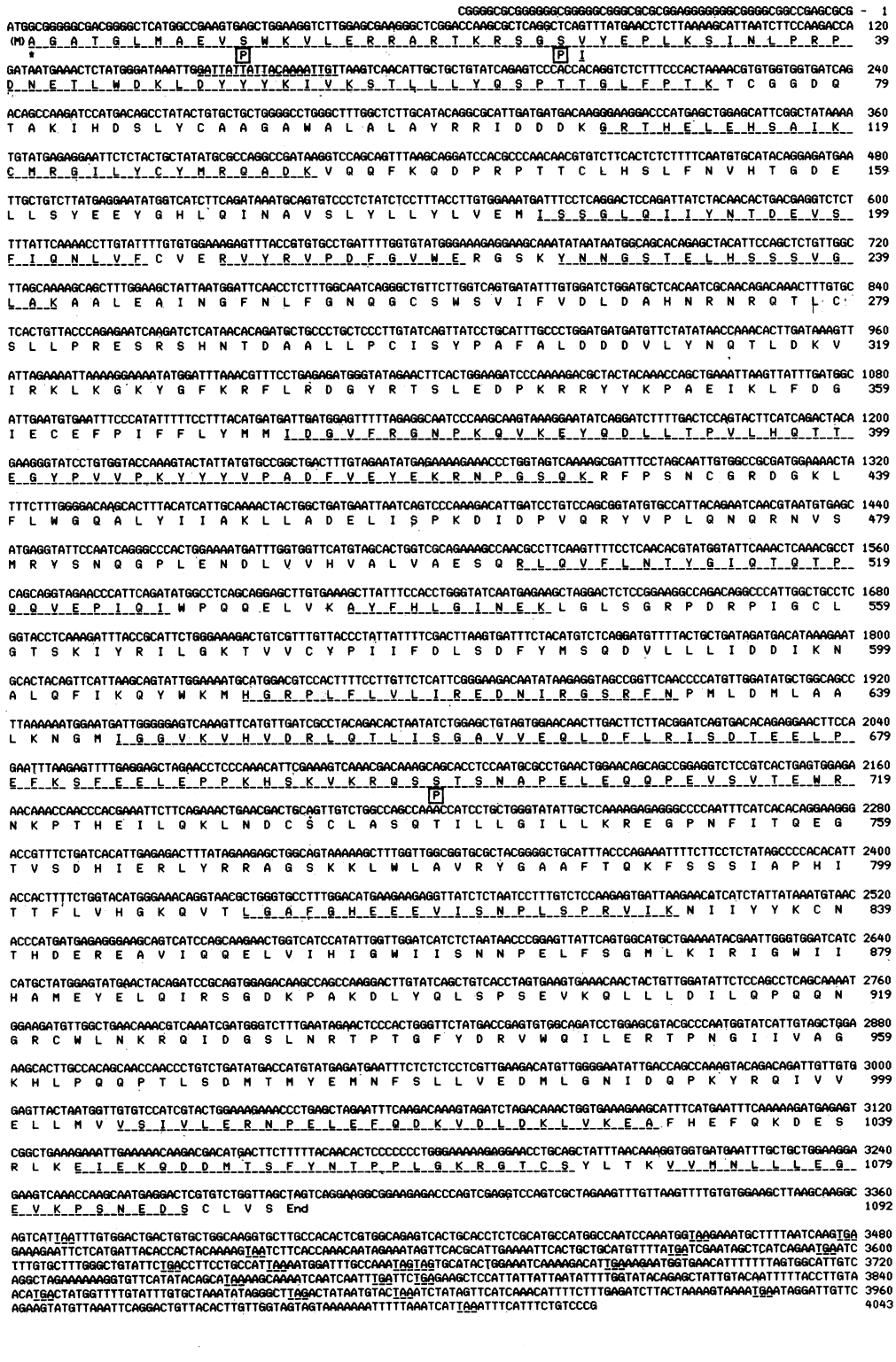

FIG. 1. Nucleotide sequence of cDNA βPhk-D1N and deduced amino acid sequence (single letter code) of βPhk. Nucleotides are numbered positively beginning with residue 1 of the ATG initiator codon and ending with the last residue before the oligo(dC) tail; they are numbered negatively beginning from the 5′ side of residue 1 to the residue adjacent to the oligo(dG) tail. Amino acid number 1 is the alanine following the initiator methionine removed in the mature subunit. The asterisk indicates the acetyl group that blocks the N terminus. Underlined peptide sequences are confirmed by Edman degradation. Underlined nucleotide sequences are the recognition site of the oligonucleotide probe and in-frame stop codons in the 3′ untranslated sequence. Serine residues subject to autophosphorylation are indicated below by a boxed P. A valine/isoleucine heterogeneity exists in position 27 (see text).

a poly(A) signal sequence, ATTAAA, begins 22 nt upstream from its 3′ end. Therefore, probably only a few nucleotides from the 3′-terminal mRNA sequence are not represented in the cDNA. As RNA blot hybridization (below) yields an estimated mRNA length of 4200 nt, the cDNA molecule seems to be an almost complete copy.

A long open reading frame begins with the first methionine codon within a sequence of moderate similarity to the initiator consensus (7). It accommodates all peptide sequences determined by Edman degradation (they cover 39% of the total sequence) without any mismatch. The deduced and the published (8) experimental amino acid compositions of the polypeptide are in good agreement (not shown). We confirm the valine/isoleucine polymorphism in position 27 reported by Cohen *et al.* (9). Our cDNA yields valine; however, we have isolated peptides having either amino acid in this position. Their sequences are otherwise identical, and this partial sequence is unique within the βPhk molecule. The G + C richness of the 5′ flanking sequence breaks down within the reading frame after a few codons. The G + C percentage of the coding sequence is 43.8% overall and 44.0% in third codon positions, very similar to the α subunit.

Posttranslational Processing of the N Terminus. The N terminus of the mature β subunit is blocked (8). The N-terminal peptide was isolated as a Lys-C fragment resistant to Edman degradation. By fast-atom bombardment mass spectrometry, the molecular mass of the complete protonated peptide was determined to be 1362 Da. This corresponds to a peptide with the composition $Ala_3GluGly_2MetLysLeuSer$-ThrTrpVal (derived from phenylthiocarbamoyl-amino acid analysis, data not shown) with an additional acetyl group. From the mass spectrum, the sequence Ac-Ala-Gly-Ala-Thr-Gly-Leu-Met-Ala-Glu-Val-Ser-Trp-Lys was deduced. After cleavage of the peptide with cyanogen bromide, the sequence of the N-terminal subfragment was confirmed analogously by fast-atom bombardment mass spectrometry, and the sequence of the C-terminal subfragment was confirmed by Edman degradation. Thus, in the mature β subunit, the initial methionine is removed and the amino group of the following alanine, as in many other proteins, is blocked by an acetyl group.

RNA Blot Analysis. A Northern blot probed with the whole βPhk-D1N cDNA reveals a major band with an estimated size of 4200 nt (DNA standards) and a minor band of 4500 nt (Fig. 2).

DISCUSSION

Homology Between the α and β Subunits. Sequence comparison shows that the two polypeptides are homologous, except for an N-terminal sequence that is unique to the β subunit and two segments in the middle of the molecule that are unique to the α subunit (Figs. 3 and 4). The profiles of hydropathy and β-sheet potential are very well conserved in region B (Fig. 4). Although sequence similarity of α and β subunits decreases toward the C terminus in the second half of region B, the hydropathy and β-sheet potential profiles remain similar. Sequence similarity also drops in the second half of region F. Here, a well-conserved C-terminal section seems to be linked to the first half of the region F through a nonconserved hinge. α-Helix potential (not shown) and length (88 vs. 86 amino acids) are well conserved between α and β subunits in the second half of region F, suggesting homology also in this part of the molecules.

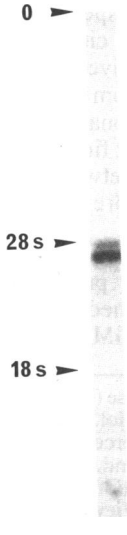

FIG. 2. RNA blot analysis of βPhk mRNA. Poly(A)$^+$ RNA (1 μg) from rabbit psoas muscle was glyoxylated and resolved on a 1% agarose gel. Positions of origin and glyoxylated rRNA standards are indicated.

The domains that are unique or of low sequence similarity are hydrophilic. This may indicate that they are exposed on the surface of the protein and may participate in mediating its interactions with the environment.

The nontranslated parts of the α and β subunit mRNAs, as far as they are available from the cDNA, are dissimilar in sequence as well as apparently in length and give no indication of common structural elements that might be involved—e.g., in coordinating the biosynthesis of the two subunits.

Phosphoserine Localization in Unique Domains. Remarkably, 9 of the 10 known phosphorylation sites on α and β subunits are located in sequences unique to either subunit. Thus, in the course of evolution, different regulatory domains were apparently spliced to a common primordial molecule.

We have detected three autophosphorylation sites on the β subunit (serines 11, 26, and 700). At least serine 700 is partially occupied by endogenous phosphate. There are several additional sites of endogenous phosphate that have not yet been sequenced representing ≈50% of the total endogenous phosphate (refs. 13 and 14; unpublished data). Serine residues 26 and 700 fulfill the target sequence requirements of cAMP-dependent protein kinase (basic residues to the N-terminal side, ref. 15), whereas serine 11 is in the type of environment preferred by protein kinase C (basic residue to the C-terminal side, ref. 16) or casein kinase I (acidic residue two positions to the N terminus, ref. 17). Thus, as in the α subunit (6), various classes of sites are accepted in the autophosphorylation of phosphorylase kinase. Serine 26 is known to become phosphorylated by cAMP-dependent protein kinase in response to adrenaline *in vivo* (18).

Serine residues 11 and 26 reside in region A that is unique to the β subunit. Serine 700 is located on the very edge of region B, where sequence similarity to the α subunit is largely degenerated. The cluster of four immediately adjacent serine and threonine residues surrounding serine 700 seems to be conserved in the α subunit, but the cluster of basic residues that makes it a potential target for cAMP-dependent protein kinase in the β subunit is not.

Reciprocal to the β subunit, the cluster of seven phosphorylation sites that we have described previously in the α subunit also turns out to be unique to this subunit. We have detected additional endogenous phosphate in the second half of region E, C-terminal to serine 1030, but we have not yet located it. This situation is reminiscent of the clustering of phosphorylation sites on rhodopsin (19) and especially glycogen synthase (20). In further analogy with glycogen synthase, the C-terminal half of region E (46 amino acids from serine 1018) is rich in serine (28%) and proline (11%) and very hydrophilic. Several of these closely spaced and highly exposed phosphoserines might have similar regulatory properties or their phosphorylation might occur in an interactive process as in glycogen synthase (21). However, of the seven phosphoserine residues on the α subunit, only the phosphorylation of serine 1018 has as yet been shown to be functionally relevant and to occur *in vivo* (9, 18). It is not yet known whether autophosphorylation occurs *in vivo* or whether the

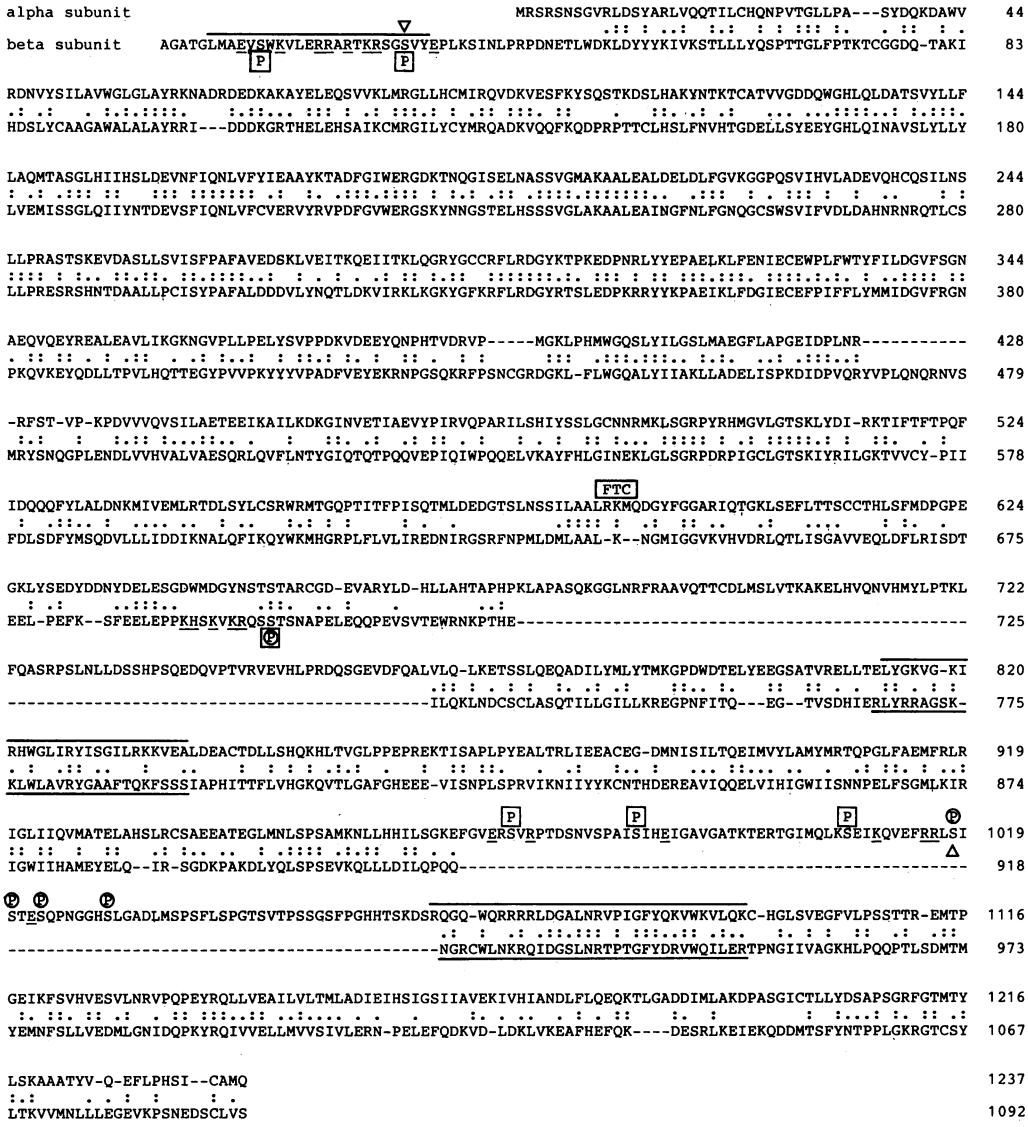

FIG. 3. Sequence alignment of the α (upper) and β (lower) subunits of phosphorylase kinase (one-letter amino acid notation). Sequence identities are indicated by colons, conservative replacements (10) are indicated by single dots, and gaps are indicated by dashes. Serine residues subject to autophosphorylation are marked by a boxed P, those carrying endogenous phosphate are marked by a circled P, and those that are phosphorylated by cAMP-dependent protein kinase (9) are marked by a triangle. Charged residues in the vicinity of the phosphoserine residues that are potentially relevant as substrate recognition signals are underlined. Putative calmodulin-binding sequences are over- or underlined. FTC marks lysine 588 in the α subunit that is specifically labeled by fluorescein isothiocyanate (6).

endogenous phosphate residues influence the properties of the enzyme, but at least serine 700 in β is both endogenously phosphorylated and autophosphorylated.

Putative Calmodulin-Binding Sites. Phosphorylase kinase can be activated by exogenous calmodulin (δ') and cross-linking experiments have suggested that this additional calmodulin molecule binds to subunits α and β, with a preference for the β subunit (22). We have previously (6) proposed the α subunit sequences of amino acids 810–840 and 1060–1100 as potential calmodulin-binding sites, which are rich in basic, hydrophobic, and aromatic residues with a central tryptophan. We now find that both sequences are conserved in the β subunit, retaining the exact positions of all aromatic residues and the regular spacing of basic and hydrophobic residues that provides the potential to form a basic amphipathic helix. In the β subunit the first sequence additionally possesses serine residues (792–794) that are found at the C termini of several calmodulin-binding peptides (23). With its regular pattern of hydrophobic and basic residues, the absence of acidic amino acids and proline, and only two glycine residues, it is probably the best candidate for a high-affinity calmodulin-binding site. Unique to the β subunit, the N-terminal domain A also seems to have the potential to bind calmodulin. Because it serves also as a substrate for phosphorylation, this could provide the molecular basis for a regulatory interplay between its phosphorylation and its interaction with the δ subunit, exogenous calmodulin, or troponin C. To date, only the γ subunit has been shown to interact with the δ subunit (22). Phosphorylase kinase is unique among the Ca^{2+}/calmodulin-dependent protein kinases in that it retains calmodulin as an integral subunit even in the absence of Ca^{2+}. The action of additional calmodulin-binding sites on the α and/or β subunits may be responsible for this.

No Homology to Other Proteins. Affinity ligand binding, differential effects on protein substrate specificity by Ca^{2+}, antibodies and affinity ligands (literature cited in ref. 6), and the observation of an enzymatically active proteolytic fragment that was presumed to be derived from the β subunit (24) have led to speculations that an additional catalytic center might reside on the β subunit. We do not find in its sequence any of the similarities that are shared between the catalytic domains of protein kinases and therefore believe that this possibility is unlikely.

In general, we did not observe similarities with any other protein sequences, and, in particular, protein kinase sequences, in the data bases that we searched.¶ The regulatory domains/subunits of the cAMP- and cGMP-dependent pro-

¶EMBL/GenBank Genetic Sequence Database (1987) EMBL Nucleotide Sequence Data Library (Eur. Mol. Biol. Lab., Heidelberg), Release 12.0; Protein Identification Resource (1987) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 13.0; and EMBL/GenBank Genetic Sequence Database (1988) GenBank (IntelliGenetics, Mountain View, CA), Release 54.0.

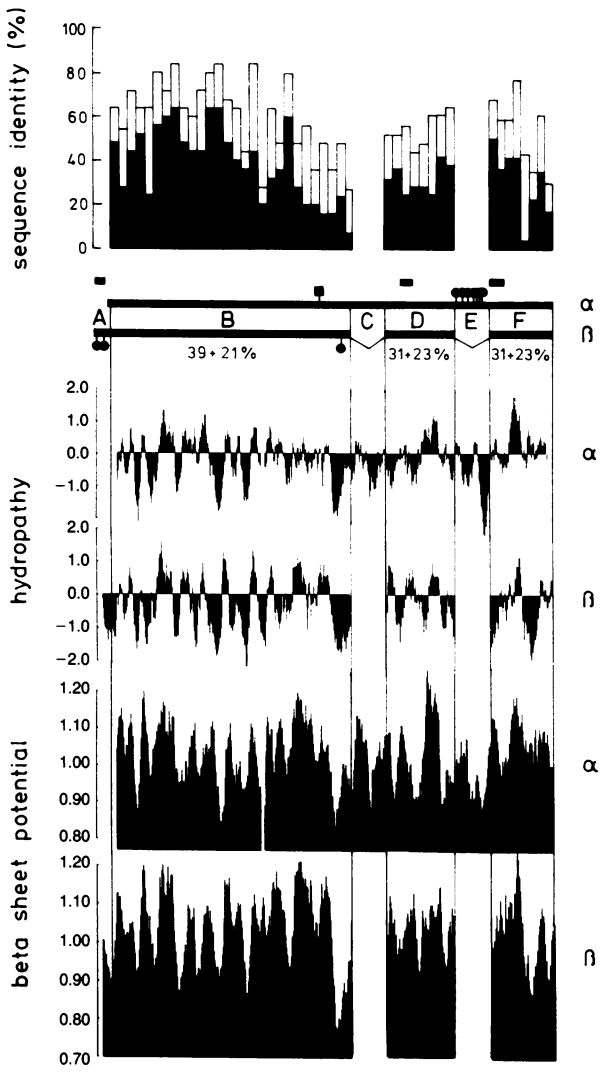

FIG. 4. Homologous and unique regions of α and β subunits. The bar diagram gives the percentage of amino acid identities (solid bars) and conservative replacements (open bars) in the homologous regions. Segment width per bar is 25 residues except in region F (23 residues) and the last segment of region B (15 residues). The scheme below gives an overview of the homologous and unique regions of α and β subunits. Phosphorylation sites are marked by solid circles, the FTC-labeled site of α-Phk is marked by a solid square, and putative calmodulin-binding sites are marked by solid bars above. The first figure below the homologous regions signifies the percentage of overall amino acid identities; the second figure signifies additional conservative replacements. The diagrams below are the hydropathy (11) and β-sheet potential (12) profiles (window 20 in both cases).

tein kinases, myosin light chain kinase, protein kinase C, and brain calmodulin-dependent kinase II (α and β subunits) did not reveal any significant similarities to the α and β subunits of phosphorylase kinase that might have indicated sequences commonly involved in the interaction between regulatory and catalytic domains/subunits of protein kinases.

ATP affinity ligands were found to label the β subunit, and ADP was observed to have an allosteric effect on enzyme activity and influence the conformation of the β subunit (25). We do not find the perfect consensus motif Gly-Xaa-Gly-Xaa-Xaa-Gly-Lys of nucleotide-binding proteins in the β subunit sequence, but the two similar sequences that we have pointed out in the α subunit are conserved: (Gly-Cys-Leu-Gly-Thr-Ser-Lys: 556–562; Gly-Met-Ile-Gly-Gly-Val-Lys: 643–649 in β). However, whereas in the α subunit, lysine 588, which is very close to the second site (Gly-Tyr-Phe-Gly-Gly-Ala-Arg: 592–598 in α), is labeled specifically by fluorescein isothiocyanate (6), its putative counterpart in the β subunit, lysine 641, does not react with fluorescein isothiocyanate.

Sequence comparison of the α and β subunits defines an architecture of structural domains for these molecules and suggests that their genes evolved through gene duplication and subsequent divergence and exon shuffling. The homology of the two subunits may indicate that they reside in similar positions within the quaternary structure (26). We hope that this structural analysis will guide future experiments to define the functional roles of the various domains. From characterization of the isoenzyme cDNAs, we expect additional insights into how structural variations of the subunits fine-tune the properties of phosphorylase kinase to the regulatory requirements of specific cell types.

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